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**BATCH AND CONTINUOUS FERMENTATION
METHODS FOR THE PRODUCTION
OF DEXTRANSUCRASE**

A thesis submitted by
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for the degree of Doctor of Philosophy

THE UNIVERSITY OF ASTON IN BIRMINGHAM
April 1991

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THE UNIVERSITY OF ASTON IN BIRMINGHAM.

BATCH AND CONTINUOUS FERMENTATION METHODS FOR THE PRODUCTION OF DEXTRANSUCRASE

PhD

RUTH DIANE PENNELL

1991

SUMMARY

The available literature concerning dextransucrase and dextran production and purification has been reviewed along with the reaction mechanisms of the enzyme. A discussion of basic fermentation theory is included, together with a brief description of bioreactor hydrodynamics and general biotechnology. The various fermenters used in this research work are described in detail, along with the various experimental techniques employed.

The micro-organism *Leuconostoc mesenteroides* NRRL B512 (F) secretes dextransucrase in the presence of an inducer, sucrose, this being the only known inducer of the enzyme. Dextransucrase is a growth related product and a series of fed-batch fermentations have been carried out to extend the exponential growth phase of the organism. These experiments were carried out in a number of different sized vessels, ranging in size from 2.5 to 1,000 litres. Using a 16 litre vessel, dextransucrase activities in excess of 450 DSU/cm³ (21.67 U/cm³) have been obtained under non-aerated conditions. It has also been possible to achieve 442 DSU/cm³ (21.28 U/cm³) using the 1,000 litre vessel, although this has not been done consistently.

A 1 litre and a 2.5 litre vessel were used for the continuous fermentations of dextransucrase. The 2.5 litre vessel was a very sophisticated MBR MiniBioreactor and was used for the majority of continuous fermentations carried out. An enzyme activity of approximately 108 DSU/cm³ (5.20 U/cm³) was achieved at a dilution rate of 0.50 h⁻¹, which corresponds to the maximum growth rate of the cells under the process conditions. A number of continuous fermentations were operated for prolonged periods of time, with experimental run-times of up to 389 h being recorded without any incidence of contamination. The phenomenon of enzyme enhancement on hold-up of up to 100% was also noted during these fermentations, with dextransucrase of activity 89.7 DSU/cm³ (4.32 U/cm³) being boosted to 155.7 DSU/cm³ (7.50 U/cm³) following 24 hours of hold-up. These findings support the recommendation of a second reactor being placed in series with the existing vessel.

Keywords :- Dextransucrase, Dextran, *Leuconostoc mesenteroides*, (continuous) fermentation.

Dedicated to my parents

LUCIE AND COLIN PENNELL

for all their love, support (financial and
otherwise!) and guidance throughout
all my studies

and in memory of

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who gave me my first opinion of
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1. INTRODUCTION.

This chapter outlines some of the basic points of the project, including a brief discussion of some of the theory involved, along with the aims of the project. This section also contains a brief description of the various chapters included in this thesis.

1.1. Dextranucrase.

This research work is basically concerned with the production of the enzyme dextranucrase using the principle of fermentation, whereby an organism, such as *Leuconostoc mesenteroides* NRRL B512 (F), that is capable of producing the desired product, is grown in a nutritious medium rich in a substrate that will allow, by induction or otherwise, the formation of the product. Sucrose is a substrate that is known to induce the formation of dextranucrase in *Leuconostoc mesenteroides*, with the enzyme then being secreted from the interior of the cell into the surrounding medium. Dextranucrase is, therefore, an inducible extracellular enzyme, with sucrose being the only known substrate, to date, that will allow its induction. Dextranucrase acts as a biological catalyst for the conversion of sucrose to dextran, a variable length polysaccharide, consisting of D-glucose molecules, joined with α -1,6 glucosidic bonds. Dextran has several uses other than pharmaceutical, namely as a gelling agent in the food industry and as chromatographic media, such as Sephadex. However, the dextran produced by *Leuconostoc mesenteroides* NRRL B512 (F) dextranucrase has uses in the pharmaceutical industry, such as being used in an iron-dextran form to treat iron-deficiency anaemia and in a partially hydrolysed form as a blood plasma substitute for the treatment of shock.

1.2. Production of Dextranucrase.

Dextranucrase is secreted from *Leuconostoc mesenteroides* cells under certain, well-defined process conditions in terms of operating temperature and pH. The enzyme can be formed between temperatures of 20 and 30°C, with the higher temperatures encouraging improved cell growth, although not necessarily better enzyme elaboration. A pH of between 6.0 and 6.9 can be used for dextranucrase production, although optimum operating conditions have been found to occur at a temperature of 23 - 24°C and a pH of 6.65 - 6.70. The enzyme is a growth-related product, which means that it is predominantly secreted during the exponential growth phase of the cells. It is

therefore desirable to operate the fermentation in such a way as to extend this exponential period in order to increase dextransucrase production rates. This can be done either by feeding extra substrate into the medium during a batch fermentation, or by operating the fermentation continuously at high growth rates. These techniques are commonly known as fed-batch and continuous fermentations respectively. There are two ways in which dextransucrase can be used to produce clinical dextran and these are the whole cell and the cell-free fermentation methods. Currently, dextran is manufactured industrially via the whole cell method, whereby the *Leuconostoc mesenteroides* cells are grown in a sucrose-rich medium so that the dextransucrase is induced and immediately converted by the excess sucrose into dextran. The cell-free method involves two separate stages, with the first stage concerning simply the production of the enzyme. This enzyme-containing broth, with or without any purification or cell removal stages, is then transferred to a non-sterile sucrose solution where the enzyme catalyses the sucrose-to-dextran reaction. These two methods are discussed in detail in Section 2.7.2, along with a comparison of the two techniques.

There has been a great deal of work carried out on dextran and dextransucrase, with particular emphasis on enzyme purification, although this always leads to a loss of enzyme activity. Several researchers have investigated methods to concentrate the enzyme, whilst others have attempted immobilization of dextransucrase, with some work having been carried out on dextransucrase production from immobilized *Leuconostoc mesenteroides* cells. Currently, a great deal of research is being carried out at Aston University, using a novel continuous chromatographic reactor separator, which produces dextran and fructose from sucrose and simultaneously separates the two products using the theory of chromatography.

1.3. Aims of the Project.

The aims of this project are listed below :-

1. To obtain, and improve upon, dextransucrase yields reported in the literature and to confirm these results.

2. To optimize the dextransucrase fed-batch production process by altering various process parameters, such as pH, temperature and medium composition.
3. To purchase and commission a fermenter for the purpose of carrying out continuous fermentations of dextransucrase.
4. To continuously produce dextransucrase such that the levels achieved previously in the literature could be achieved and, if possible, to improve upon these results by altering certain process parameters.
5. To scale-up the fed-batch fermentation to industrial scale size in order to produce a more economically viable technique for manufacturing dextran, as opposed to the traditional whole cell method.
6. To produce a computer simulation model of the continuous process.

1.4. Outline of the Thesis.

Chapter 2 contains a review of all the available, relevant material on dextransucrase, dextran and *Leuconostoc mesenteroides*.

Chapter 3 presents some basic fermentation principles of batch, fed-batch and continuous cultures and some bioreactor hydrodynamics theory.

Chapter 4 contains some basic principles of general enzyme biotechnology.

Chapter 5 reviews all the apparatus used during the course of this project, along with all experimental and analytical techniques carried out.

Chapter 6 details the results obtained from batch and fed-batch fermentations carried out in both laboratory and industrial scale vessels.

Chapter 7 discusses the findings from Chapter 6.

Chapter 8 contains the results obtained from the continuous fermentations carried out over the duration of this research work.

Chapter 9 discusses the findings from Chapter 8 and includes a description of the simulation program used to model the continuous process.

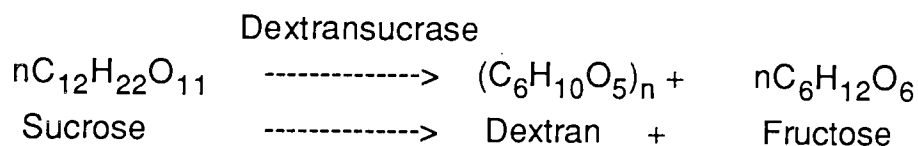
Chapter 10 presents the overall conclusions from the project along with a number of recommendations for future work.

2. DEXTRANSUCRASE LITERATURE REVIEW

2.1. Introduction.

Dextranucrase (Sucrose 1,6- α -D glucan 6- α -glucosyltransferase EC 2.4.1.5) is an extracellular enzyme that is secreted from organisms belonging to the family Lactobacteriaceae, tribe Streptococceae, and genus *Leuconostoc* - namely *Leuconostoc mesenteroides*, and *Leuconostoc dextranicum*.⁽¹⁾ The third species of *Leuconostoc* - *Leuconostoc citrovorum* - does not produce dextranucrase. Certain strains of *Streptococcus* - namely *Streptococcus bovis* ⁽²⁾ and *Streptococcus DS Strain 50* ⁽³⁾ - along with *Betabacterium vermiforme* ⁽⁴⁾ and *Streptobacterium dextranicum* ⁽⁵⁾, can also secrete dextranucrase. The studies in this project have concerned dextranucrase secreted from *Leuconostoc mesenteroides* B-512 (F), which, along with *L.mes* B-512, was developed by the Northern Utilisation Research Development Division of the U.S. Department of Agriculture at Peoria, Illinois.

Dextranucrase is responsible for the synthesis of dextran and fructose from sucrose, according to the following Equation:-



"Dextran" is a collective term given to a group of bacterial polysaccharides, consisting of a chain of D-glucose units joined together with α -1,6 glucosidic linkages. The structure, degree of branching and the molecular weight of the dextran vary, depending on the enzyme-synthesizing bacteria used.

A major characteristic of dextranucrase is its specificity for sucrose. No other sugar has been found to act as a donor of glucosyl groups for the formation of the polymer dextran - with even the similar isomer α -D-galactopyranosyl- β -D-fructofuranoside being inactive with this reaction. This specificity for sucrose is due to a phenomenon known as induction, whereby the micro-organism evolves new enzyme(s) to act on foreign substrates - in this case, dextranucrase acting on sucrose. Many investigations have been carried out to see if it is possible for *Leuconostoc mesenteroides* to grow on different carbon sources, and to produce the enzyme. It was found that similar levels

of cell growth were obtained from sucrose, glucose, fructose and maltose carbon sources, although dextransucrase activity was detected only with sucrose as substrate.

2.2. Production of Dextransucrase.

The earlier work carried out on dextransucrase involved the production of the enzyme followed by immediate dextran synthesis. A discussion of this whole-cell production of dextran will be given in Section 2.7.2.1. Later research was carried out into the separate production of dextransucrase from dextran. The dextran is thus produced in a separate stage - this is known as the cell-free production of dextran. The majority of work regarding dextransucrase production has been carried out using the organism *Leuconostoc mesenteroides* NRRL B512 (F).

Various workers have attempted to grow the organism on different carbohydrate sources, and monitor dextransucrase production (if any) in each. Whiteside-Carlson and Carlson (1949) ⁽⁶⁾ cultured several different strains of *Leuconostoc* in various media containing glucose, fructose or sucrose. The strains employed in this work were not necessarily all dextran producing, for example *Leuconostoc dextranicum* 8086 (ATCC). They found that *Leuconostoc* grows preferentially in sucrose-based media, although some growth did occur when fructose and glucose were substituted as carbon sources. Neely and Nott (1962) ⁽⁷⁾ grew *Leuconostoc mesenteroides* in medium containing one of seventeen different sugars, with reasonable cell growth only being detected with sucrose, invert sugar, glucose, maltose, melibiose and fructose as substrate. Certain carbohydrates, such as mannose and galactose supported some cell growth, whilst others, such as lactose and cellobiose allowed barely any growth. However, dextransucrase activity was detected only in the sucrose medium, confirming the fact that dextransucrase is sucrose specific.

Tsuchiya *et al* (1952) ⁽⁸⁾ varied the sucrose concentration in the medium to investigate the effect on dextransucrase activity. They found that higher sucrose levels induced better enzyme production although, at levels of above 3% w/v (30 g/l) sucrose, the culture contained so much dextran that cell removal became very difficult. They concluded that a concentration of 2% w/v sucrose was the optimum value for enzyme production, with *Leuconostoc mesenteroides* NRRL B512. However, it must be mentioned that no pH

control took place, and the culture pH was allowed to drop to a value of 5.0 which is the optimum value for dextran synthesis. Tsuchiya and co-workers (8) also found that glucose, fructose and maltose were incapable of inducing dextransucrase formation, and where these sugars were used in the medium with sucrose, enzyme yield was proportional to sucrose concentration. Neely and Nott (1962) (7) came to a similar conclusion, although they attempted to boost enzyme activity by the addition of a second amount of sucrose once the first batch had been utilized by the cells. They found that at the low pH of 5.0, there was no increase in activity, but, when the pH was readjusted to 7.0, enzyme production proceeded at a maximum rate, indicating still further that dextransucrase is an induced enzyme.

Tsuchiya *et al* (1952) (8) also considered the effect of pH on the production of dextransucrase. They carried out experiments in which the culture pH was controlled at values of 6.1, 6.3, 6.7 and 7.0, ± 0.1 unit, with 5M NaOH being added continuously to control the pH at the required value. They obtained the maximum enzyme yield at pH 6.7, although they found that 35% of the activity was lost at pH 6.67 in 1 hour, whereas enzyme activity could be retained at pH 5.0 to 5.3 for at least 24 hours at 25°C. Maintaining the pH at a value of 6.7 is a factor that stimulates high enzyme production, although dextransucrase is quickly inactivated at a pH of 6.7. The pH was controlled both by the presence of a high concentration of phosphate buffer, and the continuous addition of alkali. Whiteside-Carlson and Carlson (1949) (6) used a concentration of 5 g/l of NH_4Cl in their basal medium for the cultivation of various strains of *Leuconostoc*, but by 1951, Whiteside-Carlson and Rosano (9) had observed that the ammonium ion was detrimental to the growth of *Leuconostoc*, and replaced the NH_4Cl buffer with potassium acetate (5 g/l). Tsuchiya *et al* (8) investigated the effect of the ammonium ion, by controlling the pH with a mixture of KCl and $(\text{NH}_4)_2\text{HPO}_4$, and observed the same detrimental effect on the organism. Consequently, they recommended that either NaOH or KOH could be used to control the pH, but not NH_4OH .

Schneider (10), working on behalf of Fisons, at Battelle (1978) also carried out experiments to investigate pH dependency on enzyme yield, and confirmed that maximum activity occurred at a pH of 6.7 (Figure 2.1) (10). He also altered the substrates used, and found that, although glucose supported cell growth as well as sucrose, no enzyme was induced, and that fructose supported less growth than either sucrose or glucose. Using the theory that substrate

analogues can be better inducers than the natural substrate, he used sucrose palmitate as substrate (known to be a good inducer of invertase), but very little growth and no enzyme production were observed. No growth was obtained using glycerol substrate. These conditions of pH and substrate type have since become acknowledged as being those most likely to bring about maximum enzyme yields.

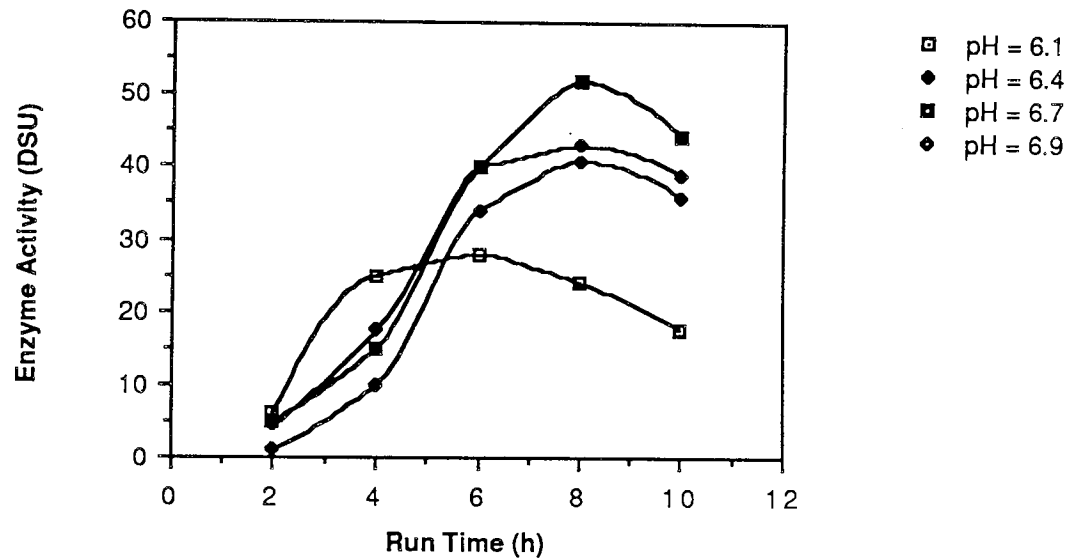


Figure 2.1: Effect of pH on Dextranucrase Activity

Tsuchiya *et al* (1959) (8) also investigated temperature effect on dextranucrase production. They carried out experiments with a medium pH of 6.7, at temperatures of 20, 23, 26 and 29°C, finding a maximum enzyme yield at 23°C, with very little at 29°C. Schneider (1978) (10) also considered the influence of temperature, carrying out experiments at 19, 23 and 27°C. Growth rate was increased with increasing temperature, although maximum growth level was relatively unaffected. However, the enzyme yield obtained at 27°C was considerably lower than the high growth rate would seem to indicate dextranucrase production being growth related. These findings agreed with those of Tsuchiya *et al* (8), in that, although high enzyme yields may be produced at higher temperatures, decomposition of the enzyme would occur at temperatures above 27°C (see Figure 2.2.) (8). The optimal temperature for dextranucrase production is, therefore, 23°C.

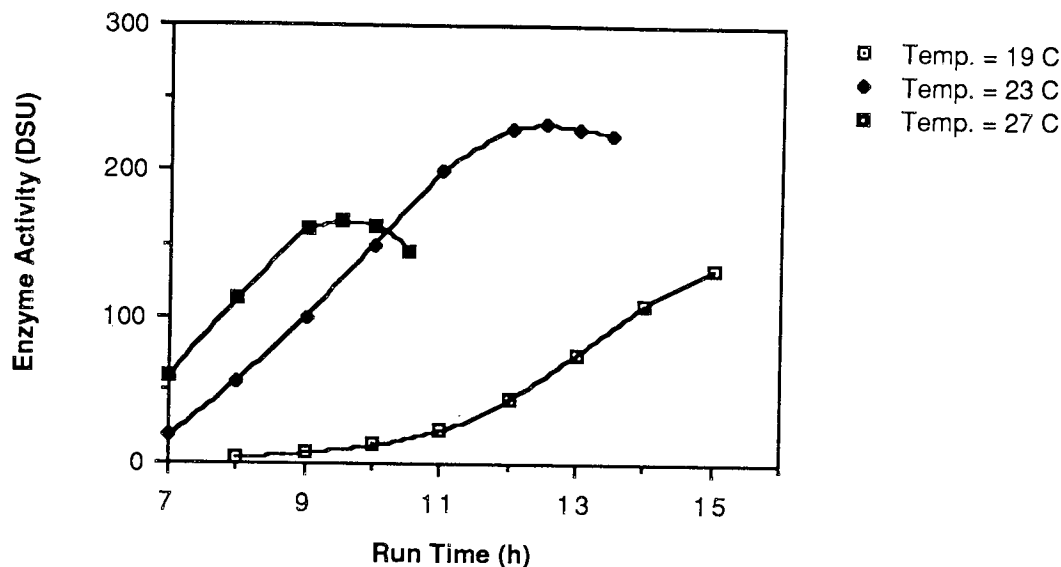


Figure 2.2: Enzyme Production at Varying Temperatures

As dextransucrase production is growth related, attempts were made to increase the maximum cell concentration thus far obtained. Schneider (10) found that the growth of the culture stopped at an OD₅₉₀ value of 1.2 (see Section 5.4.1.), and tried to explain this by suggesting that cessation of growth was caused by the lack of some essential nutrient in the medium, the presence of an inhibitor produced during the fermentation, or the physiological state of the culture. He carried out various experiments investigating these phenomena, but could not increase the biomass level above approximately 9 g/l. He concluded that there were probably physiological limitations for further growth.

Schneider (10) also carried out experiments to vary the aeration rate into the culture, and claimed that the specific growth rate of the cell is dependent on the aeration conditions, with the highest growth rate being obtained at high dissolved oxygen concentrations. However, these high growth rates did not correspond to high biomass levels, and enzyme yields, implying that high oxygen levels inhibit the cell growth above levels of 4.5 g/l and hence impair enzyme levels. With no aeration, he claimed that the biomass level decreased, as did enzyme yield. The dissolved oxygen concentration, as determined by Schneider, for maximum enzyme activity, should therefore be controlled at 40 - 50% of saturation.

Ajongwen-Numfor (1988) (11) attempted to confirm these findings by carrying out fermentations, with the dissolved oxygen level controlled at the level mentioned by Schneider, with constant aeration and variable agitation rate. He also carried out corresponding fermentations with no aeration and low agitation, and found that, although reasonably high levels were obtained with the conditions specified by Schneider, greatly increased levels were obtained with no aeration and low agitation.

Schneider (12) compared Difco and Yeatex yeast extracts, believing that the final enzyme activity was also a function of the type of yeast extract used. He found that much higher levels of dextransucrase were obtained using the Difco brand.

2.2.1. Development of Fed-Batch Fermentations.

Initial attempts to produce dextransucrase have been carried out by batch fermentation (see Section 3.8), where there is no addition of cells or medium to the reaction vessel. Using this technique Tsuchiya *et al* (1952) (8) obtained 120 Dextransucrase Units/cm³ in medium containing 5.0% (w/v) sucrose, 2.0% corn steep liquor, 2.0% KH₂PO₄ and 0.5% by volume R* Salts. They defined one Dextransucrase Unit as the amount of enzyme that converted 1 mg of sucrose to dextran in 1 hour at pH 5.2 and 30°C. This experiment incorporated no pH control, no sucrose addition and, as a result of the high initial sucrose concentration, the resulting mixture was extremely viscous due to dextran production. With a 2.0% sucrose solution, they carried out a batch fermentation, obtaining 70 Dextransucrase Units/cm³ after 16 hours. Control of the pH greatly increased the initial rate of secretion of the enzyme, indicating that high enzyme yields could be obtained if the pH were to be controlled at 6.7 throughout the course of a full fermentation. Neely and Nott (1962) (7) carried out a fermentation whereby the initial medium contained 2.0% sucrose, and, after monitoring the pH drop down to 5.2, readjusted it to 7.0 and added a further 2.0% sucrose to the medium. This served to greatly boost enzyme activity, and was an early example of a fed-batch fermentation.

Schneider (1978) (10) also recognized the importance of sucrose addition during the fermentation, and, in a similar experiment to that carried out by Neely and Nott, added 20 g/l sucrose to a fermentation where the pH had dropped from 7.2 to 5.6 and then readjusted to 7.0. Before sucrose addition they had 30 DSU/cm³ (1.44 U/cm³), whereas by the end of the fermentation

they had obtained 55 DSU/cm³ (2.65 U/cm³)- with one DSU being defined as the amount of enzyme which will convert 1 mg sucrose to dextran in 1 hour at pH 5.2 and 25°C and one U being the I unit for enzyme activity, defined as one micromole of reducing sugar produced per minute at 25°C and pH 5.2. Neely and Nott obtained approximately 60 DSU/cm³ (2.89 U/cm³) for their similar experiment.

Schneider carried out three fermentations, with pH at 6.7, temperature at 23°C and aeration at 4 vvm (volume of air/volume of medium/minute). In one experiment, he had an initial sucrose concentration of 20 g/l and after six hours, added sucrose continuously for 4 hours at a rate of 5 g/l. In a second experiment he used an initial sucrose concentration of 40 g/l, with no subsequent sucrose addition. In the third experiment, he used 10 g/l sucrose initially and after 5 hours added 6 g/l continuously for five hours. He determined that it was most advantageous to start a fermentation with a low sucrose concentration and to add small amounts of sucrose continuously as productivity per micro-organism increases as initial sucrose level decreases. He then carried out a fermentation whereby the sucrose level in the medium was measured and maintained at approximately 1-5 g/l in the fermenter. Under these conditions, he obtained an activity in excess of 200 DSU/cm³ (9.63 U/cm³) in the fermenter, with an OD₅₉₀ value of 1.2.

He later claimed (1979) (12), together with various co-workers, that it was possible to reach a maximum activity of 395 DSU/cm³ (19.02 U/cm³) under highly aerated conditions and using Difco yeast extract as the nitrogen source. They also managed to scale up the process to a 300 litre pilot plant fermenter and with the same conditions as used for the laboratory scale fermentations. They obtained an activity of 300 DSU/cm³ (14.44 U/cm³) with the sucrose level in the medium being maintained at 8-10 g/l.

Lopez and Monsan (1980) (13) carried out fermentations under a mild aeration rate of 0.06 vvm and an agitation rate of 280 rpm. They fed sucrose into the fermenter at a rate of 20 g/l and obtained an activity of 180 DSU/cm³ (8.67 U/cm³). Schneider obtained similar results with an unaerated fermentation.

Brown and McAvoy (1986) (14) attempted to improve the fed-batch fermentation of dextransucrase. They noticed that, with certain yeast extract

types as nitrogen source, the cell growth and enzyme production were severely limited towards the end of the fermentation. They changed to using Continental yeast extracts, namely Ohrlly and Gist-Brocades, and obtained excellent growth and enzyme production. They monitored CO₂ and O₂ levels in the effluent gases of these fermentations, as they believed that the rate of production of CO₂ and uptake of O₂ could indicate the metabolic activity of the cells. They found that *Leuconostoc mesenteroides* NRRL B512 (F) suffers some severe limitation late in its exponential growth phase, and attempted to discover the exact source of this limitation. They determined that the addition of the vitamin mixture of thiamine, folic acid, riboflavin, pantothenic acid, nicotinic acid and biotin to the culture recovered its metabolic rate and, by a process of elimination, they claimed that the addition of 0.0206 g/l folic acid would restore the metabolic rate of the culture, as it was this component that was limiting in the yeast extract.

Brown and McAvoy (1989) ⁽¹⁵⁾ carried out controlled fed-batch fermentations with the pH being controlled with a NaOH/sucrose mixture in the molar ratio of 1.4. They used the same basic medium as that specified by Schneider, namely various concentrations of sucrose, 40 g/l Bovril Yeatex, 20 g/l K₂HPO₄, and 0.5% v/v R* salts. They controlled the dissolved oxygen concentration in the range 40-50% saturation. They obtained a maximum enzyme activity of 301 DSU/cm³ (14.49 U/cm³) which corresponded to a total cell dry weight of 10.9 g/l. They found that the fermentation proceeded in a linear, rather than an exponential manner, after the initial non-linear lag and acceleration phases. This linearity was determined by the sucrose usage results, which were measured by a digital balance. They obtained a linear sucrose usage rate of 57.1 g for a 4.9 litre fermentation, although they noticed that when cell growth ceased, so did enzyme production. It seemed that some vital medium component had become limiting at this point, although the rate of sucrose consumption slowed down, but did not cease. This linear, rather than exponential rate, was attributed to the scavenging of a trace nutrient, perhaps present in the yeast extract, with subsequent limitation within the cells, although the nutrient was still unknown.

More recently, Landon and Webb (1990) ⁽¹⁶⁾ attempted to scale up the dextransucrase production process to a 1,000 litre fermenter. They used medium containing 40 g/l sucrose, 20 g/l yeast extract and 16 g/l Na₂HPO₄. They did not control the pH, although the temperature was controlled at 26°C.

There was no aeration or agitation. Under these conditions, an activity of 142 DSU/cm³ was achieved in the laboratory (1 DSU being defined as the amount of enzyme that converts 1 mg of sucrose to dextran in one hour at pH 5.2 and 30°C in a 10% sucrose solution) in a 200 cm³ culture. This process was carried out in the 1,000 litre fermenter, with the only differences being the use of 20 g/l K₂HPO₄, instead of Na₂HPO₄, and the agitation of the fermentation broth at 60 rpm for one minute before sampling. They achieved an activity of only 45 DSU/cm³ (40 DSU/cm³ in a 6 litre laboratory vessel) and claimed that the scale-up process was difficult due to problems encountered with the inoculum medium sterilization, use of K₂HPO₄ and mixing during the fermentation. A 6 litre fermentation was carried out, with continuous agitation and the attempted elimination of all the other factors, and an activity of only 99 DSU/cm³ was achieved. They claimed it was not possible to scale-up the dextransucrase fermentation process without better defined mixing conditions. They also considered operating a fed-batch fermentation system, with the pH controlled at 6.1, where, according to Tsuchiya *et al* (1952) (8) initial enzyme production was greatest, although this would mean a higher degree of dextran formation. They attempted to limit this conversion by more accurate control of the sucrose concentration in the medium.

2.2.2. Continuous Fermentations.

There has not been a great deal of previous research into the continuous production of dextransucrase. Lawford and co-workers (1979) (17) carried out studies using a New Brunswick BioFlo C30 Bench Top Chemostat - as described in Section 5.3.1. They controlled the pH at 6.7 ± 0.1, temperature at 25°C, agitation rate at 300 rpm and aeration rate at 0.5 vvm. They used a medium composition of 1 g/l potassium phosphate, 4 g/l Difco yeast extract, 10 g/l casein hydrolysate, 10 g/l sucrose, 5 cm³/l R* salts and 0.1 cm³/l antifoam, with the sugar being autoclaved separately. They claimed a theoretical critical dilution rate of 0.67 h⁻¹ based on the maximum specific growth rate of a mid-exponential-phase batch culture with either glucose or sucrose as an energy source. However, they found that, in practice, the culture washed out at a dilution rate of 0.4 h⁻¹, although they claimed to have achieved maximum enzyme activity at a dilution rate of 0.53 h⁻¹ (when the culture was already being washed out). They reported a maximum dextransucrase activity of approximately 160 g reducing sugar/min/cm³ which corresponds to approximately 9 DSU/cm³ (0.43 U/cm³). Kurt and Curt (18) carried out experiments with a very low dilution rate of 0.09 h⁻¹, and obtained

enzyme with an activity of 25 DSU-Units/cm³. (One DSU-Unit is defined as the amount of enzyme that will produce 1 mg of fructose in 1 hour at 23°C and pH 5.2). This gives a value of approximately 48 DSU/cm³ (2.31 U/cm³), although the DSU is determined at 25°C, and the DSU-Unit at 23°C. They used their enzyme for the continuous conversion of sucrose to dextran, where they achieved 80-90% conversion of sucrose. In 1983, Paul *et al* (19) attained the best results thus far achieved in the literature, when they reached an activity of 70 DSU/cm³ (3.37 U/cm³) with a dilution rate of 0.40 h⁻¹. They used medium that contained 2% w/v sucrose and 2% w/v yeast extract.

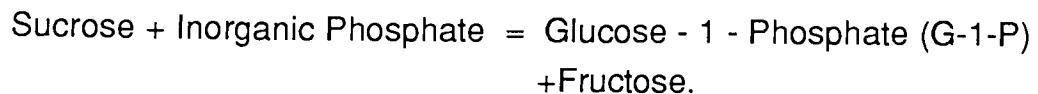
2.3. Leuconostoc Mesenteroides.

Leuconostoc mesenteroides is a member of the Streptococcaceae family and is of the Genus *Leuconostoc*. These Gram positive, non-motile cells are sometimes spherical but often lenticular - particularly on agar - and often found in pairs or chains. Bergey (20) has found that the cell colonies are small, usually less than 1 mm diameter, and are round and greyish white. They are chemoorganotrophs (organisms that obtain energy for growth by oxidation of reduced compounds of sulphur, hydrogen, ammonia or nitrite) and require rich media, often having complex growth factor and amino acid requirements. It is generally recognized that nicotinic acid, thiamine, pantothenic acid and biotin are required by all species of *Leuconostoc*. Whiteside-Carlson and Carlson (1949) (6) found that folic acid is required by certain strains, such as *L. mesenteroides* ATCC 683, although they claimed that biotin is only essential in glucose and fructose media - not in the sucrose medium as used for dextransucrase production. They also noted that, especially with sucrose as the carbohydrate source, there were certain inhibitory effects with various strains in the cases of riboflavin, biotin, folic acid, p-aminobenzoic acid, pyridoxal and pyridoxamine. It was also found that the elimination of any given vitamin had no adverse effect on either dextran yield or acid production (cell growth), unless that vitamin was one of those essential for growth. Stacey (1947) (21) claimed that the factor that stimulated dextran synthesis by *Leuconostoc* was p-aminobenzoic acid. In further studies by Whiteside-Carlson and Carlson (1949) (22) they found that low levels of p-aminobenzoic acid failed to stimulate dextran synthesis and higher concentrations retarded polysaccharide formation by the various strains of *Leuconostoc* they used.

Leuconostoc is a facultative anaerobe, with an optimum temperature between 20 and 30°C and is non-pathogenic both to animals and humans. The genus of Leuconostoc can be divided into two groups - the first consisting of five species which are closely related and difficult to separate from each other and which initiate growth between pH 5.5. and 6.5, but rarely below 5.0. They are found in slimy sugar solutions, fermenting vegetables, and in milk and dairy products. It is sometimes difficult to separate species of Leuconostoc from the gas-forming Lactobacilli. No known Leuconostocs hydrolyse arginine and all form only D(-)-Lactic Acid (20). Gas forming Lactobacilli form DL-Lactic Acid, although D(-)-Lactic Acid is sometimes produced and many hydrolyse arginine. However, some common and widely distributed gas-forming Lactobacilli form dextran and share other properties with the Leuconostocs they are often confused with. These aspects will be discussed in Sections 7.3.4/5 - concerning the effect of different yeast extract types (and different Gistex Batches) on enzyme production where the vitamin, amino acid and sugars analyses of various yeast extracts carried out during the course of this project will be discussed.

2.3.1. Sucrose Metabolism in *Leuconostoc Mesenteroides*.

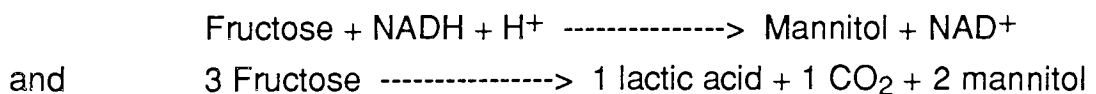
Sucrose phosphorylase (E.C. 2.4.1.7)⁽²³⁾ is produced from only a small number of microorganisms, such as *Leuconostoc mesenteroides*, *Pseudomonas saccharophila*, *Pseudomonas putrefaciens*, *Clostridium pasteurianum*, *Acetobacter xylinum* and *Pullularia pullulans*. This enzyme is responsible for the following reversible reaction:-



Vandamme and co-workers (1987) ⁽²³⁾ studied the production of this enzyme from *L. mesenteroides* ATCC 12291 using 10 and 20 litre anaerobic or microaerophilic batch fermentations. They carried out their fermentations at 30°C - conditions that do not favour dextransucrase (and hence, dextran) production - with a minimum amount of agitation. The fermentation pH was not controlled and it decreased throughout the fermentation from an initial value of 7.6. Sucrose phosphorylase is an intracellular enzyme and so to maximize its yield it would be necessary to improve the cell growth of the organism. Vandamme *et al* found that the organism utilizes the sucrose completely up to a concentration of 40 g/l, with the sucrose apparently being

phosphorolysed into glucose-1-P, which is immediately metabolized, and into fructose. The fructose section of the sucrose molecule is partially metabolized and partly converted into mannitol once the sucrose level in the medium has declined to less than 10% of its initial value. Increasing the initial sucrose level causes an increase in mannitol accumulation, with the cells not metabolizing the mannitol at all. G-1-P would, in this case, be used for cell maintenance.

Leuconostoc mesenteroides lacks a phosphoenolpyruvate:hexose phosphotransferase system, so it phosphorylates (forming ATP from ADP) glucose and fructose via ATP-dependent enzymes. Glucose is fermented anaerobically by the phosphoketolase pathway, generating 1 molecule of ATP. Ethanol, lactic acid and CO₂ are also produced, in equal molar amounts (see Figure 2.3.). In *Leuconostoc mesenteroides*, sucrose fermentation differs from that of glucose in that a major end product of sucrose fermentations is mannitol, which is formed when the intracellular, NADH-dependent enzyme, mannitol dehydrogenase, acts on fructose. This mannitol production maintains the redox balance of the cell, and so not all acetyl phosphate is reduced to ethanol (see Figure 2.3.), with the additional ATP being available for the cell under both aerobic and anaerobic conditions, when grown in sucrose medium. Mannitol dehydrogenase (E.C.1.1.1.67) converts fructose into mannitol as shown below.



This, then, explains the build-up of acetic and lactic acids and CO₂, with ethanol also being produced via the phosphoketolase pathway (Figure 2.3.).

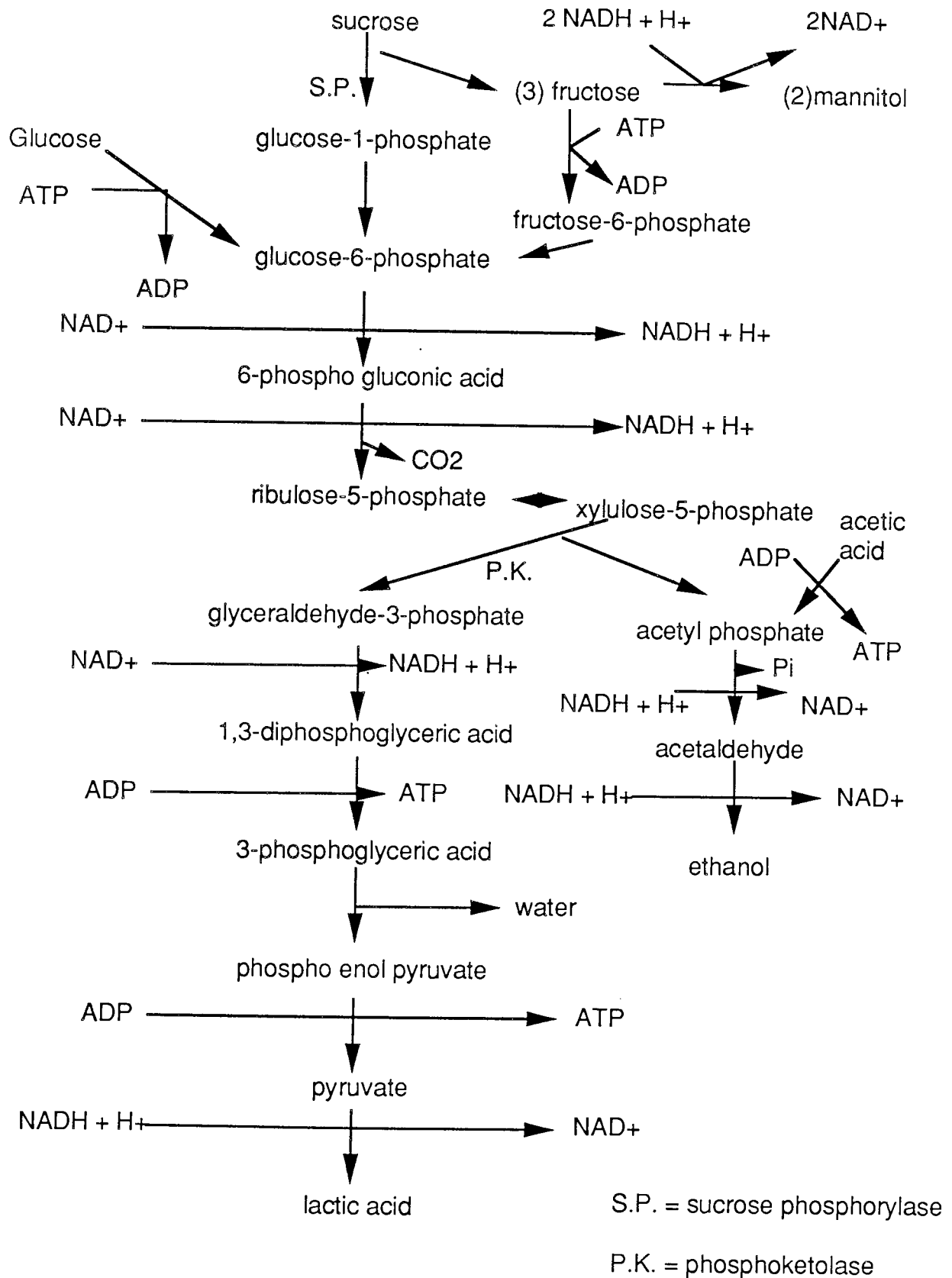


Figure 2.3

Metabolism of sucrose, glucose and fructose by *Leuconostoc mesenteroides* (phosphoketolase pathway)

Sucrose phosphorylase action is rarely detected in the fermentation broth, being almost exclusively confined to the cells. Tests carried out by Vandamme *et al* (1987) (24) indicate that the enzyme is almost certainly intracellular - as is mannitol dehydrogenase. Therefore, sucrose is phosphorolysed within the cell, with G-1-P preferentially utilized and excess fructose being secreted and accumulating in the medium. The remaining fructose is converted to mannitol and secreted in turn. *L. mesenteroides* ATCC 12291 forms sucrose phosphorylase constitutively, as sucrose presence is not a pre-requisite for enzyme synthesis - which is not the case for dextransucrase.

During the Vandamme studies on sucrose phosphorylase, it was necessary to minimize dextran production, which causes increased medium viscosity and hence makes cell recovery more difficult. Increasing the pH of the fermentation to values above 7.0 restricted dextran formation (see Section 2.3.2.), but in certain cases it was necessary to run the fermentations at pH values which would optimize dextran synthesis. Monsan and Lopez (1981) (25) found that the addition of maltose terminated the growth of linear dextran chains from the dextransucrase molecule (see Section 2.6), and so Vandamme *et al* added 1 mmol/dm³ of maltose to the reaction mixture and observed an appreciable decrease in viscosity, allowing much easier recovery of cells and enzyme from the fermentation broth - although there was still a loss of sucrose (carbon source) as short dextran chains were being formed.

Leuconostoc mesenteroides produces a number of enzymes as it grows. It forms sucrose phosphorylase constitutively - i.e. it is synthesised under all conditions of growth, and does not require a specific substrate to induce its synthesis - whereas dextransucrase is an induced enzyme. Dextransucrase is an extracellular enzyme and as such is detected in the fermentation broth, but sucrose phosphorylase is intracellular and as such is much harder to detect. The optimum conditions for sucrose phosphorylase production, under anaerobic conditions, are temperature of 32°C and pH of 7.3. At such a high temperature, dextransucrase will be degraded, although at such a high pH it would not be formed to a great extent anyway (see Section 2.3.2.). These conditions minimize dextran and mannitol production, which prevent a loss of carbon source. At conditions designed for optimal dextransucrase production, i.e. temperature of 25°C and pH of 6.7, the sucrose was

completely metabolised, with low fructose and high mannitol levels accumulating in the medium. Under these conditions, low levels of sucrose phosphorylase are reached, but they drop away by the end of the fermentation.

This section, therefore, explains why there is mannitol and fructose present in the medium during a dextransucrase fermentation - the presence of the sugars being detected by HPLC analysis.

2.3.2. Transmembrane Proton Gradient Theory.

David Otts, in his 1987 PhD thesis (26), discussed the regulation of dextransucrase secretion in *Leuconostoc mesenteroides* by proton motive force. This work was based upon the chemiosmotic theory, which is a biochemical mechanism suggested as the route of phosphorylation (formation of ATP) linked to electron transport. According to this hypothesis, protons are pumped outside the cell as a result of the passage of electrons (usually through the electron transport chain). An electric potential is generated across the membrane, with the cytoplasm being negative - this corresponds to the formation of a pH gradient with the cell interior alkaline with respect to the exterior. These two gradients have a contributory effect on the electrochemical potential of protons, $\Delta\mu_{H^+}$, which is the force that pulls protons back across the membrane into the cell. The free energy change (ΔG) as protons flow down these two gradients is equal to:-

$$\Delta G = \Delta\mu_{H^+} = F\Delta\psi - 2.3RT \left(\frac{\log [H^+]_{in}}{[H^+]_{out}} \right) \quad 2.1$$

where $\Delta\mu_{H^+}$ is the difference in electrochemical potential of protons across the cytoplasmic membrane (kcal)
 $\Delta\psi$ is the membrane potential
 R is the gas constant
 T is the temperature (Kelvin)

Equation 2.1 can be converted to units of electrical potential by dividing all terms by the Faraday's constant (F) so that :-

$$\frac{\Delta\mu_{H^+}}{F} = \Delta p = \Delta\psi - \frac{2.3RT}{F} \left(\log \frac{[H^+]_{in}}{[H^+]_{out}} \right) \quad 2.2$$

The logarithmic factor is equivalent to the pH difference across the cell membrane, i.e. $\Delta\text{pH} = \text{pH}_{\text{out}} - \text{pH}_{\text{in}}$. Also, $(2.3RT/F)$ is given the abbreviation Z and at 25°C (298 K) is equal to 59 mV. Therefore Equation 2.2. becomes:-

$$\Delta p = \Delta\psi - 59 \Delta\text{pH} \quad 2.3$$

where Δp is the proton motive force (units mV).

Otts, in association with Day (27), investigated the relationship between proton motive force and the secretion of dextransucrase in *Leuconostoc mesenteroides* ATCC 10830 [NRRL B512(F)]. They found that the organism was able to maintain a constant proton motive force of -130 mV when grown in batch culture at pH values of 5.8 to 7.0. The contribution to this value of $\Delta\psi$ and ΔpH (see Equation 2.3) varies depending on the pH of the fermentation. Between pH values of 5.5 and 7.0 dextransucrase secretion is dependent on proton motive force. Optimal rates of dextransucrase transport require Δp to be greater than -90 mV. Above this Δp value there is no appreciable increase in dextransucrase secretion, i.e. there is a saturation effect of the dextransucrase secretion system by Δp . However, at pH values above 7.0, the enzyme levels in the fermentation broth had dropped considerably, although Δp was greater than -90 mV - at pH 7.5, $\Delta p = -112$ mV. It is known that dextransucrase is severely inactivated at a pH of 8.0, and at a pH of 7.5, extracellular enzyme levels were depressed by greater than 90% as compared to optimum levels attained at pH 6.7 - 7.0. The proton gradient had become inverted, i.e. the cell interior was acidic with respect to the exterior - compared again to the situation below pH 7.0. The addition of nigericin to the fermentation at a pH of 7.5 had the effect of partially dissipating the inverted pH gradient, resulting in a four-fold increase in dextransucrase secretion. The internal cell pH remains relatively constant at pH 7.0 when the external medium pH is varied from 6.7 to 7.5, effectively eliminating internal pH as a control factor in dextransucrase secretion. So, dextransucrase is dependent on the presence of a proton gradient, where the interior is alkaline with respect to the exterior.

Leuconostoc mesenteroides maintains a level of proton motive force that is typical for organisms that utilize a strictly fermentative metabolism, e.g. *Clostridium thermoaceticum*. The contribution of the membrane potential and

the pH gradient to the proton motive force varies according to the external pH. Usually, as the external pH decreases, the membrane potential also decreases, which is accompanied by an increase in pH gradient to maintain the proton motive force. This interconversion is thought to prevent the large build-up of charge across the membrane caused by the expulsion of protons from the cell. If the charge ratio generated across the membrane is too large, then the secretion of protons from the cell would not be thermodynamically feasible. In many bacteria, the decrease in membrane potential is usually accomplished through the uptake of cations, particularly K^+ (from K_2HPO_4 or KH_2PO_4). *Leuconostoc mesenteroides* probably uses cation transport to buffer Δp . An increase in the K^+ concentration of the medium decreased $\Delta\psi$ with a corresponding increase in $-\Delta pH$. It would seem that *Leuconostoc mesenteroides* utilises K^+ as the buffering cation for Δp .

The production of dextransucrase is not necessary for sucrose metabolism by *Leuconostoc mesenteroides*. Although dextransucrase secretion is severely inhibited at a pH of 8.0, growth of the organism in sucrose medium is not greatly affected. It is probable that it relies on sucrose phosphorylase production for sucrose utilization at high pH values (see Section 2.3.1). Although proton motive force is vital for efficient protein secretion in bacteria, it is not a requirement for cell growth - and this is also the case with *Leuconostoc mesenteroides*.

2.4. Purification of Dextransucrase.

At the conclusion of a dextransucrase fermentation, the broth contains not only the enzyme but several impurities including cells, unused nutrients, salts, proteins and other byproducts such as fructose, glucose, mannitol and dextran. It is necessary to remove these impurities, but this process is always accompanied by a loss of enzyme activity.

The first stage in dextransucrase purification is the removal of the used cells, a relatively simple process as the desired enzyme is not located within the cells. This step is usually carried out via either batch or continuous centrifugation, although high centrifugal forces can lead to a loss in activity at 4°C and 15 minutes centrifuging time (28, 29).

Following this step, other purification stages can take place including precipitation, ultrafiltration and gel permeation chromatography. Hehre

(1946) ⁽³⁰⁾ was one of the first to purify dextransucrase by adding ammonium sulphate and then washing the precipitate three times with half saturated ammonium sulphate in 0.1% acetic acid, followed by enzyme extraction at a pH of 6.3 with citrate buffer, and precipitation in alcohol. Braswell and Stern (1959) ⁽³¹⁾ purified the enzyme by precipitation at 1°C in 18% ethanol; separating this precipitate by centrifugation and suspending it in acetate buffer at pH 5.0, then allowing it to stand overnight at 1°C, after which it was again centrifuged and the precipitate dissolved in a phosphate buffer at pH 7.0. This solution was centrifuged and the sediment discarded. The pH of the supernatant was dropped to 5.0 with HCl, rendering the enzyme insoluble. Finally, it was centrifuged and resuspended in a smaller volume of pH 5.0 acetate buffer. This lengthy process resulted in a 900-fold purification of the dextransucrase. By 1962, these workers, in collaboration with Goodman ⁽³²⁾, had modified their process and, after cell removal by centrifugation, with subsequent chilling of the solution at 0°C, retaining all the enzyme activity, they added cold ethanol, dropwise, to the cold centrifugate, until a concentration of 33% precipitated out the enzyme along with any dextran present. They centrifuged this solution at 0°C, separating 90% of the active enzyme into the precipitated phase. Subsequently, the alcohol concentration was brought to 38% to ensure quantitative separation of the enzyme. The precipitated enzyme was redissolved in 0.1 M acetate buffer at pH 5.0 and then adsorbed on a calcium phosphate gel, removing all the activity from the solution. The gel was then centrifuged and the supernatant discarded; followed by washing with distilled water until all carbohydrates had been removed. The gel was then stirred at 0°C with a 0.5M solution of NaH₂PO₄, which extracted the enzyme quantitatively. The active eluent was dialysed against distilled water and lyophilised, with the fluffy enzyme powder containing undetectable amounts of dextran. However, this purification method was again long and expensive, and it became necessary to develop cheaper and simpler processes to retain maximum activity.

Lawford *et al* (1979) ⁽¹⁷⁾ precipitated out the enzyme with the addition of ammonium sulphate to the cell-free culture fluid, and then desalted the crude enzyme by gel-filtration with Sephadex G-25, before applying it to a column of DEAE - cellulose equilibrated with 50 mM potassium acetate buffer, pH.5.5, containing 0.2 mM CaCl₂. This process allowed 90% of the active enzyme to bind to the ion exchanger, with the enzyme being eluted with buffer containing 250 mM NaCl.

Robyt and Walseth (1979) ⁽³³⁾ devised a method that was subsequently modified by Kaboli and Reilly (1980) ⁽³⁴⁾, whereby the enzyme was first concentrated by ultrafiltration, and then purified by GPC on Ultrogel AcA 34, with total yields of up to 54.2% being reported. Monsan and Lopez (1981) ⁽²⁵⁾ avoided the concentration stage by applying 250 cm³ of the centrifuged broth directly on a 100 x 4 cm³ GPC column containing 1 l of Ultrogel AcA 34, using 0.01M pH 5.0 citrate buffer as elution agent. They also found that the addition of 0.05 M CaCl₂ to the purified enzyme resulted in yields of up to 96.4%.

Paul and co-workers (1983, 1985) ^(28,29) purified the centrifuged enzyme fluid by aqueous two-phase partition between the dextran present in the solution and polyethyleneglycol ($M_w = 1500$). The enzyme was obtained in the dextran-rich phase, with successive phase partition steps increasing the final yield to 95%.

Schneider (1979) ⁽¹²⁾, during his work for Fisons at the Battelle Research Centre, carried out a great deal of research into enzyme recovery procedures. He found that the addition of up to 2 g/l of sucrose to the centrifuged enzyme resulted in yields of up to 80% following enzyme precipitation with the addition of 30% alcohol (95% ethanol, 5% methanol). He proposed an enzyme recovery scheme whereby, following centrifugation, 4 g/l sucrose was added to the broth, after which the enzyme was precipitated out with a diluted alcohol solution at temperatures below 8°C. The enzyme was then recovered by centrifugation at 0°C, with the wet enzyme precipitate being stored in a frozen state (-18°C) as a 5 - 10% solution in 0.05 M acetate buffer at pH 5.2.

Miller *et al* (1985) ⁽³⁵⁾ added lyophilised dextranase to the centrifuged culture fluid, and then dialysed it overnight against 0.02 M sodium acetate (pH 5.2), 0.05M NaCl. The dialysate was then applied to a DEAE-cellulose column and equilibrated, followed by washing with the same buffer. The active fractions thus obtained were passed to a Sephadex G-200 column, which was washed with 0.02M imidazole-HCl (pH 6.7), 0.4M NaCl, then with 0.05M imidazole-HCl (pH 6.7) without NaCl, and finally eluted with the same buffer containing 3M urea. This final eluent was then applied to a column of DEAE-Trisacryl M equilibrated with the same urea buffer, and then eluted

with 1M NaCl, while maintaining 3M urea in the eluent. Purified dextransucrase was obtained after dialysis against 0.02M imidazole-HCl (pH 6.7), 0.2M NaCl, to remove the urea.

More recently Otts and Day (1988) (27) purified dextransucrase using an aqueous two-phase partition technique. They mixed the culture supernatant with a 10% dextran T-500 solution, and then added 20% polyethylene glycol before centrifugation. The lower, dextran-rich phase was then diluted with distilled water, and centrifuged after a further addition of polyethylene glycol. This procedure was repeated twice, and the final dextran phase was then diluted with 0.1 M sodium acetate buffer, to pH 5.0. This procedure recovered dextransucrase with a yield of 95% - with a 1,500-fold purification of the culture supernatant.

2.5. Immobilization of Dextransucrase.

Very little work has been carried out to date on the immobilization of dextransucrase, with Kaboli and Reilly (1980) (34) using various supports such as Whatman No. 1 Filter Papers, certain Enzacryl products, DEAE-cellulose, DEAE-Sephadex A.25 and A.50, and a range of SP-Sephadex - although they did not manage to obtain yields greater than 10% with any of these products. They managed to obtain yields of 20% when they bound the enzyme to alkylamine silica and porous Spheron beads. However, Monsan and Lopez (1981) (25) managed to improve the situation somewhat when they immobilized the enzyme onto an amino-spherosil support activated with glutaraldehyde. They found that the specific surface area of the silica was one of the most important factors for dextransucrase immobilization; the lower the specific surface area, the higher the coupling efficiency. The pore sizes of the support also had to be wide enough to allow not only enzyme fixation, but also the diffusion of dextran and sucrose. The higher the specific surface area, the lower the pore size, so that the best results were obtained when immobilizing dextransucrase onto a 6 m²/g silica, with a pore size of 550 nm. They found that as a result of immobilization, the average molecular weight of the dextrans produced seemed to increase. However, in the presence of acceptors such as maltose, lower molecular weight dextrans were formed, suitable for clinical dextran use. The molecular weight distribution of the dextran produced in the presence of an acceptor was less disperse with immobilized dextransucrase than when clinical dextran was produced by acid hydrolysis of high molecular-weight dextran.

More recently, in a series of papers by El-Sayed and co-workers (1990) (36-38), *Leuconostoc mesenteroides* cells were immobilized on a number of supports and used to produce dextransucrase and dextran. The culture type used during these experiments was *Leuconostoc mesenteroides* ATCC 10830, which is identical to culture type NRRL B512 (F) as used during this research work. During fed-batch fermentations, the cells were immobilized on three types of celite for enzyme, followed by dextran, production. As with previous workers, they found that enzyme formation was greatly affected by the pore size of the support, along with particle size. They found that immobilizing the culture on Celite R648, which has an average particle radius of 200 μm and pore size of 0.14 μm , produced the highest total enzyme activity of all the celite types used. They also claimed that a culture of free cells, in standard fed-batch fermentation mode, produced about 18% more enzyme activity than immobilized cells in calcium aginate beads, but about 64% less than immobilized cells on Celite R630. The dextran yields from all the immobilized cultures prepared were, in all cases, higher than those obtained from free cultures under similar conditions. During further studies concerning *Leuconostoc mesenteroides* immobilization in calcium aginate beads, they found that the cells produced only 93% of the activity obtained from the cells in a bubble column, with the immobilized cells utilizing sucrose at a lower rate than the free cells. They also claimed that an immobilized cell culture in an airlift bioreactor produced considerably more soluble enzyme than in a bubble column and in a greatly reduced time, with a faster sucrose utilization rate. A continuous feed of 5 g/l of sucrose to the immobilized cells in the airlift bioreactor increased enzyme production by about 107% compared to the conventional batchwise operation of the reactor. The maximum enzyme activity achieved using this method was approximately 51.9 DSU/cm³ (2.5 U/cm³). The order of decreasing enzyme productivity in the various reactors tested was fed-batch immobilized-cell bioreactor > batch immobilized-cell bioreactor > batch bubble column with free cells > batch bubble column with immobilized cells, with the order holding for both soluble enzyme activity and intact enzyme activity. The advantages of immobilized cells over free cells may be related to the effects of reduced shear rate and more controlled metabolism in the former case. The advantage of the airlift bioreactor over the bubble column is most likely due to superior pH control in the former, with the gentler bubbling in the airlift bioreactor obviously having some effect - particularly as the work carried out by Ajongwen-Numfor (11) claimed optimum enzyme production under conditions of no

aeration. These workers then carried out a series of experiments on enzyme production followed by dextran formation from immobilized cell cultures with a semi-continuous sucrose feed. The enzyme was produced in three sequential cycles of semi-continuous fed-batch operation, with each cycle consisting of an enzyme production period of 24 hours, followed by a dextran production segment, also of 24 hours, with both sections involving fed-batch operation. The free cells were only used in one cycle and, hence, had a total reaction time of only 48 hours. An increase in sucrose feed rate from 5 to 10 g/l led to increases of the total enzymic activity by about 88% with immobilized cells and about 100% with free cells, although at the higher rate the fermentation medium eventually became quite viscous due to the formation of dextran (about 15 - 25 g/l). During dextransucrase fed-batch semi-continuous fermentations total enzyme activity produced by immobilized cells were 1.35 and 1.56 times greater than those produced by free cells with sucrose feed rates of 10 and 5 g/l respectively. It should be noted again, though, that the immobilized cell process was operated for a time period three times longer in duration than the free cell process. Generally, El-Sayed *et al* found that immobilized cells produced more dextransucrase and dextran than did free cells under similar process conditions, although the period of operation was significantly longer. A major advantage of immobilized cells over free cells is that they can produce the enzyme and the final product with reduced contamination of the said product by the cells and, hence, reduced downstream processing costs. Immobilized cells produced a maximum concentration of soluble enzyme of about 235.5 DSU/cm³ (11.34 U/cm³) of working volume (medium plus beads) during the three cycles of operation, compared with only 119.2 DSU/cm³ (5.74 IU/cm³) in the case of free cells over a single cycle. It should be noted here that the fermentation medium used during these experiments consisted of 20 g/l yeast extract ; 20 g/l sucrose ; 2 g/l KH₂PO₄ ; 2 g/l Tween 80 ; 2.5 g/l CaCl₂.2H₂O ; 0.2 g/l MgSO₄.7H₂O ; 0.01 g/l NaCl ; 0.01 g/l FeSO₄.7H₂O ; 0.01 g/l MnSO₄.H₂O and 1 cm³ silicone emulsion antifoam, along with aeration of 0.5 vvm - conditions not designed to produce maximum enzyme yields compared to those obtained during the course of this project. According to El-Sayed *et al*, dextran production was also considerably higher with immobilized cells compared to free cells, even taking into account the much longer time period required with the immobilized cells.

This work is the most extensive study on dextransucrase production using immobilized cells and although the overall yields are poor, they could well be increased with the medium composition used by Ajongwen-Numfor (11) and during this work.

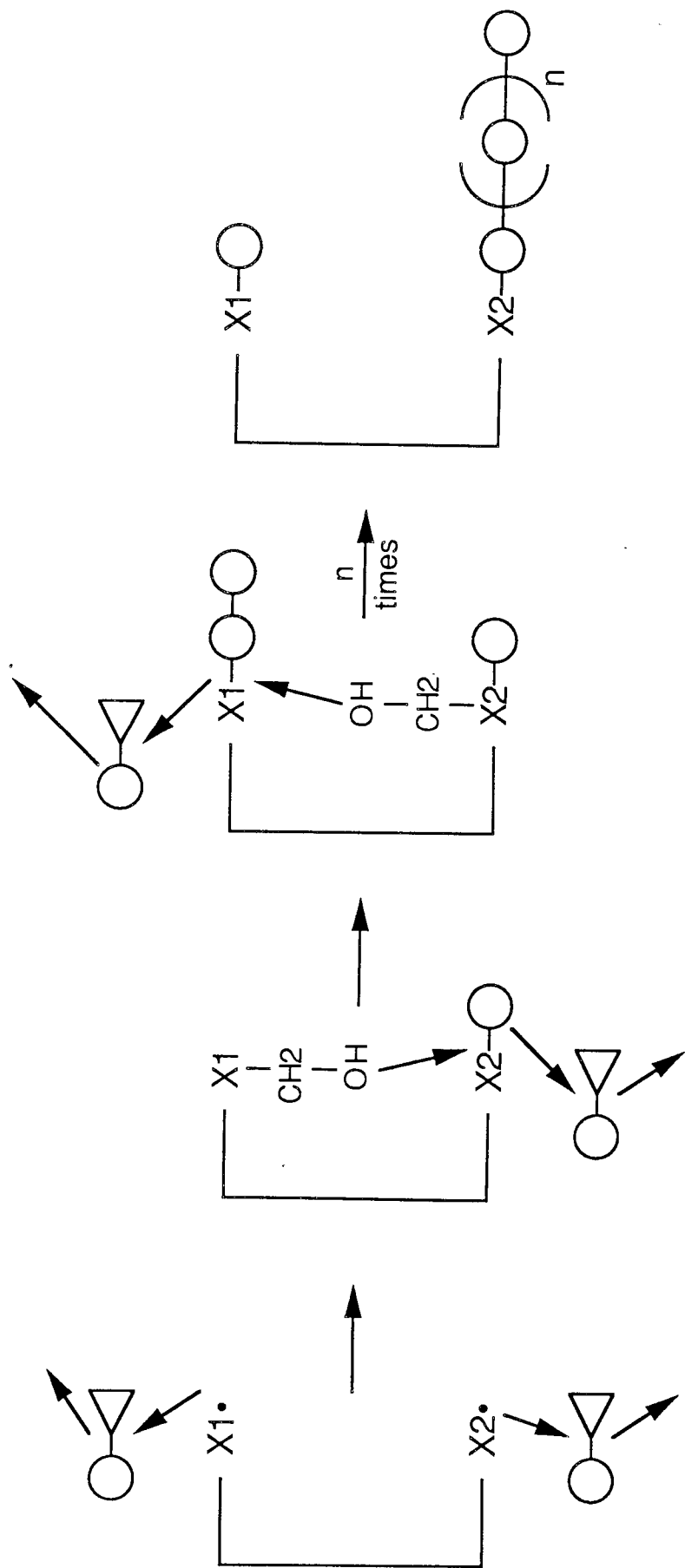
2.6. Reaction Mechanisms of Dextransucrase.

Several workers have carried out investigations into the mechanism of formation of the dextran molecule and the reasons for the presence of branches. Stacey (1943) (39) observed that the enzyme remains in combination with the polysaccharide during synthesis, a view supported by Hehre (1951) (40). Jeanes *et al* (1953) (41) reported that dextrans produced from the B-512 and B-512 (F) strains of *Leuconostoc mesenteroides* contained 95% 1,6-linkages and 5% 1,3-linkages, and that these branches occurred at random, at an average of every 23 glucose units. Bovey (1959) (42) in the first of three papers on dextran formation, initially believed that branching was caused by a so-called "branching enzyme" that was more heat stable than the main dextransucrase enzyme. Tsuchiya *et al* (1953/5) (43,44) showed that the addition of certain glucosyl acceptors, such as isomaltose, maltose, fructose and low molecular weight dextrans, to the dextran-synthesizing system caused a reduction in the amount of high molecular weight dextran being produced, along with an increase of very low molecular weight dextran. In the third paper by Bovey (1959) (45), a kinetic scheme was proposed which claimed that in the absence of an acceptor, dextran molecules were built up by a single chain process, in which one enzyme molecule presided over the transfer of $10^5 - 10^6$ glucosyl units from sucrose to a growing polymer chain, with the chain supposedly being terminated by the dissociation of the enzyme. It was thought that the enzymatic chain reaction was initiated very inefficiently by sucrose molecules playing the role of primer, whereas the propagation of the reaction was very efficient as the enzyme molecule remained in contact with the polymer chain, with the activation energy required for glucosyl transfer being retained after each step and being used in the following step. By this time, Bovey (46) had ceased to believe in the presence of a branching enzyme, thinking it likely that the polymerizing enzyme carried out the branching by glucosyl transfer from sucrose to the 3-position of the glucose units of the polymer chain. Larm *et al* (1970) (47) claimed that 40% of the B-512 dextran side chains contained only one glucose unit, at least 45% were two glucose units long, and the rest were longer than two such units. Bourne *et al* (1966) (48) claimed that essentially;

all the side chains in dextrans NRRL B-1375, B-1415 and B-1416 consisted of single glucose units.

Robyt, Kimble and Walseth (1974) (49) found that dextran was biosynthesized by the transfer of glucose from sucrose to the reducing end of the growing dextran chains. They proposed that dextransucrase forms an enzymatically active covalent complex with glucose and dextran and that the glucose is inserted between the enzyme and the dextran by a nucleophilic attack of the C₆-OH of glucose onto the C₁ of the dextran, forming an α -1,6 glucosidic bond. This releases one of the nucleophilic groups at the active site of the enzyme, which then attacks sucrose to give an enzyme-glucosyl complex. The C₆-OH of this glucose then repeats the process of attacking the C₁ of dextran. Dextran is built up by extrusion from the enzyme when glucose units are transferred from sucrose to the active site and inserted between the enzyme and the reducing end of the dextran polymer. Figure 2.4 shows a schematic representation of this mechanism. Robyt and Taniguchi (1975) (50) also explained the formation of branches in dextran (see Figure 2.5) by claiming that the C₃-hydroxyl group of an acceptor dextran molecule makes a nucleophilic attack onto the C₁ of either the glucosyl- or dextransyl-enzyme complex to form an α -1,3 branch linkage which releases either the glucose or dextran from the enzyme. Acceptor dextran is produced when the dextran attached to the enzyme is released by low molecular weight acceptor molecules such as glucose or fructose.

Robyt and Walseth (1977) (51) proposed the mechanism of acceptor reactions of *Leuconostoc mesenteroides* B-512F dextransucrase. They claimed that acceptor molecules act as nucleophiles in which a hydroxyl



X1 and X2 represent nucleotides at the active site; represents sucrose;
 is glucose; is fructose; indicates glucose residues joined by
 α -1,6-glucosidic linkages

Figure 2.4

Mechanism proposed for the synthesis of dextran by *Leuconostoc mesenteroides* B-512F dextransucrase

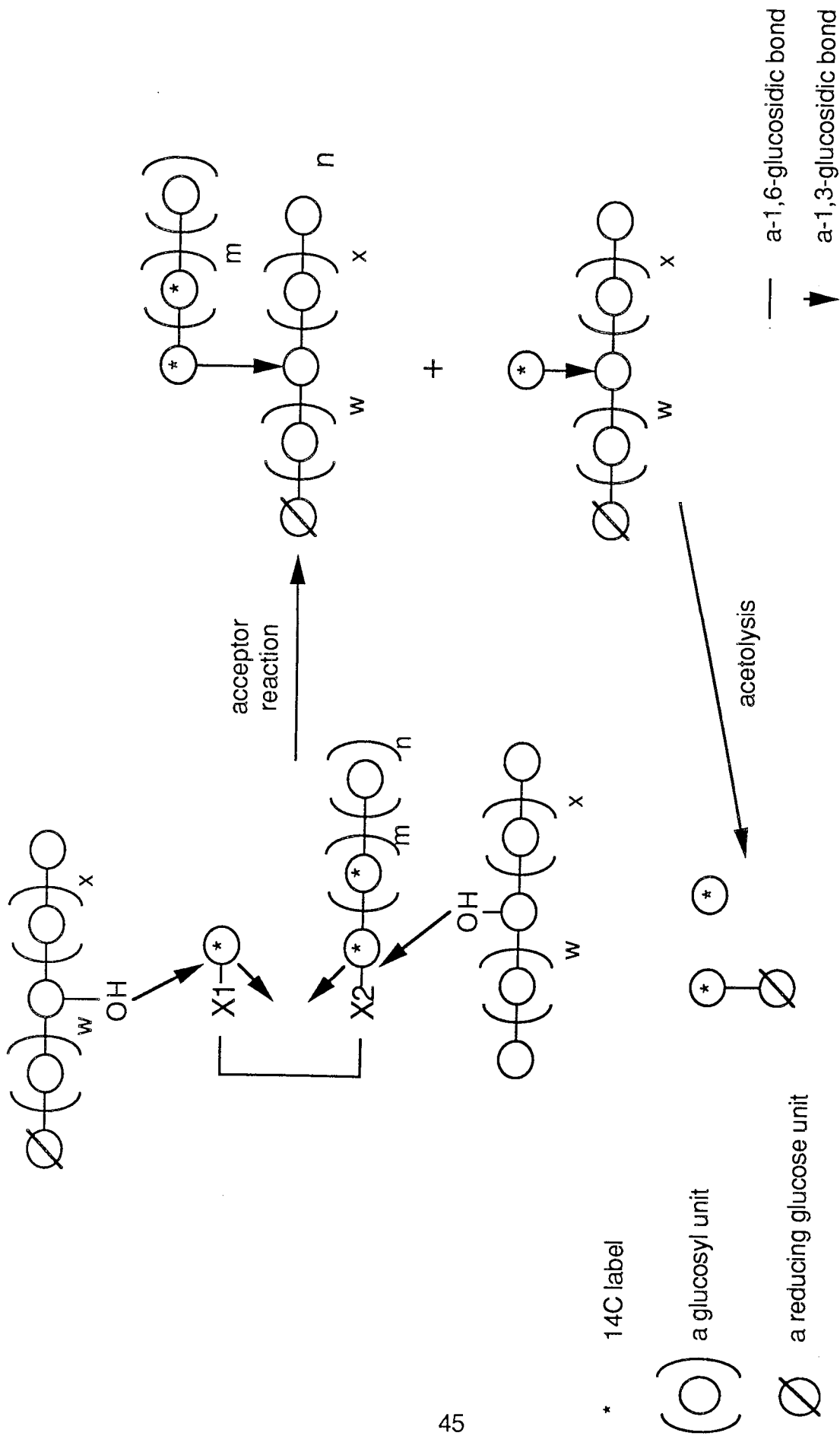
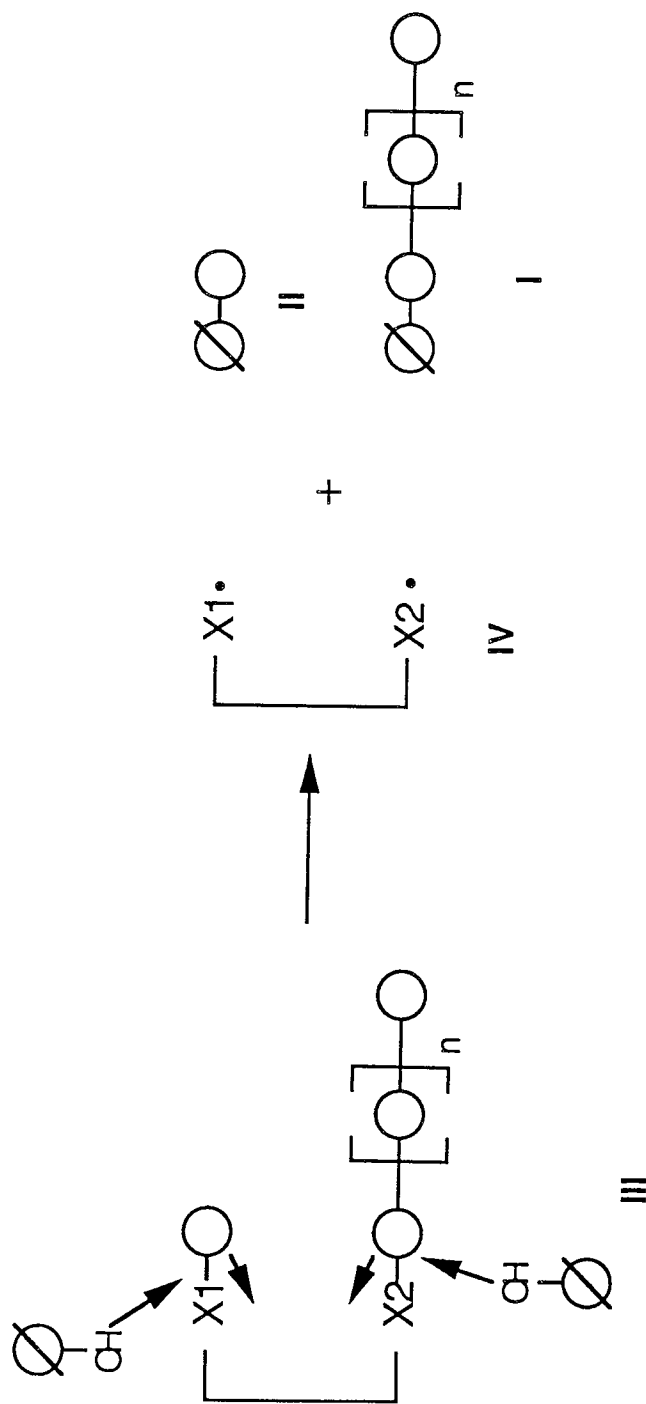


Figure 2.5

Mechanism of Branch Formation in Dextran



X1 and X2 represent nucleophilic groups at the active site of the enzyme; \bigcirc represents two glucose residue units linked by an α -1,6-D-glucosidic bond; \bigcirc -OH denotes a ^{14}C -labelled acceptor. I is a Fraction I, reducing-end labelled dextran acceptor-product; II is a Fraction II reducing-end labelled oligosaccharide acceptor-product; III is a labelled acceptor displacing glucosyl and dextranosyl groups from the charged enzyme; and IV is the enzyme.

Figure 2.6

The Mechanism for the Acceptor Reaction with Charged Dextrantransucrase in the absence of sucrose

group (C₆-OH for D-glucose and maltose, and C₅-OH for D-fructose) attacks the C₁ of the glucosyl or dextranosyl groups in the enzyme complex. Fraction I products (dextrans) are obtained by the displacement of the dextranosyl group, and Fraction II products (oligosaccharides) are obtained by the displacement of the D-glucosyl group (see Figure 2.6). This acceptor reaction provides a mechanism for the termination of the dextran-chain polymerization and, by the release of dextran from the active site of the enzyme, provides a mechanism for the biosynthesis of new dextran chains. Robyt and Eklund (1982) (52) used models to create a three-dimensional picture of dextran synthesis. They believed that the glucosyl-enzyme intermediate and the growing dextranosyl chain could be stereochemically orientated to give the formation of the α -1,6 bond as required by the proposed mechanism, and that the growing dextran chain could be transferred back and forth between the two active sites of the enzyme without excessive movement of the whole chain. They also claimed that the nucleophiles (active groups) of dextranase were possibly phosphate or pyrophosphate groups, as they would give covalent intermediates that are relatively stable and yet have sufficient energy for the synthesis of the α -1,6 linkage.

2.7. Dextran.

2.7.1. Introduction.

Dextran is the name given to a family of α -1,6 glucans synthesized from sucrose by the enzyme dextranase. They have a general molecular formula (C₆H₁₀O₅)_n. The main properties of dextrans are their viscous and near-Newtonian behaviour and the fact that they contain no charged groups.

Dextran has many uses in various industrial and medical applications, one of the greatest being in the latter field, where partially hydrolyzed dextrans have been used as blood plasma substitutes in the treatment of shock (required molecular weight in the range of 50,000 - 100,000 daltons). It is currently being manufactured by the pharmaceutical industry as a blood volume expander (molecular weight 70,000 daltons) or as a blood flow improver (molecular weight 40,000 daltons) and it has been used as an iron-complex to produce iron-dextran, which is used to treat iron deficiency and anaemia in humans and animals (53). Other uses include those of secondary recovery of petroleum, and as protective coatings for seeds, deflocculants in paper products, and in the food industry, for stabilizing and imparting viscosity to

syrops. Dextran is also increasingly being used for the preparation of separation membranes (e.g. Sephadex). Neely (1960) ⁽¹⁾ includes a list of patents issued on dextran in his paper on Dextran : Structure and Synthesis.

2.7.2. Production of Dextran.

Dextran can be produced in two ways. The first method is known as the whole cell fermentation method and is the one currently in use in industry. The second technique - the cell free fermentation method - involves the production of dextransucrase by fermentation and the subsequent transfer of the enzyme to a reaction vessel containing a sucrose medium where the biosynthesis of dextran takes place.

2.7.2.1. Conventional Fermentation of Dextran.

The traditional method for producing dextran has been to grow the required organism (eg. *Leuconostoc mesenteroides*) in medium containing high concentrations of sucrose along with other required nutrients, salts, vitamins and minerals. This medium is inoculated with cells at a neutral pH, and the fermentation takes place, without any subsequent pH control, to completion. Three stages take place during such a fermentation with the growth phase of the organism, the enzyme producing phase and the enzyme/sucrose reaction phase. The initial pH of approximately 7.0 is the optimum for cell growth; as it drops to around 6.0 to 6.9, it favours dextransucrase production; and at pH values down to 5.2, there is optimum dextran synthesis. During an industrial scale dextran fermentation, such as those carried out by Fisons plc Pharmaceutical Division, the medium used contains 17% sucrose and 90% of this sucrose is converted to dextran over a period of some 20 hours. The dextran is then processed to produce native dextran as described in 2.7.3.

2.7.2.2. Enzymatic Synthesis of Dextran.

A great deal of work has been carried out on the production of dextran from a reaction involving cell free dextransucrase and sucrose. Section 2.2 has discussed in detail a great deal of the work that has been carried out to optimize dextransucrase production. Schneider (1978) ⁽¹⁰⁾ working on behalf of Fisons plc, produced dextran using this cell free method, by adding 15 cm³ of purified enzyme, with an activity of 554 DSU/cm³ (26.67 U/cm³), i.e. a total of 8,310 DSU (400.10 U) of enzyme, to 2,100 cm³ of a 10% sucrose solution in 0.05 M acetate buffer, at a pH of 5.2 and temperature of 25°C. The final dextran yield of this reaction was 88% and when they compared this result to

the dextran yield obtained from a conventional process, they obtained the results seen in Table 2.1.

Table 2.1.
Comparison Between Conventional and Enzymatic Processes for Dextran
Production as Carried out by Schneider (1978).

Parameter	Conventional Process	Enzymatic Process
Total Sucrose Used	850 g	34g for enz. prod. + 816 g for dext. prod. <u>850g total sucrose</u>
Yeast Extract	7.5 g	17.1g
Volumes	5 l	0.85 l for enz. prod. 8.16 l for dext. prod. <u>9.01 l total volume</u>
Duration	87 hrs	18 hrs. for enz. prod. 35 hrs for dext. prod. <u>56 hrs total duration</u>
Dextran Obtained	265.8 g	340.1 g.
Yield	66%	84.6% (88% for dext. prod. alone).

Nett increase in dextran production by the enzymatic route + 28%.

Schneider also found (1979) (12) that the dextran produced enzymatically was identical - from a structural point of view - to the dextran currently being produced by Fisons plc. He found the yields obtained to be significantly higher, reaching 93% with 8% sucrose, or 87-88% with 10% sucrose, indicating that the enzymatic route is a much more efficient means of obtaining high quality dextran.

Landon and Webb (1990) ⁽¹⁶⁾ also recommended the enzymatic route for the production of dextran, and attempted to scale up the process. They determined that, to maintain current dextran productivity (as obtained by Fisons), a dextransucrase activity of around 5 DSU/cm³ (0.24 U/cm³) would be required in the main 10,000 litre fermenter (located at Holmes Chapel - the Fisons site), which corresponded to an activity of 50 DSU/cm³ (2.41 U/cm³) to be obtained in the 1,000 litre seed vessel at Holmes Chapel. In the laboratory they obtained an enzyme activity of 142 DSU/cm³, but on scale-up they only achieved 45 DSU/cm³, not quite sufficient for adequate dextran synthesis. However, they did not allow any mixing during their enzyme production step and they believed that improved agitation conditions would lead to increased enzyme activity and hence make the cell-free production of dextran a much more viable proposition.

2.7.3. Manufacture of Commercial Dextran.

During the Industrial Production of Dextran, as described in Section 2.7.2, the polymer is further processed to produce a final product known as "Native Dextran". Following the fermentation stage, the dextran is then purified by a series of alcohol precipitation, acid hydrolysis and spray drying techniques ⁽⁵³⁾. These downstream processes obviously contribute a great deal to the cost of manufacturing dextran and so the production process should be optimized so as to minimize these costs. The alcohol precipitation stage is quite a critical one, as is shown in Figure 2.7⁽⁵³⁾, whereby the percentage yield of dextran precipitated with ethanol varies with varying sucrose strengths. The yield can be divided into high and low molecular weight dextran, depending on the alcohol strength at which it is precipitated. It is known that an optimum sugar concentration for maximum weight yield of high molecular weight dextran in a given fermentation vessel is 17.9% (see Figure 2.8)⁽⁵³⁾, although if yield is translated onto a direct operating cost basis for a given plant (see Figure 2.9) a different sugar concentration optimum of 12.5% is obtained, and this, along with other factors, must be taken into account when deciding upon optima for dextran fermentations.

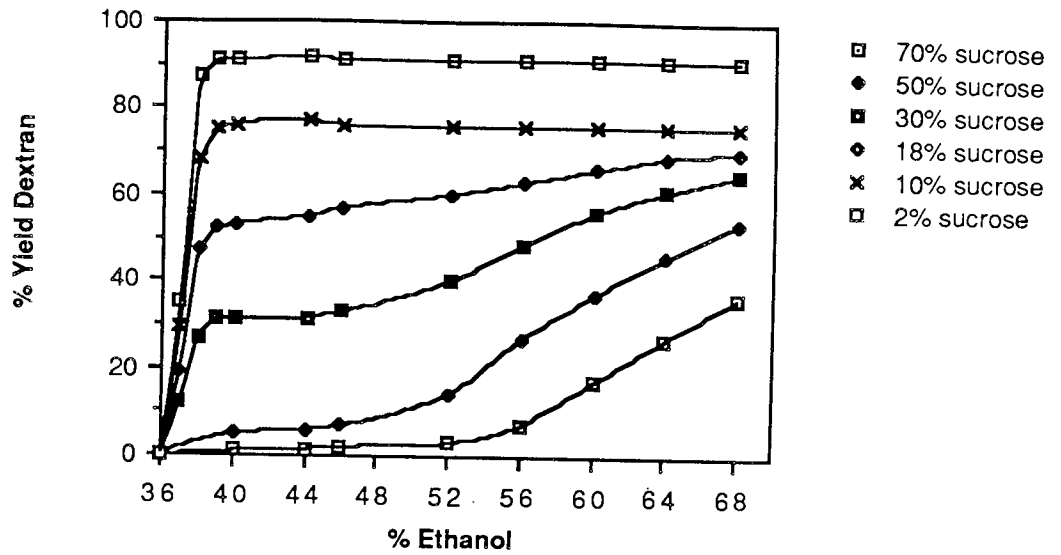


Figure 2.7: Dextran Yield with Varying Sucrose and Ethanol Levels

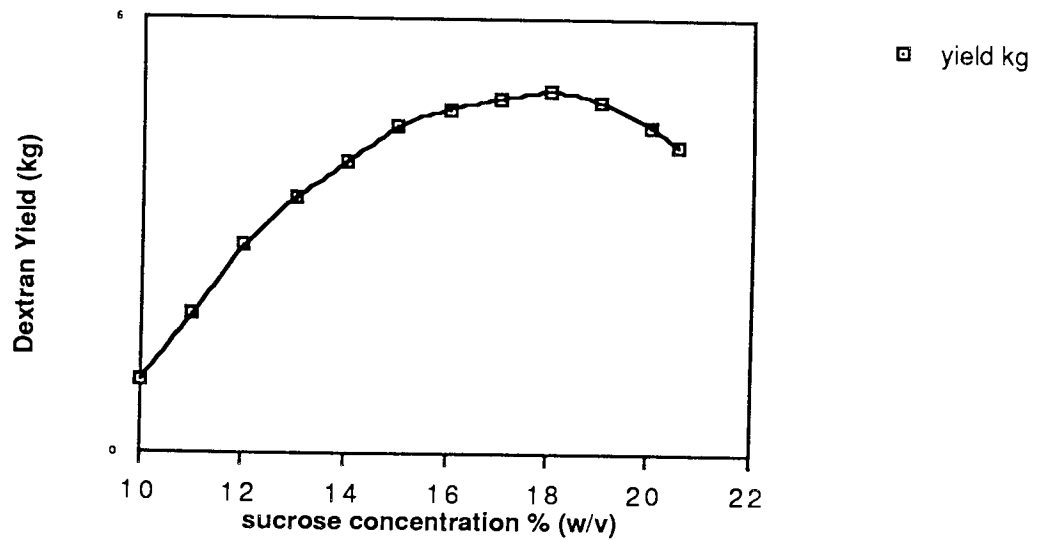


Figure 2.8: Dextran Yield as a function of Sucrose Concentration

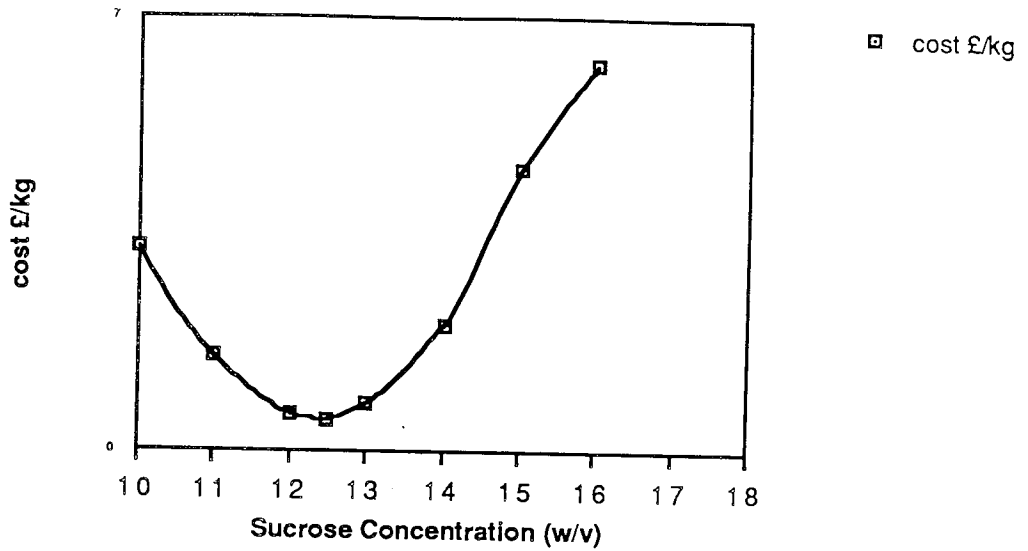


Figure 2.9: High Molecular Weight Dextran Costs

Following the spray-drying stage of the native dextran process, the final product passes to a final unit where it is prepared into final pharmaceutical dosage forms - completing the commercial production of dextran.

3. FERMENTATION THEORY.

3.1. Introduction.

The term "fermentation" can be defined in two ways. The original definition (54) applies to the metabolism of microbial cells, and concerns the degradation of organic substances by organisms to provide chemical energy in the form of ATP in reactions that do not require molecular oxygen. In anaerobic cells, this is the only energy producing process. However, more recently, another definition of "fermentation" has come to the fore (55), as a description of processes in which cells or other microorganisms are cultured in a container (bioreactor or fermenter) in liquid or solid medium for experimental or commercial purposes. Originally, this term was applied only to anaerobic cultures, in an attempt to be consistent with the first definition, but has since become a general description of microorganisms or cells being cultured both anaerobically and aerobically.

Fermentation processes are becoming increasingly common in industry due to the relative simplicity of the reaction compared to a similar chemical process (56). Fermentations are reasonably cheap to carry out as they take place at or around body temperature and usually at atmospheric pressures. A fermentation vessel needs to be designed such that it will be able to withstand the harsher conditions of the sterilization cycle (57), but as a temperature of 121°C and a pressure of 2.5 bar for about half an hour is the most extreme situation it will experience, this is not too much of a problem. A fermentation can also be designed to produce a very specific product, which is not always the case with a chemical process. However, complete fermentations are not always cheap to carry out, as the various downstream processes involved are quite expensive and there is a great deal of uneconomic "down-time" as most fermentations are batch processes and so are only running for a percentage of the available time.

A fermentation process can usually be divided into six basic component parts, each of which needs to be correctly carried out in order to ensure a successful fermentation.

(i) The formulation of the medium to be used in culturing the process organism during the development of the inoculum and in the production vessel.

- (ii) The sterilization of the medium, fermenters and ancillary equipment.
- (iii) The production of an active, pure culture in sufficient quantity to inoculate the production vessel.
- (iv) The growth of the organism in the production fermenter under optimum conditions for product formation.
- (v) The extraction of the product and its purification.
- (vi) The disposal of effluents produced by the process.

This complete process, which can be shown diagrammatically in Figure 3.1, will be discussed briefly, in the individual sections, during the course of this chapter.

3.2. Preparation of Fermentation Media.

The culture medium used during various aspects of the process (including inoculum propagation stages, as well as the fermentation itself) has to be designed to meet both the requirements of the microorganism and the economical needs of the industrial process when considering the cost of the various downstream processes involved. The media components chosen must be capable of producing the maximum yield of biomass and/or product per gram of substrate used at the maximum rate of formation. All components must be cheap, readily available at all times and of a consistent quality and must allow the minimum yield of undesired products, which should minimize extraction and purification processes hence reducing the overall cost of the fermentation.

The basic cell requirements from a fermentation medium are shown in Equation 3.1.

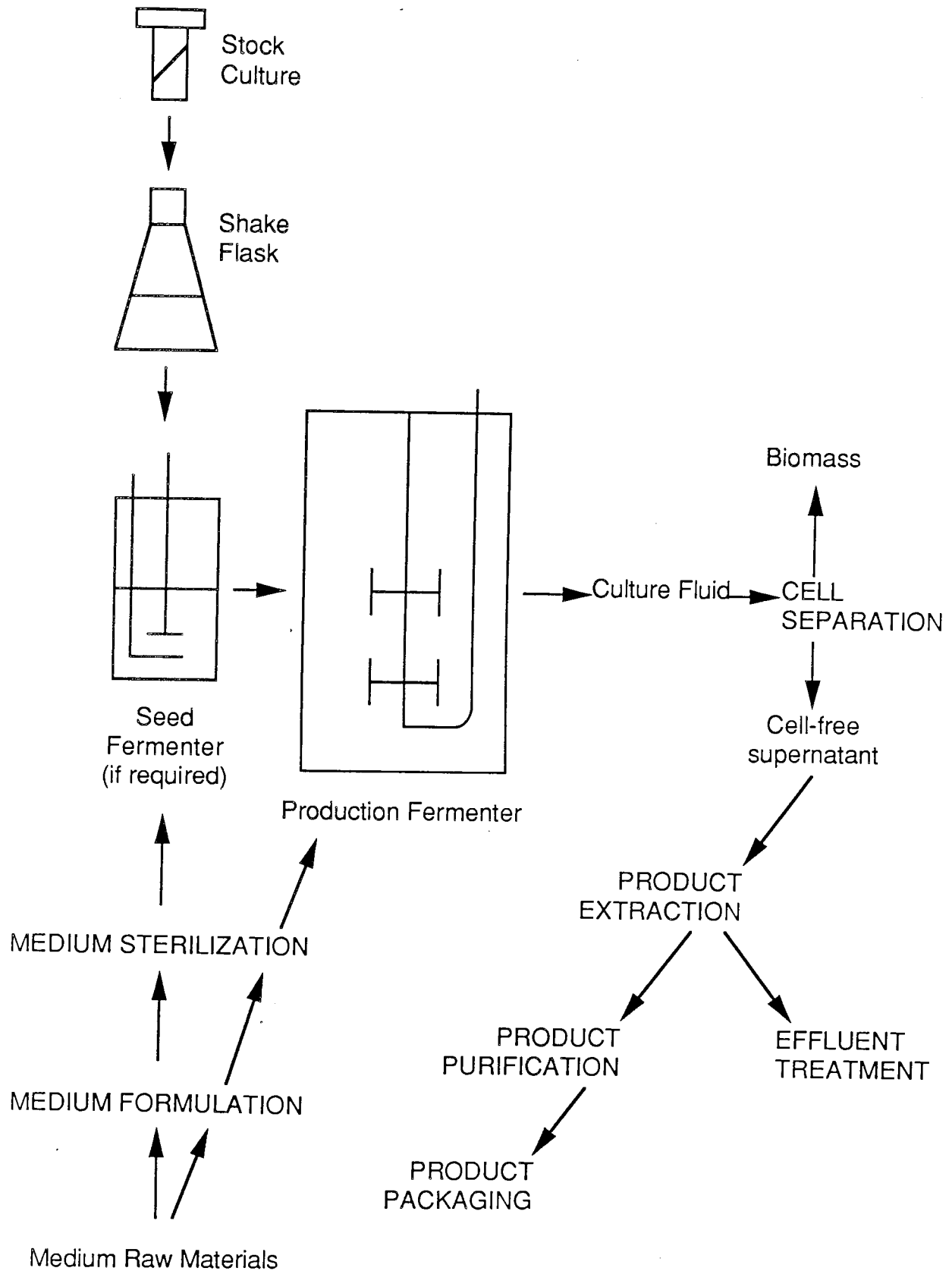
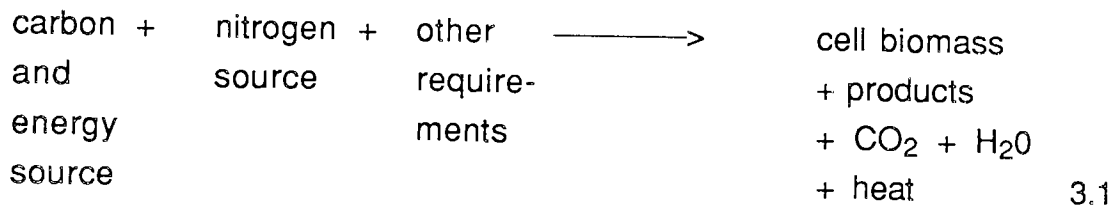


Figure 3.1

**A Generalized Schematic Representation
of a Typical Fermentation Process**



It is necessary that all the individual elements that provide the composition of the required microorganism are included in the fermentation medium. Herbert (1976) (58) gave the percentage dry weight elemental composition of various types of microorganisms, with that of bacteria shown in Table 3.1.

Table 3.1.
Elemental Composition of Bacteria (% by dry weight).

Carbon	50 - 53
Hydrogen	7.0
Nitrogen	12 - 15
Phosphorus	2.0 - 3.0
Sulphur	0.2 - 1.0
Potassium	1.0 - 4.5
Sodium	0.5 - 1.0
Calcium	0.01 - 1.1
Magnesium	0.1 - 0.5
Chloride	0.5
Iron	0.02 - 0.2

The energy for the growth of the microorganism usually comes from the media carbon source and is commonly a carbohydrate. The carbon source is often used as the substrate for the product formed during the course of the fermentation. Sucrose can be obtained from both sugar cane and sugar beet and is a particularly common carbon source in fermentations, being cheap, plentiful and easy to obtain. It can be used in its refined form, or in a very impure form as beet or cane molasses, which are the residues left after the crystallization of sugar solutions in sugar refining. Catabolic enzymes, such as dextransucrase, are frequently inducible, being formed in the presence of, or in response to, an inducing agent, which is often the substrate of the enzyme, or a structurally similar compound. In this case, sucrose is the only known inducer of dextransucrase.

The carbon source can have an effect on product formation (59). The rate at which the carbon source is metabolized can often influence the formation of biomass or other metabolic by-products. Fast growth due to high concentrations of rapidly metabolized sugars is often associated with the formation of unwanted metabolites (eg. dextran). This problem can be solved by the continuous or semi-continuous feeding of glucose or sucrose into the fermenter, thus maintaining a low concentration of the carbon source in the medium.

Another important medium factor is the nitrogen source. Most industrially used microorganisms can utilize both organic and inorganic sources of nitrogen, with inorganic nitrogen being supplied as ammonia gas, ammonium salts or nitrates and organic nitrogen supplied as amino acids, protein or urea. Pure amino acids are quite expensive and are usually included in the medium as components of more complex organic sources, such as yeast extract, corn steep liquor, casein hydrolysate and fish meal, all of which are non-homogeneous and readily available.

Other components required in the medium are magnesium, phosphorus, potassium, sulphur, calcium and chloride (see Table 3.1) and because of the concentrations required, they are often added as distinct components. Other elements are required, but are usually present as impurities in other major ingredients.

Vitamins are required in small amounts (60) as cofactors for certain enzymes, although many of the natural carbon and nitrogen sources contain several of the required vitamins and amino acids. However, it is often preferable to supplement the medium with a quantity of a pure vitamin that is lacking in the carbon or nitrogen source, rather than using a larger bulk of a less expensive multiple vitamin source, as it is important to avoid the addition of unwanted precursors or inhibitors that may have undesirable effects on the fermentation product. Certain precursors and inhibitors may indeed be required in the medium; precursors being chemicals which, when added to certain fermentations, are directly incorporated into the desired product and inhibitors

being chemicals that could enhance the production of a certain product, or accumulate a reaction intermediate that is usually metabolized.

The control of pH is extremely important in most fermentations (57) and compounds are often added to the medium to act specifically as a buffer. Often phosphates, which are parts of many media, play an important role in buffering. The pH can also be externally controlled with the addition of alkalis such as ammonia or sodium hydroxide and acids such as sulphuric acid.

The presence of oxygen is also an important medium component in fermentations as its availability can control growth rate and product formation. Medium rheology is important here as the individual components of the medium can influence the viscosity of the final broth and, hence, affect oxygen transfer. Most fermentation media tend to produce foam, which can be reduced by the addition of such antifoaming agents as silicones, fatty acids and esters. These agents also serve to reduce oxygen transfer in the medium.

The other very important medium component in fermentations is water, with some of the factors that need to be considered including pH, dissolved solids and effluent contaminants. The mineral content of water is often quite crucial in many fermentations.

3.3. Sterilization Theory.

A fermentation product is produced by the culture of a certain organism in a nutrient environment. Sterile conditions are essential in fermentation processes as they involve the use of pure cultures. Sterilization, therefore, prevents the growth of undesirable organisms (contaminants). Totally sterile conditions are required for the majority of industrial fermentations, as a contaminated medium can suffer in many ways. The available nutrients can be consumed by the contaminants and be converted to unwanted cells and products; the contaminant may degrade the desired product, or produce compounds that may make the extraction of the final product difficult, and if the fermentation is a continuous one, the contaminant may outgrow the production organism, and displace it from the fermenter.

A contaminant can be avoided in a fermentation by using a pure inoculum to start the fermentation (see Section 3.4), sterilizing the nutrient medium,

sterilizing the fermenter vessel, sterilizing all materials and ancillary equipment to be used during the fermentation and maintaining aseptic conditions throughout the whole process. The main methods of sterilization (61) are moist heat (steam) sterilization; dry heat - pasteurization and tyndallization; chemical sterilization; ionising radiation and filtration. The heat treatment methods, particularly using steam, are by far the most common and it is these methods which will be discussed below.

Heat sterilization is carried out to cause the death of the microorganisms present. Death is defined as an irreversible loss of the cells' ability to grow and divide, ie. loss of viability. The loss of viability with time when cells are exposed to lethal conditions is exponential and is shown in Figure 3.2. The destruction of microorganisms by heat is a first order reaction as shown by Equation 3.2.

$$\frac{-dN}{dt} = KN \quad 3.2$$

where N is the number of viable organisms present,
 t is the time of the sterilization treatment and
 K is the specific death rate of the organism.

Equation 3.2 can be integrated to give

$$\frac{N_t}{N_0} = e^{-Kt} \quad 3.3$$

where N_0 is the number of viable organisms present at the start of the sterilization cycle and
 N_t is the number of viable organisms present after a treatment time, t .

Equation 3.3 can be plotted on a log, linear scale to give a graph shown in Figure 3.3, whereby a value of K can be found. The death rate of the organism depends on the sterilization temperature, as in Equation 3.4.

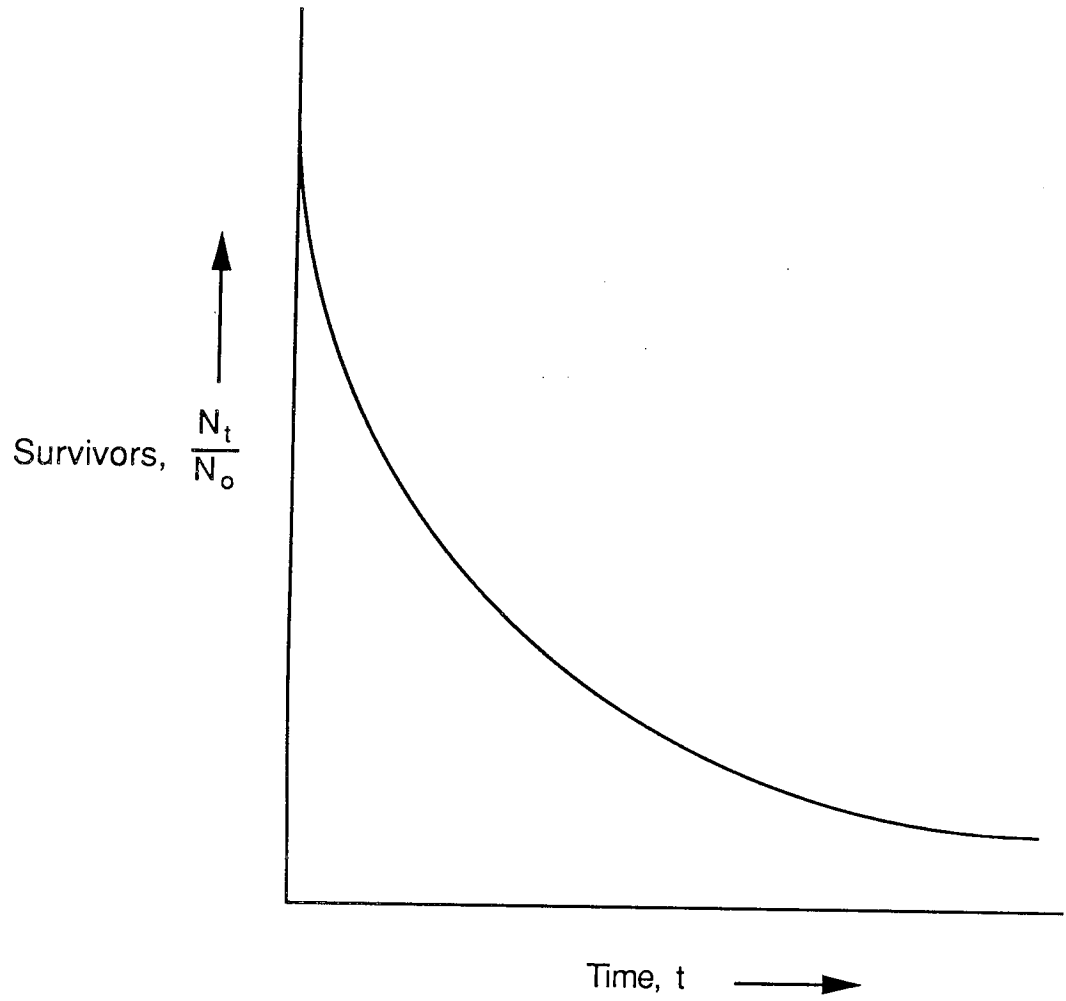


Figure 3.2

Loss of Cell Viability with Time

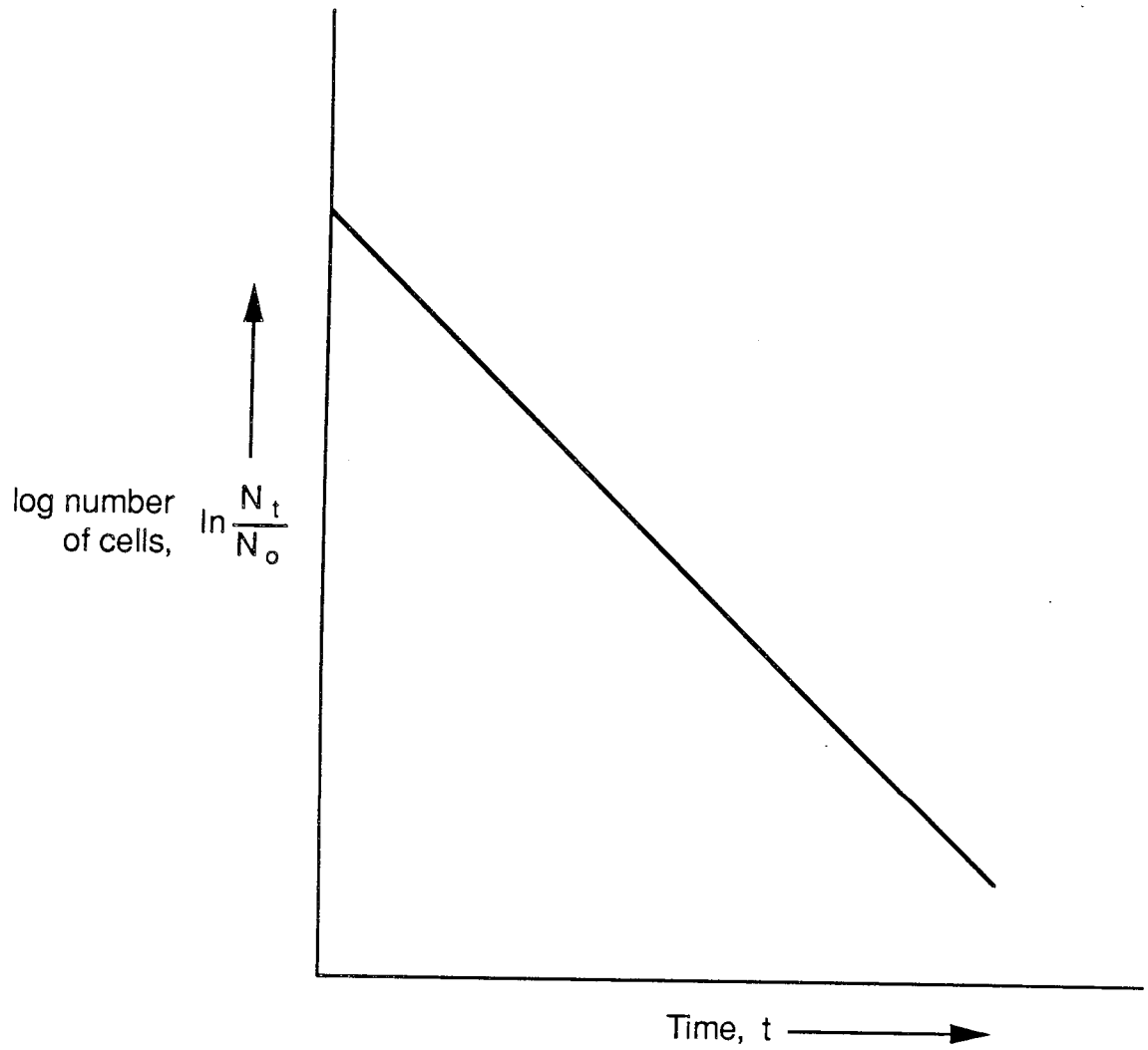


Figure 3.3

Log Number of Cells versus Time

$$\frac{d \ln K}{dt} = \frac{E}{RT^2} \quad 3.4$$

where E is the Activation Energy,
R is the Gas Constant, and
T is the Absolute Temperature.

In studying the effect of temperature, the decimal reduction time, D, is used. D is the time required to reduce the cell population by 10 fold, ie.

$$D = \frac{N_t}{N_o} = \frac{1}{10} \quad 3.5$$

or, from Equation 3.3:

$$D = \frac{2.303}{K} \quad 3.6$$

The dependence of D upon the temperature is shown in Figure 3.4. The thermal destruction curve can be used to determine the time required to kill all the cells in medium at a specific temperature, starting with a known cell number, defined as F.

$$F = n.D \quad 3.7$$

where n is the number of log cycles between start and finish.

For example, in many fermentations, the probability of contamination of 10^{-6} is often thought to be sufficient for most purposes. If the initial count of contaminants is taken as 10^{11} , and D at 120°C is 1.5 min. for the most resistant spores of many contaminants, then

$$n = 11 + 6 = 17, \text{ and} \\ F_{120^\circ\text{C}} = 17 \times 1.5 = 25.5 \text{ minutes.}$$

In practice, time is required for the system to reach the lethal temperature (almost always $116 - 121^\circ\text{C}$) and so heating and cooling periods must be taken into account when determining sterilization time, to avoid any medium degradation that may occur at prolonged high temperatures.

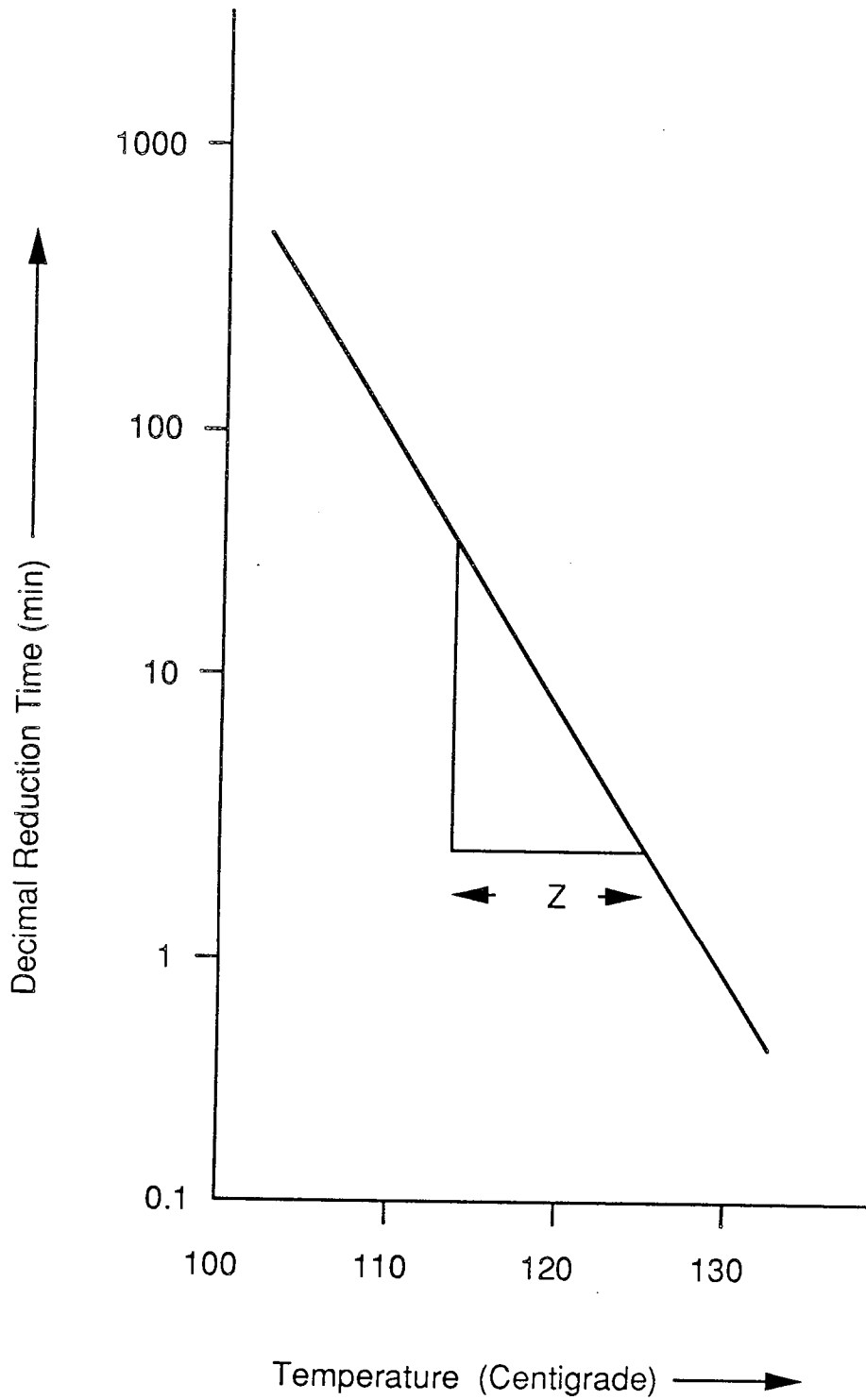


Figure 3.4

The Effect of Temperature on the Decimal Reduction Time

It is important that all aspects of the fermentation are sterile. The medium is usually sterilized *in situ* with the vessel and any steam sterilizable probes. Any further equipment, including tubing and inoculum bottles, should be sterilized in an autoclave.

3.4. Preparation of Inoculum.

A fermentation involves the growing of a particular organism in medium to an extent that it will produce high yields of a desired product (56, 57, 62). A culture used to inoculate a fermentation must be in a healthy, active state to minimize the length of the lag phase in the subsequent fermentation. It must also be available in sufficiently large volumes to provide an inoculum of optimum size and it must be in a suitable morphological form, free of contaminants and capable of forming the required product. The lag time in a fermentation is minimized by growing the culture in the "final-type" medium, with inoculum media being generally less nutritious than production media and containing a lower level of carbon.

The quantity of inoculum normally used is between 3 and 10% of the fermentation volume, so starting from a slope of the culture, the inoculum must be built up in a number of stages in vessels of increasing size, until the required volume for the production vessel is reached. It is important that a bacterial inoculum should be transferred from one stage to the next in the logarithmic state of growth, when the cells are still metabolically active. A schematic representation of a typical inoculum development process is shown in Figure 3.5. The growth cycle of the cells during each inoculum stage is that of a typical batch culture (See Section 3.8.1).

3.5. Instrumentation and Control.

Fermentation products are usually only formed under certain restricted environmental conditions and so the success of a fermentation usually depends upon well controlled factors such as temperature, pH, degree of agitation, dissolved oxygen concentration and other factors that may have to be monitored during the process. The criticality of temperature, pH and DOC on dextransucrase production have been discussed in a previous chapter.

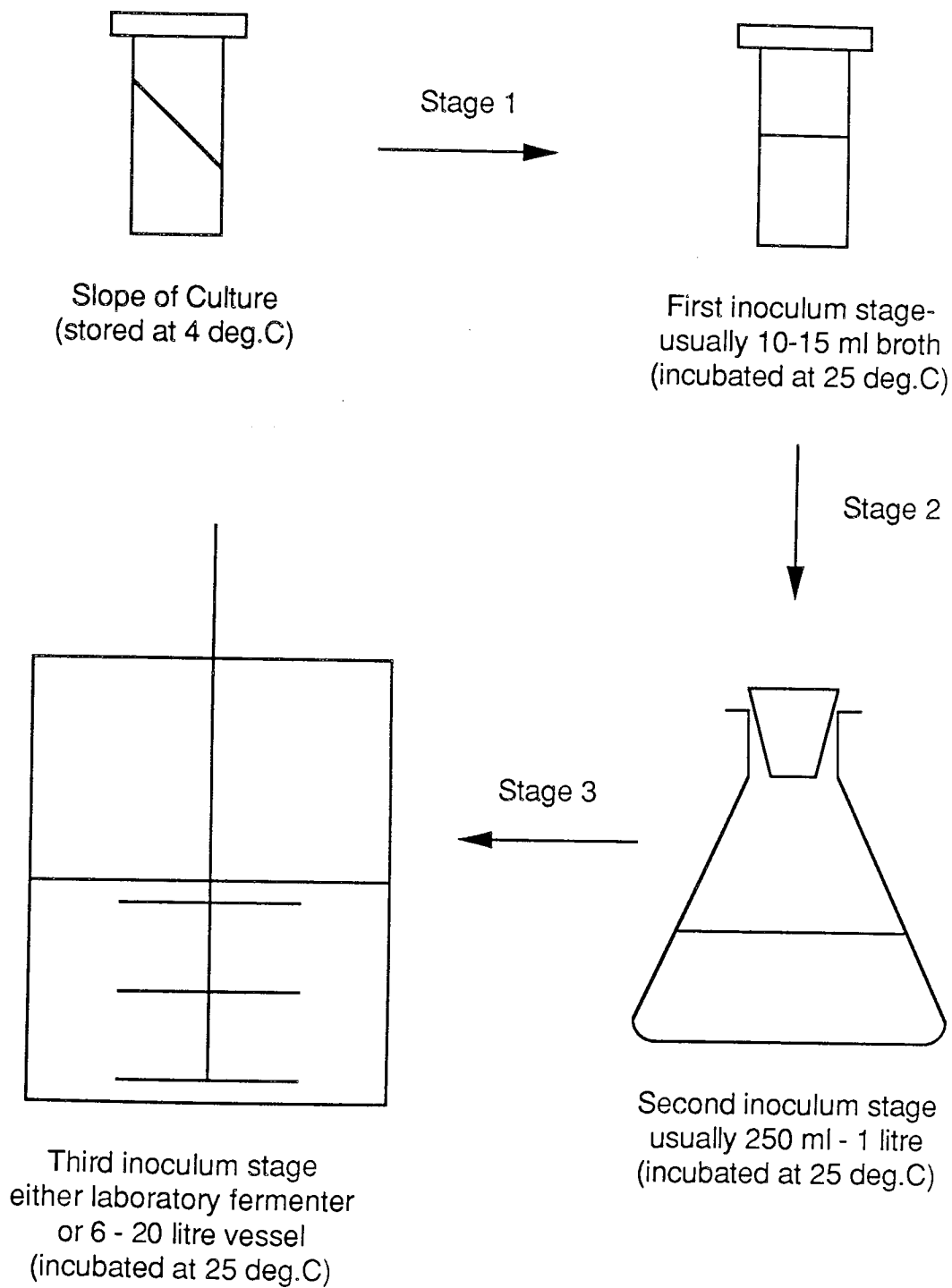


Figure 3.5

**Schematic representation of a typical three stage
inoculum development programme**

Several of the environmental parameters mentioned above can be monitored using analytical probes and, once the signals from them have been amplified, they can be used to actuate control procedures. For example, signals from thermistors or temperature probes can be used to regulate the flow of cooling water during the fermentation, or of steam during sterilization. The pH can be controlled with the use of a pH electrode that initiates the injection of acid or alkali into the fermenter. The dissolved oxygen concentration within the fermenter can also be controlled. Low dissolved oxygen levels can inhibit aerobic cultures and high levels can inhibit oxygen dissolutions and so waste energy. A dissolved oxygen probe is used to monitor the DOC, and the signal from such a device can influence the air flow into the fermenter, or the agitation intensity. It is important that the fermenter contents are well stirred so that the probe reading at one point is indicative of the whole fermenter contents.

In these days of increasingly sophisticated control systems (63, 64), it is becoming more common for a fermentation module to be linked to a computer system, the simplest task for a computer being data logging. The computer can be programmed in such a way that, at pre-determined intervals, it will scan all the signals from the appropriate sensors and log them in a data store. As well as on-line measurements being recorded, analytical data that has been separately determined can be logged into the data store for specific known times, providing the operators with a complete record of the process, along with an overall model of the fermentation, which may be used to analyse data in a number of ways. This is known as data analysis and is becoming increasingly common.

3.6. Product Recovery and Purification.

Fermentation broths are complex, aqueous mixtures of cells, soluble extracellular products, intracellular products, and unconverted substrate. A complete fermentation process should include, as well as the actual reaction stage itself, a subsequent product recovery section. A typical recovery procedure that is carried out to obtain a highly purified product could be as follows:-

3.6.1. Removal of Cells and Other Particulates.

The most common processes here are centrifugation, filtration and/or settling, sedimentation and decanting (65). Size and density are usually the features that allow separation of cell-sized (0.3 to 10 μm) particles by filtration, centrifugation and/or settling. Sedimentation involves the settling of particulates in a simple gravitational field, whereas centrifugation requires the production of enhanced settling velocities by centrifugal forces. Currently, a number of new techniques are emerging for cell recovery, including the flotation method (55) for small particles, where solids are removed by attachment to rising air bubbles. Electrokinetic deposition uses voltage gradients of 1,050 volts/cm to produce solid biomass with densities up to 40% w/v, with the cost of this power input being offset by subsequent savings in dewatering or drying stages.

3.6.2. Primary Isolation.

The best known techniques here include solvent extraction, sorption, precipitation and ultrafiltration (66). During these stages the desired product concentration increases considerably and substances of widely differing polarities are separated from the product. Extraction techniques involve the presence of two liquid phases. Aqueous-organic two-phase systems tend to predominate in antibiotic recovery, as antibiotics are extremely soluble in organic solvents which are nearly water immiscible. An extraction process, alternating aqueous to organic, then organic to aqueous can provide both concentration and purification. Aqueous liquid-liquid extraction systems have been developed which allow for protein recovery without causing damage to enzymes or other proteins present in the broth. Dextran, even in crude forms, is the key item for product recovery here. Sorption involves the partitioning of a solute between a bulk solution phase and a typically porous, or high surface area, solid, with an example being the sorption of streptomycin by ion-exchange. Precipitation involves the removal of the required substance in solution from a liquid, by treating the solution such that the required compound forms an insoluble precipitate; removing the solid material by subsequent filtration or centrifugation. Ultrafiltration techniques are used for the separation or concentration of colloidal substances, using an ultrafilter, with molecule sizes from 10 \AA to 500 - 1,000 \AA usually being removed by this method.

3.6.3. Purification.

Purification operations are often selected for impurity removal as well as for further product concentration. Techniques used here include fractional precipitation, many types of chromatography and adsorption. Chromatography involves the column resolution of a small batch of flowing multicomponent fluid into separate fluid volumes of nearly pure solute solutions. Different types of chromatography are often defined in terms of the principle of separation, for example, ion-exchange chromatography where the solution is passed over a fixed-bed column containing ion-exchange resin onto which the proteins will bind by electrostatic forces. Affinity chromatography is based on the natural specificity of some biopolymers to certain substances, which are subsequently packed within the column. This technique is often used in monoclonal antibody production. Other chromatographic methods used are absorption and permeation chromatography, with developments currently being made in sophisticated automated chromatographic techniques including gas/liquid chromatography (GLC) and high performance liquid chromatography (HPLC).

3.6.4. Final Product Isolation.

The last steps must provide the desired product in a form suitable for final formulation and blending, or for direct shipping. Processes here include centrifugation and subsequent drying of a crystallized product, drum or spray drying, freeze drying (lyophilization) or organic solvent removal.

The product recovery operations mentioned above each provide a significant increase to the final cost of the required product and so to minimize these costs it appears necessary to improve the upstream fermentation process so that it yields a final broth of higher product concentration. Ultimately it is the downstream purification costs that determine the overall viability of a fermentation process and, hence, whether it will take place at all.

3.7. Principles of Scale-Up.

The development of microbiological processes is usually carried out in three stages or scales ⁽⁶⁷⁾; the first being the bench scale where basic screening procedures are carried out; the second being the pilot plant where optimal operating conditions are determined; and the third being the plant scale where the process is brought to economic fruition. These steps are shown in Figure 3.6.

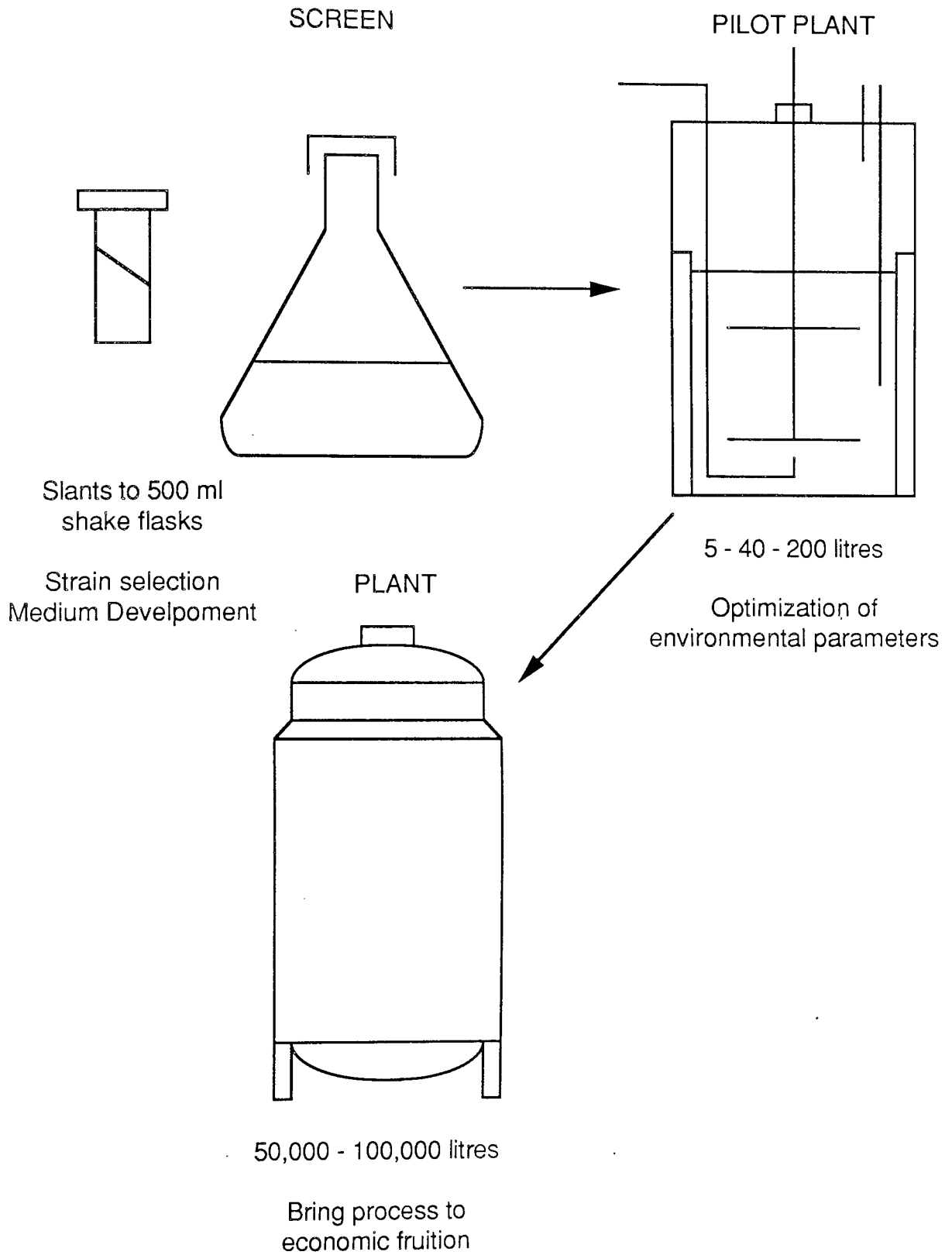


Figure 3.6

Scale-up of a Laboratory Fermentation to the Industrial Level

There are many factors that could be selected as the basis for a scale-up procedure (65, 69). These include constant volumetric oxygen transfer rate, $k_L a$, constant impeller tip speed, $TTND_i$, constant volumetric power input, P_g/V , equal mixing times, similar N_{Re} or momentum factors; and feedback control to try and ensure as nearly as possible, constant values of key environmental factors. For fermentations where dissolved oxygen concentration is an important control factor a constant $k_L a$ is a good scale-up factor, being an excellent way to compare two pieces of fermentation equipment. Certain fermentation systems, particularly highly mycelial ones, are affected by shear or impeller tip speed. A number of fermentation companies attempt to maintain a constant impeller tip speed on scale-up, with the tip speed range generally thought satisfactory for scale-up being 250-550 cm/s. If tip speed and $k_L a$ are to be maintained constant on scale-up it is not possible to also maintain geometric similarity. However this is not crucial as shear is relatively insensitive to scale change while being almost directly proportional to the power per unit volume as is the mass transfer coefficient, $k_L a$. *Therefore only a slight manipulation of impeller to tank diameter ratio is necessary to achieve both constant $k_L a$ and impeller tip speed on scale-up.* A further problem that is often encountered on scale-up is the non-uniformity of larger vessels. Whilst small fermenters (< 500 l) are essentially well mixed, the very large vessels (> 5,000 l) tend to be poorly mixed, causing differences in dissolved oxygen levels within the bigger vessels. The mixing time of a fermentation increases quite significantly with increasing scale. Heat transfer is a further factor that needs to be taken into consideration during scale-up. During a fermentation, heat is produced by the organisms as well as by agitation. This heat is normally removed using cooling coils or jackets. On scale-up, heat removal systems have to be modified to maximize the cooling area with, for example, the inclusion of baffles as well as jackets and cooling coils.

Generally scale-up is not a simple process with other factors such as a constant Reynolds Number and feedback control being considered. However, generally the normal criteria chosen for scale-up are constant power/unit volume, constant gas flowrate/unit volume and constant geometric similarity of the vessel, although not necessarily of the agitator.

3.8. Microbial Growth Kinetics.

For microorganisms to grow and maintain themselves, they must be able to produce ATP - high energy adenosine triphosphate - which is used in the many essential energy-consuming processes of the cells, such as transport and biosynthesis. Different types of organisms generate ATP in varying ways although the pathways are fundamentally similar in all organisms. Chemoautotrophs, such as *Leuconostoc mesenteroides*, derive ATP from the oxidation of organic compounds known as growth substrates. A microorganism will therefore grow in a culture medium, provided that the medium contains all the necessary nutrients in an available form and all other environmental factors are suitable. Microbial growth is an increase in the dry weight or bulk of an organism which involves cell division, expansion, differentiation and morphogenesis. The following sections describe various types of culture growth systems that are used both in the laboratory and in industry, and include the kinetics of the various systems.

3.8.1. Batch Culture.

A batch culture system is an example of growth in a closed system or environment (70). It consists of a vessel containing an initial limiting amount of a suitable growth supporting medium, which is inoculated with microbial cells. No fresh medium is supplied to the culture so that the cells grow until some essential component of the medium is exhausted or the environment changes due to the accumulation of a toxic product, pH change, etc.

A typical batch growth curve in a defined minimal medium is shown in Figure 3.7 (55, 57). Microbial growth is measured here by dry cell weight - X - in units of g/l. The growth curve can be divided into a number of phases described below.

Phase 1. The lag phase. This phase follows inoculation, where there is no apparent microbial growth. During this period, the cells are adapting to growth in their new environment by synthesizing the necessary enzymes for the utilization of the available substrate. The length of this lag phase depends on the previous history of the inoculum - increasing the similarity between the previous inoculum stage and the culture medium conditions will reduce the time of the lag phase. Obviously, for commercial purposes, it is necessary for there to be as short a lag phase as possible.

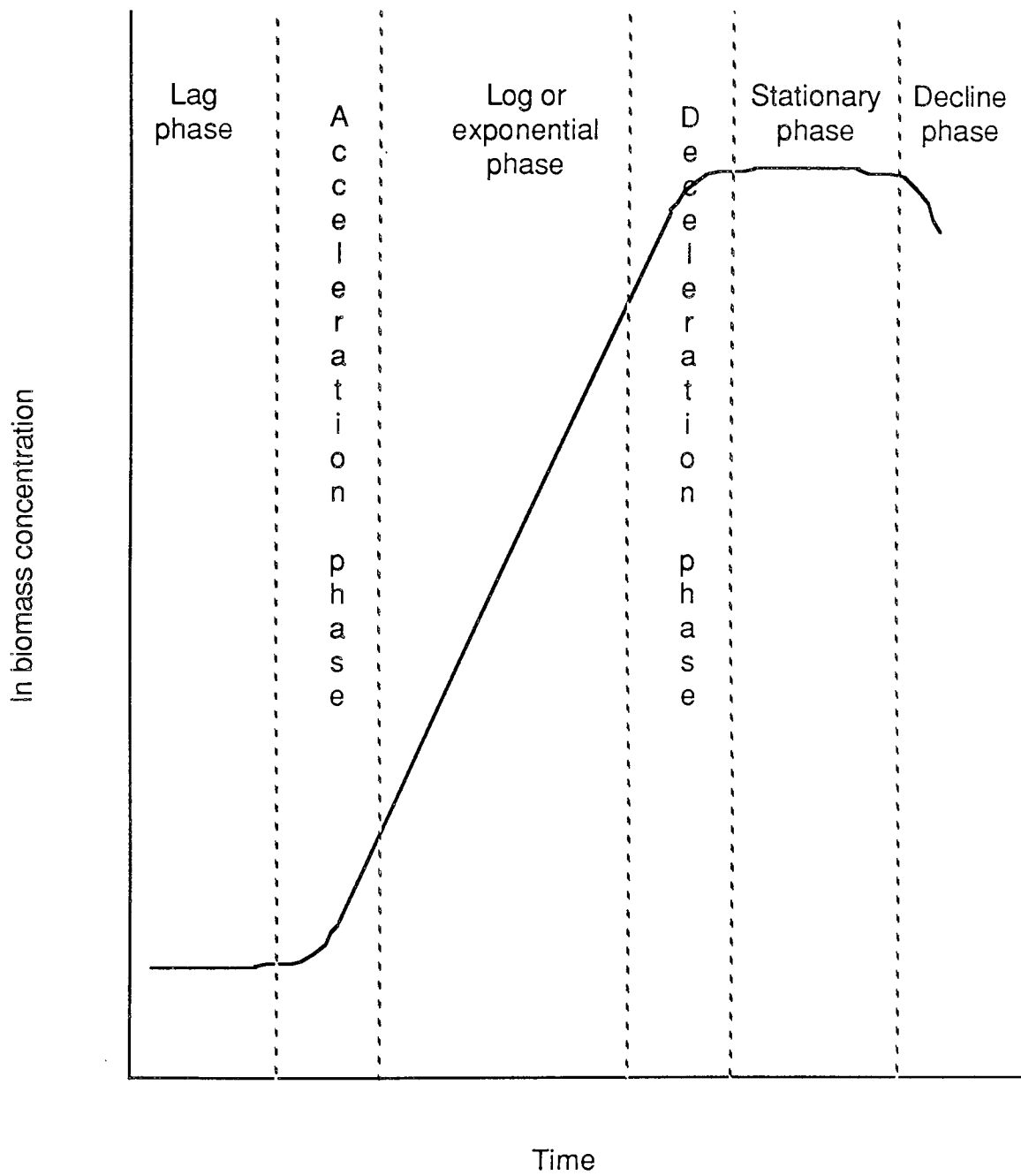


Figure 3.7

Growth of a typical microbial culture in batch conditions

Phase 2. The acceleration phase. This is a short period in the cell cycle, where the growth rate of the organism begins to increase, up to a point where it reaches its maximum growth rate - which is constant.

Phase 3. The exponential phase. This is the period in the growth cycle whereby the cells are growing exponentially, such that the synthesis of all the cell constituents increases at a constant rate so that the cell population doubles and continues to double at regular intervals. During this phase, the cell environment is continuously changing, despite the constant growth rate, as the substrates are being consumed and new products accumulated.

Phase 4. The deceleration phase. This is the period where the cell growth rate begins to decrease and does so until it reaches a value of zero.

Phase 5. The stationary phase. During this period the cell dry weight remains constant, and there is no net growth. Growth may be occurring but it is balanced by death of the cells. The cells stop growing due to the depletion of an essential nutrient, the accumulation of a toxic material or a change in the cell environment, eg. pH.

Phase 6. The decline phase. This is otherwise known as the death phase, where the death rate exceeds the growth rate, ending the microbial growth cycle.

It should be noted that the batch growth curve is not an inherent property of the organism, but occurs as a result of cells growing in a closed system.

3.8.1.1. Theory of Batch Culture.

The rate of increase in cell dry weight is proportional to the concentration of cells initially present (71). During exponential growth of a unicellular organism the cell dry weight doubles at regular intervals so that the number of doublings, n , after time, t , is given by :-

$$n = t/t_d \quad 3.8$$

where t_d is the culture doubling time.

The growth of an organism in batch culture may also be described by simple algebra, using t_d . If a culture medium were to be inoculated with one cell, it would, at the maximum growth rate, divide to produce 2, 4, 8, 16, 32, etc. cells. This increase can also be represented by expressing the cell numbers to the base two, ie. $2^1, 2^2, 2^3, 2^4$, etc. The power numbers here represent the number of generations which have passed so that, after n generations, there will be 2^n cells in the culture. If the medium were inoculated with X_0 cells, then the number of cells, X_t , present after n generations would be:-

$$X_t = X_0 \cdot 2^n \quad 3.8.$$

From Equation 3.8

$$X_t = X_0 \cdot 2^{t/t_d} \quad 3.9.$$

Taking natural logarithms gives :-

$$\ln(X_t/X_0) = \ln 2 (t/t_d) \quad 3.10.$$

Re-arranging this Equation:-

$$\frac{\ln X_t - \ln X_0}{t} = \frac{\ln 2}{t_d} = \frac{0.639}{t_d} \quad 3.11$$

The exponential phase of a batch culture can be described as :-

$$\frac{dX}{dt} = \mu X \quad 3.12$$

where μ is the specific growth rate-units h^{-1} .

Integrating Equation 3.12 gives :-

$$X_t = X_0 e^{\mu t} \quad 3.13.$$

Taking natural logarithms gives :-

$$\frac{\ln X_t - \ln X_0}{t} = \mu \quad 3.14$$

Substituting the above result in Equation 3.11 gives :-

$$\mu = \frac{0.693}{t_d} \quad 3.15$$

A plot of $\ln (X_t - X_0)$ versus time would yield a straight line of gradient $(0.693/t_d)$ or μ , as shown in Figure 3.8.

Substrates are consumed by the cells for many purposes. Energy is required for cell growth, the maintenance of cell viability and for product formation. The rate of a single substrate utilization can be determined from :-

$$\begin{aligned} \text{Substrate Accumulation} &= \text{Substrate feed} - \text{Substrate consumed for growth} - \text{Substrate consumed for product synthesis} \\ &\quad - \text{maintenance} - \text{substrate removal} \end{aligned} \quad 3.16$$

or

$$\frac{-dS}{dt} = \frac{FS}{V} - \frac{\mu X}{Y_{X/S}} - \frac{q_p X}{Y_{P/S}} - mX - \frac{F'S}{V} \quad 3.17$$

where F and F' are medium flow rates into and out of the vessel (l/h),

V is the volume of the culture (l),

s and s' are substrate concentrations going in and out of the culture (g/l),

$Y_{X/S}$ is the biomass yield coefficient (g cell dry weight formed per g substrate consumed),

$Y_{P/S}$ is the product yield coefficient (g product formed per g substrate consumed),

q_p is the product formation rate (h^{-1}), and

m is the maintenance coefficient (g substrate consumed per g cell dry weight per hour).

With batch culture, there is no substrate feed or removal, so Equation 3.17 becomes :-

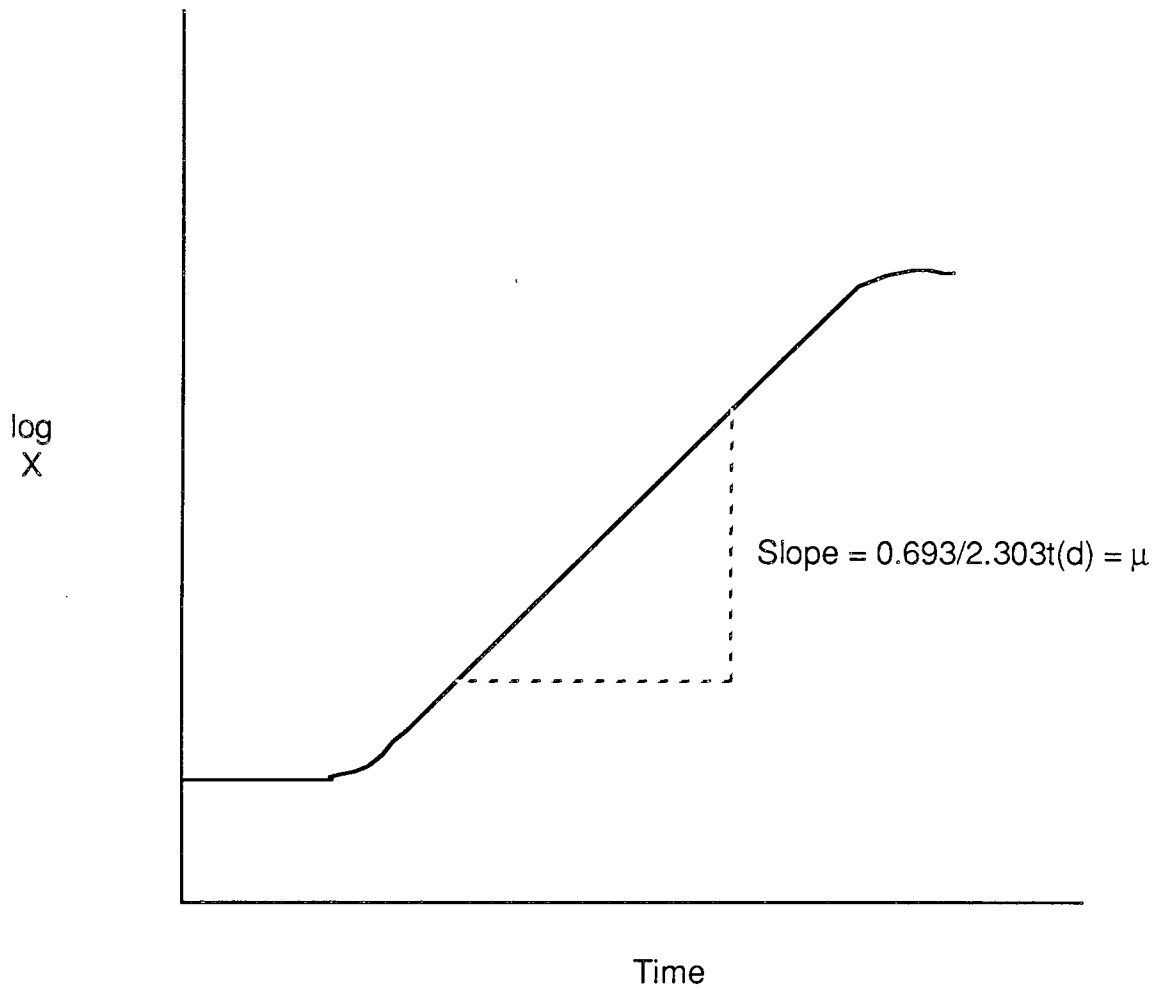


Figure 3.8

Determination of the doubling time, t(d), and the specific growth rate during a batch culture

$$\frac{ds}{dt} = \frac{\mu X}{Y_{X/S}} - \frac{q_p X}{Y_{P/S}} - mX \quad 3.18$$

This Equation can be simplified by assuming no product formation, and that the maintenance energy is negligible. So :

$$\frac{-ds}{dt} = \frac{\mu X}{Y_{X/S}} \quad 3.19$$

The specific rate of substrate utilization is given by :-

$$q_s = \frac{-1}{X} \frac{ds}{dt} = \frac{\mu}{Y_{X/S}} \quad 3.20$$

Products are formed during the growth of microbial cells, and the rate of product formation is determined from :-

$$\begin{aligned} \text{Product Accumulation} &= \text{Product Synthesis} - \text{Product Removal} - \text{Product Denaturation} \\ 3.21 \end{aligned}$$

or

$$\frac{dp}{dt} = q_p X - \frac{F}{V} P - \beta P \quad 3.22$$

where P is the product concentration (g/l) and β is the rate of product denaturation (h^{-1}).

If the product is stable ($\beta = 0$) and not removed from the vessel, Equation 3.22 becomes :-

$$\frac{dp}{dt} = q_p X \quad 3.23$$

and the specific rate of product formation, q_p , is given by :-

$$q_p = \frac{1}{X} \cdot \frac{dp}{dt} \quad 3.24$$

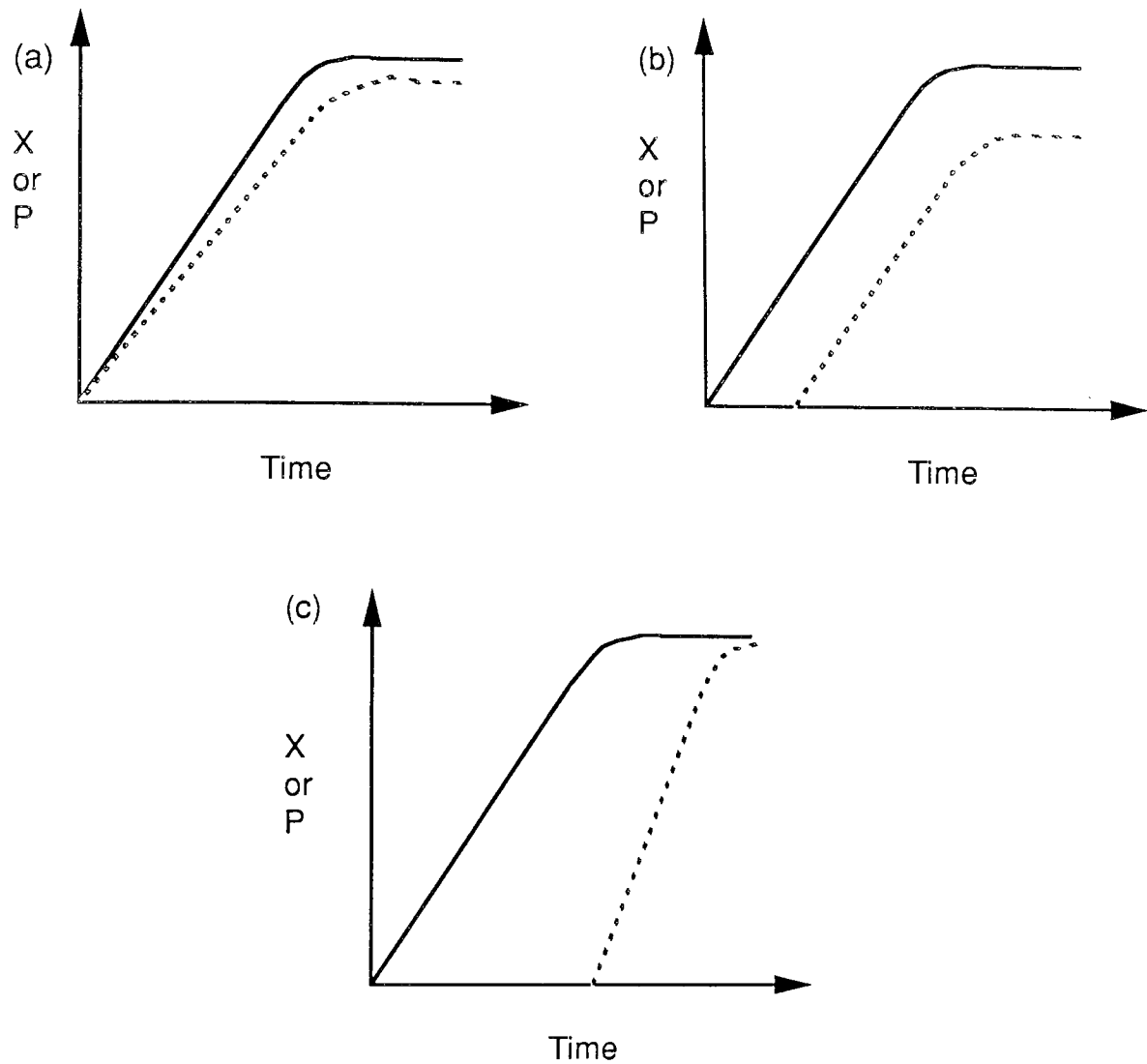


Figure 3.9

Growth and Product Formation in Batch Cultures.

(a) growth related product formation, (b) partially growth related product formation and (c) non-growth related product formation

Products of cell metabolism can be divided into three classes as shown in Figure 3.9, ie. growth related products, partially growth related products and non-growth related products. Growth related products, such as dextransucrase, are usually direct products of catabolic pathways or they are intermediates of the basic metabolic pathways. The rate of formation of such products is directly related to growth, and is described by :-

$$q_p = \frac{\mu X}{Y_{P/X}} \quad 3.25$$

where $Y_{P/X}$ is the product yield coefficient (g product formed per g cell dry weight).

The volumetric productivity of a fermentation is measured in terms of g product formed per litre per hour, although in batch cultures, the productivity has to be determined over the entire fermentation cycle.

The total batch fermentation time is given by :-

$$t = \frac{1}{\mu_{\max}} \cdot \ln \frac{X'}{X_0} + t_T + t_D + t_L \quad 3.26$$

where X' is the final cell dry weight (g/l) and t_T , t_D , and t_L are the turn around time, delay time and lag time (h) respectively.

The overall productivity, P , of a process is given by :-

$$P = \frac{\dot{X}}{\frac{1}{\mu_{\max}} \cdot \ln \frac{X'}{X_0} + t_T + t_D + t_L} \quad 3.27$$

This is shown diagrammatically in Figure 3.10.

The effect of the substrate concentration on the specific growth rate is given by the Monod Equation as shown below :-

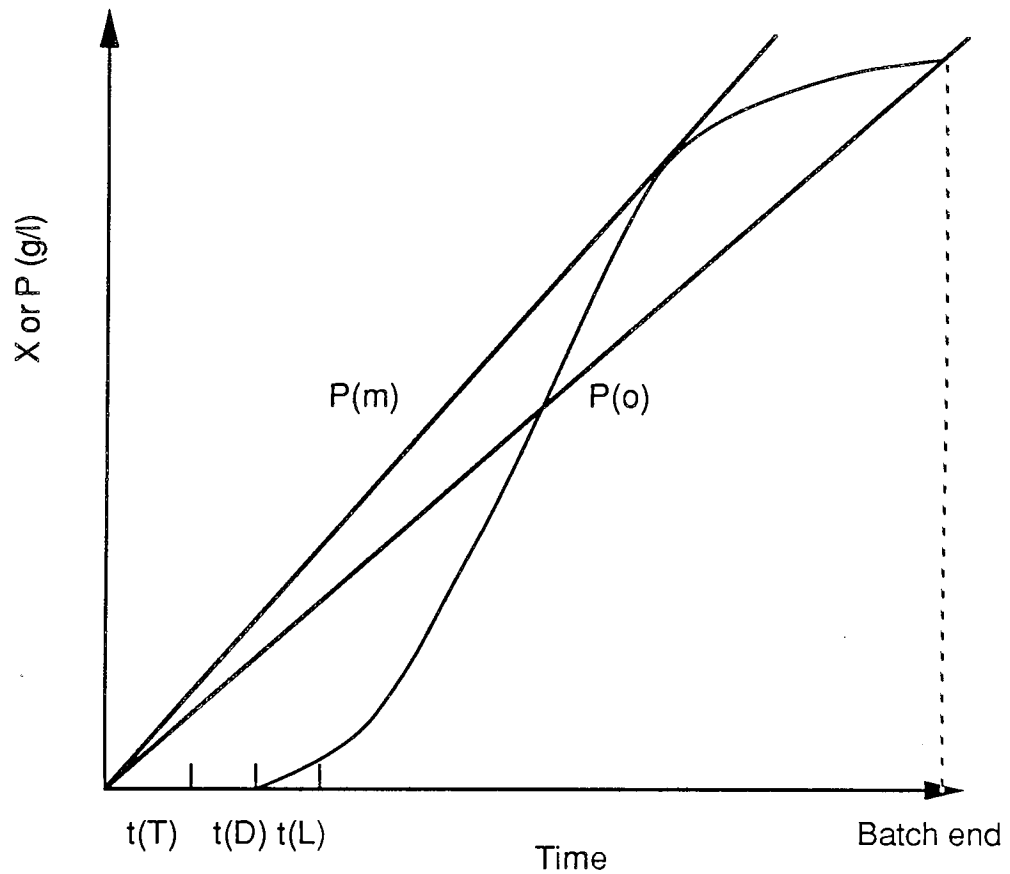


Figure 3.10

Productivity in Batch Culture

X and P represent cell dry weight (g/l) and product (g/l):
 $t(T)$, $t(D)$ and $t(L)$ represent the turnaround time, delay time
and lag time respectively: $P(m)$ and $P(o)$ represent the maximum
and overall productivity respectively

$$\mu = \frac{\mu_{\max} S}{K_s + S} \quad 3.28$$

where K_s is the saturation or Monod constant (g/l), and is related to the affinity of the organism to the substrate. (A high value of K_s represents a low affinity for the substrate).

If $S \gg K_s$ then Equation 3.28 becomes :-

$$\mu = \mu_{\max} \quad 3.29.$$

but if $S = K_s$ then Equation 3.28 becomes :-

$$\mu = \mu_{\max}/2 \quad 3.30.$$

Accurate determinations of K_s and μ_{\max} can be obtained by linearising Equation 3.28 through taking reciprocals of both the left and right hand side of the Equation and re-arranging to give :-

$$\frac{1}{\mu} = \frac{1}{S} \cdot \frac{K_s}{\mu_{\max}} + \frac{1}{\mu_{\max}} \quad 3.31$$

Plotting $1/\mu$ versus $1/S$ - the so-called Lineweaver-Burk plot - gives Figure 3.11 from which both K_s and μ_{\max} can be determined.

This represents some of the basic batch fermentation theory.

3.8.2. Fed-Batch Culture.

In fermentation processes, "fed-batch" is a technique where one or more nutrients are supplied to the vessel during cultivation and in which the products remain in the bioreactor until the end of the run (72). The basic characteristic of the fed-batch process is that the concentrations of nutrients fed into the culture liquid of the bioreactor can be controlled by manually altering the feed rate. Generally speaking, fed-batch is superior to conventional batch operation, especially when changing concentrations of a nutrient affect the yield or productivity of the desired metabolite. There are many other advantages in operating a fermentation in a fed-batch over a

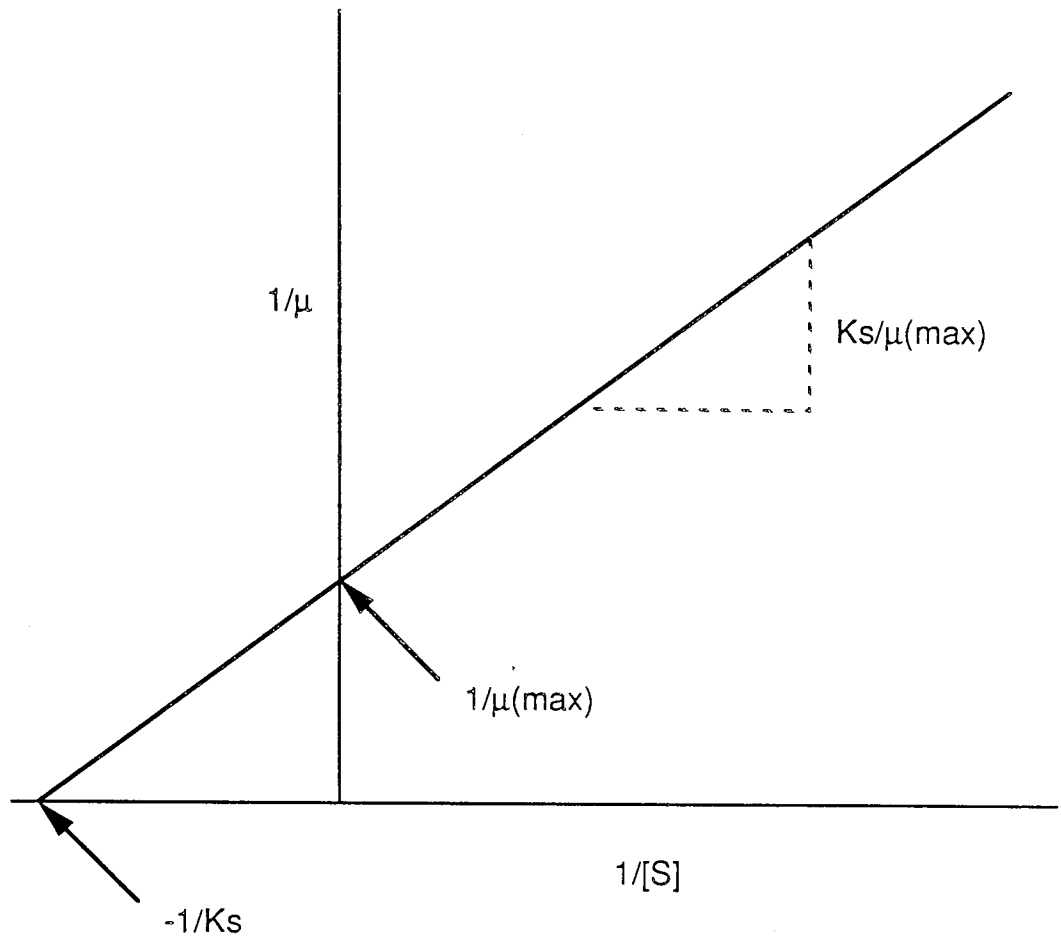


Figure 3.11

Lineweaver-Burk plot

$1/\mu$ versus $1/S$ for the determination of the kinetic constants K_s and $\mu(\max)$

batch manner, including the avoidance of any substrate inhibition which can occur at high concentrations of certain substrates. High cell concentrations require high concentrations of nutrients in the medium, which again can lead to inhibition in truly batch processes. A culture can also be operated for an extended period of time by adding nutrient(s) at the end of batch operation. If the desired metabolite is still being produced at this time, its yield will be increased by extending the culture time. A fed-batch process will also decrease the broth viscosity in the production of certain microbial polymers, eg. dextran, pullulan, or xanthan gum. The greatly increased viscosity of a batch process will serve to raise the agitation power consumption and lower the oxygen transfer efficiency.

3.8.2.1. Theory of Fed-Batch Culture.

The cell concentration of a batch culture in which growth is limited by the level of one substrate in the medium (73) can be described by the following Equation:-

$$X_t = X_0 + Y(S_R - S) \quad 3.31.$$

where Y is the yield factor (dimensionless),
 S_R is the original substrate concentration,
 S is the residual substrate concentration,
 X_t is the cell concentration after time, t, hours and
 X_0 is the inoculum concentration.

The final biomass concentration is achieved when $S = 0$ and is called X_{max} . Assuming that $X_{max} \gg X_0$, Equation 3.31 becomes :-

$$X_{max} \simeq YS_R \quad 3.32.$$

If, at the time when $X = X_{max}$, a medium feed is started, such that the dilution rate is less than μ_{max} (see Section 3.8.3.1), virtually all the substrate will be consumed as fast as it enters the culture, so that :-

$$FS_R = \mu \frac{\bar{X}}{Y} \quad 3.33$$

where F is the flow rate of the medium feed (m^3/h) and
 X is the total biomass in the culture, given by $X = XV$,
 where V is the volume of the vessel at time t .

From the above Equation, it can be seen that the input of substrate is equaled by the consumption of substrate by the cells. This implies that $(ds/dt) \simeq 0$. Although the total biomass in the culture (X) increases with time (and increasing fermentation volume), cell concentration (X) remains virtually constant, so that $(dX/dt) \simeq 0$ and therefore $\mu = D$. This situation is termed a quasi-steady-state. As time progresses, the dilution rate will decrease as the volume increases ($D = F/V$ - Section 3.8.3.1) and D will be given by :-

$$D = \frac{F}{V_0 + Ft} \quad 3.34$$

where V_0 is the original volume.

So, according to Monod Kinetics, the residual substrate level should decrease as D decreases, resulting in an increase in the cell concentration. However, over most of the range of μ which will operate in fed-batch culture, $S_R \gg K_S$ so that, for all practical purposes, the change in S would be extremely small and may be considered as zero. Therefore, provided $D < \mu_{\max}$, and $K_S \ll S_R$, a quasi-steady-state can be achieved, as shown in Figure 3.12. The major difference between the steady-state of a chemostat and the quasi-steady-state of a fed-batch culture is that μ is constant in the chemostat, but decreases in the fed-batch.

3.8.3. Continuous Culture.

In Section 3.8.1, batch culture was described as growth in a closed system and the growth cycle was described as not being an inherent property of the microbial cells, but was the result of growth in a nutrient-limiting environment. Cessation of growth is the result of the exhaustion of an essential nutrient or accumulation of toxic products. Even during the exponential growth phase the composition of the medium and cells is continuously changing and as such steady-state growth is not possible. Continuous culture (67, 74-76), on the other hand, is a system for growing cells in an open system where the microbial population is maintained in a continuous state of balanced growth

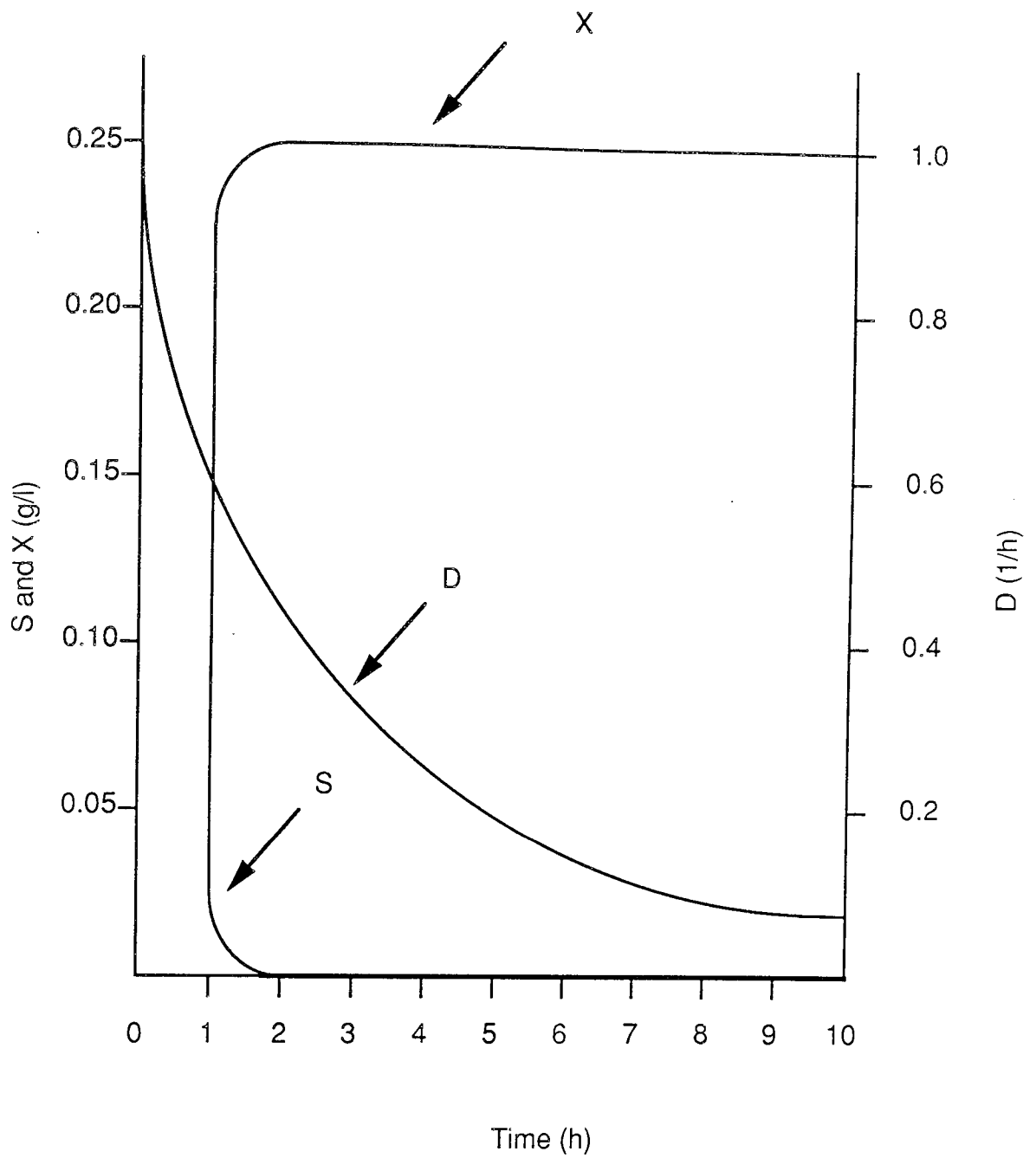


Figure 3.12

Dilution Rate, limiting substrate concentration and biomass concentration as a function of time in a fed-batch culture assuming 'quasi steady state'

by continuously removing some of the culture and replacing it with fresh medium at the same rate.

There are two types of continuous culture systems, the chemostat, and the turbidostat (70). The chemostat as used in this research is shown in Figure 3.13 and is operated by supplying an essential growth-limiting nutrient at a constant rate, with the result that the cell growth rate adjusts itself to the medium supply. The turbidostat is operated by maintaining a constant cell density by supplying fresh medium to the vessel as required.

A basic requirement for both systems is a method for maintaining a constant reactor volume. The simplest method and the method used in this project, is to use an overflow tube or weir set to a constant height within the reactor vessel so that as fresh medium is pumped into the fermenter, an equal volume of culture passes through the overflow tube to the collection vessel. In addition to controlling the medium flow into and out of the reactor, other important environmental parameters, including temperature, pH and DOC are also controlled.

3.8.3.1. Theory of Continuous Culture.

In a chemostat the growth rate is determined by the rate of supply of an essential nutrient which can be the carbon, nitrogen, phosphorus or a trace element or vitamin. All other essential nutrients are present in excess. The ability to vary the growth rate by changing the rate of supply of fresh nutrients (dilution rate) is the major advantage of continuous over batch culture (see Section 3.8.4). By maintaining the dilution rate constant, the culture can be maintained in a steady rate of growth indefinitely.

A material balance can be written for the cells in the process.

$$\text{Cell Accumulation} = \text{Cells In} - \text{Cells Out} + \text{Growth} - \text{Death} \quad 3.35.$$

$$\frac{dX}{dt} = \frac{FX_0}{V} - \frac{FX}{V} + \mu X - aX \quad 3.36$$

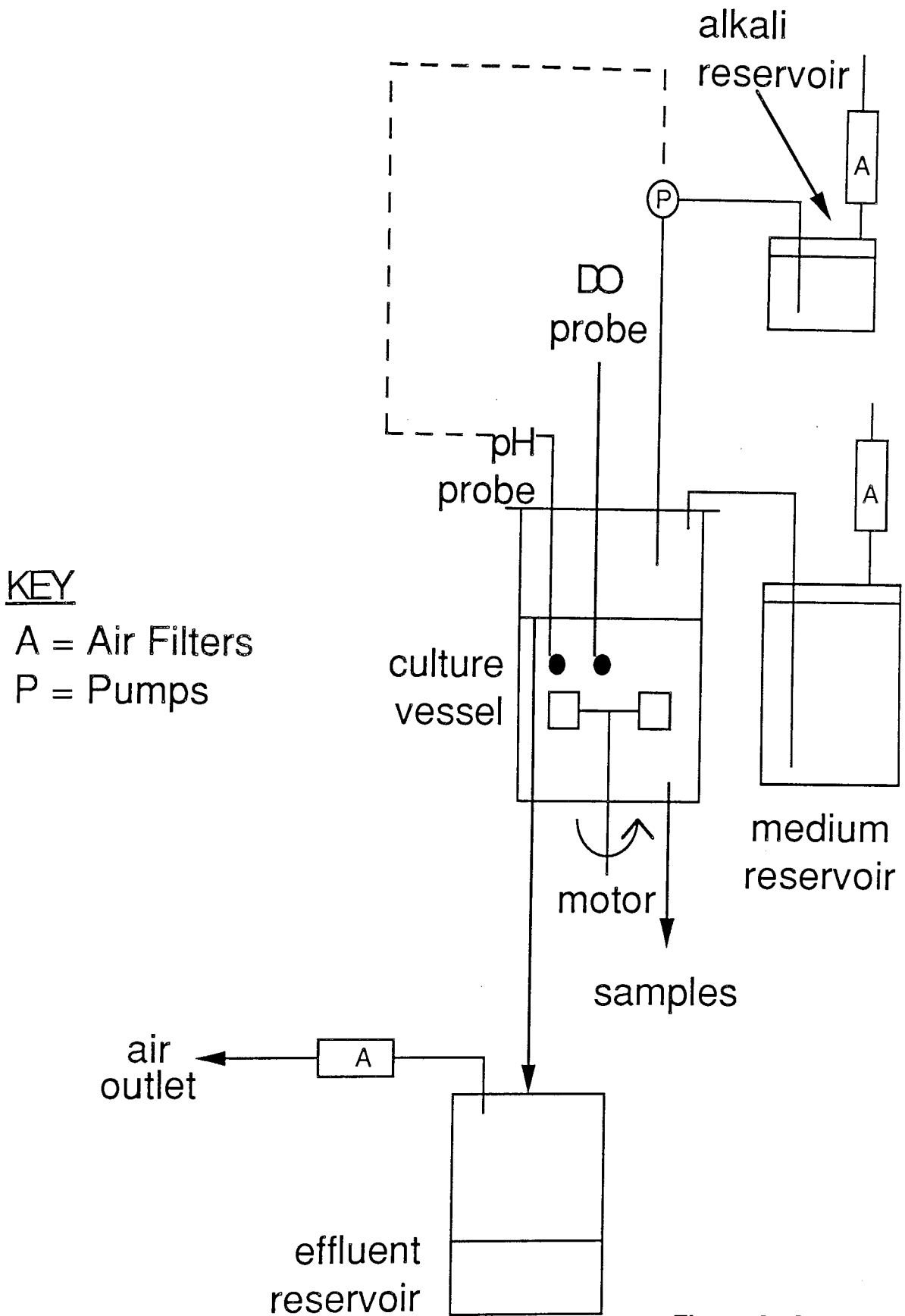


Figure-3.13

**Diagram of a Chemostat
 as used in this
 Research**

where X_0 is the cell concentration in the medium supply (g/l),
 X is the cell concentration in the reactor (g/l) and
 α is the specific death rate (h⁻¹).

With a single stage chemostat, the medium supply is usually sterile (no cells in), and $\mu \gg \alpha$. Equation 3.36 can therefore be simplified to :-

$$\frac{dX}{dt} = \frac{-FX}{V} + \mu X \quad 3.37$$

(F/V) is known as D, the Dilution Rate, and so Equation 3.37 can be written as:-

$$\frac{dX}{dt} = X(\mu - D) \quad 3.38$$

During steady-state growth (dx/dt) = 0, and therefore $\mu = D$. This means that the growth rate can be varied by altering the dilution rate. This is the case until $D > \mu_{max}$. Under these conditions the nutrient is no longer limiting and begins to build up in the vessel. As a result, there is a drop in cell concentration and subsequent wash-out of the culture. The critical dilution rate, D_c , is defined as the lowest dilution rate at which wash-out occurs. In practice, D_c is approximately equal to μ_{max} .

A material balance can be written around the chemostat on the limiting nutrient.

nutrient =	nutrient -	nutrient -	consump -	maintenance -	product
accum	in	out	tion for		formation
ulation			growth		3.39

$$\frac{dS}{dt} = \frac{FS_R}{V} - \frac{FS}{V} - \frac{\mu X}{Y_{X/S}} - mX - \frac{q_p X}{Y_{P/S}} \quad 3.40$$

where S_R is the limiting substrate concentration in medium reservoir (g/l)
and
 S is the limiting substrate concentration in the chemostat.

All the other terms in Equation 3.40 have been defined previously in this chapter.

As $mX \ll \mu X/Y_{X/S}$, this term can be neglected. When no products are formed, Equation 3.40 becomes :-

$$\frac{dS}{dt} = \frac{FS_R}{V} - \frac{FS}{V} - \frac{\mu X}{Y_{X/S}} \quad 3.41$$

At steady-state, $(ds/dt) = 0$ and $(F/V) = D$, therefore:-

$$\frac{\mu \bar{X}}{Y_{X/S}} = D(S_R - \bar{S}) \quad 3.42$$

And, at steady-state, $\mu = D$, therefore :-

$$\bar{X} = Y_{X/S} (S_R - \bar{S}) \quad 3.43$$

(At steady-state, X and S are labeled \bar{X} and \bar{S} by convention).

Equations 3.42 and 3.43 are the basic steady-state Equations that describe continuous culture. The factors X and S can be related to D by substituting values of μ in the Monod Equation, ie :-

$$\mu = \mu_m \left(\frac{S}{K_s + S} \right) \quad 3.44$$

From Equation 3.44, at steady-state $\mu = D$, and therefore :-

$$D = \mu_m \left(\frac{\bar{S}}{K_s + \bar{S}} \right) \quad 3.45$$

and solving for \bar{S} :-

$$\bar{S} = \frac{K_s D}{\mu_m - D} \quad 3.46$$

Substituting for S in Equation 3.43 gives :-

$$\bar{X} = Y_{X/S} \left(S_R - \frac{K_s D}{\mu_m - D} \right) \quad 3.47$$

These are the fundamental Equations that describe steady-state growth in a chemostat, and show that the steady-state substrate concentration, S , varies as a function of D alone, whereas biomass concentration, X , varies as a function of D and S_R (see Figure 3.14).

In continuous culture the biomass productivity, P_x (g/l.hr) is defined as :-

$$P_x = D\bar{X} \quad 3.48$$

Substituting 3.47 into 3.48 gives :-

$$P_x = D Y_{X/S} \left(S_R - \frac{K_s D}{\mu_m - D} \right) \quad 3.49$$

The relationship between productivity and dilution rate is shown in Figure 3.14.

3.8.4. Comparisons Between Batch and Continuous Techniques.

3.8.4.1. Advantages of Continuous over Batch Culture.

The major advantages are that the growth rate can be controlled and maintained indefinitely and the effect of changes in physical or chemical parameters on growth and product formation at constant growth rate can be examined. The biomass concentration can also be maintained constant by varying the dilution rate, with substrate-limited growth being maintained and changes in cell composition and metabolic activity being studied by changing the growth-limiting nutrient. Also, secondary metabolite production can often be maintained as cell growth is controlled. Continuous culture has an advantage in that values of kinetic constants, maintenance energy and true growth yields can be accurately determined. Results obtained in continuous culture are often more reliable and reproducible. There are higher productivities per unit volume, and less time lost in "down-time" involving cleaning, preparation, medium sterilization, etc. Continuous culture is often more economical due to these factors and because it is less labour intensive

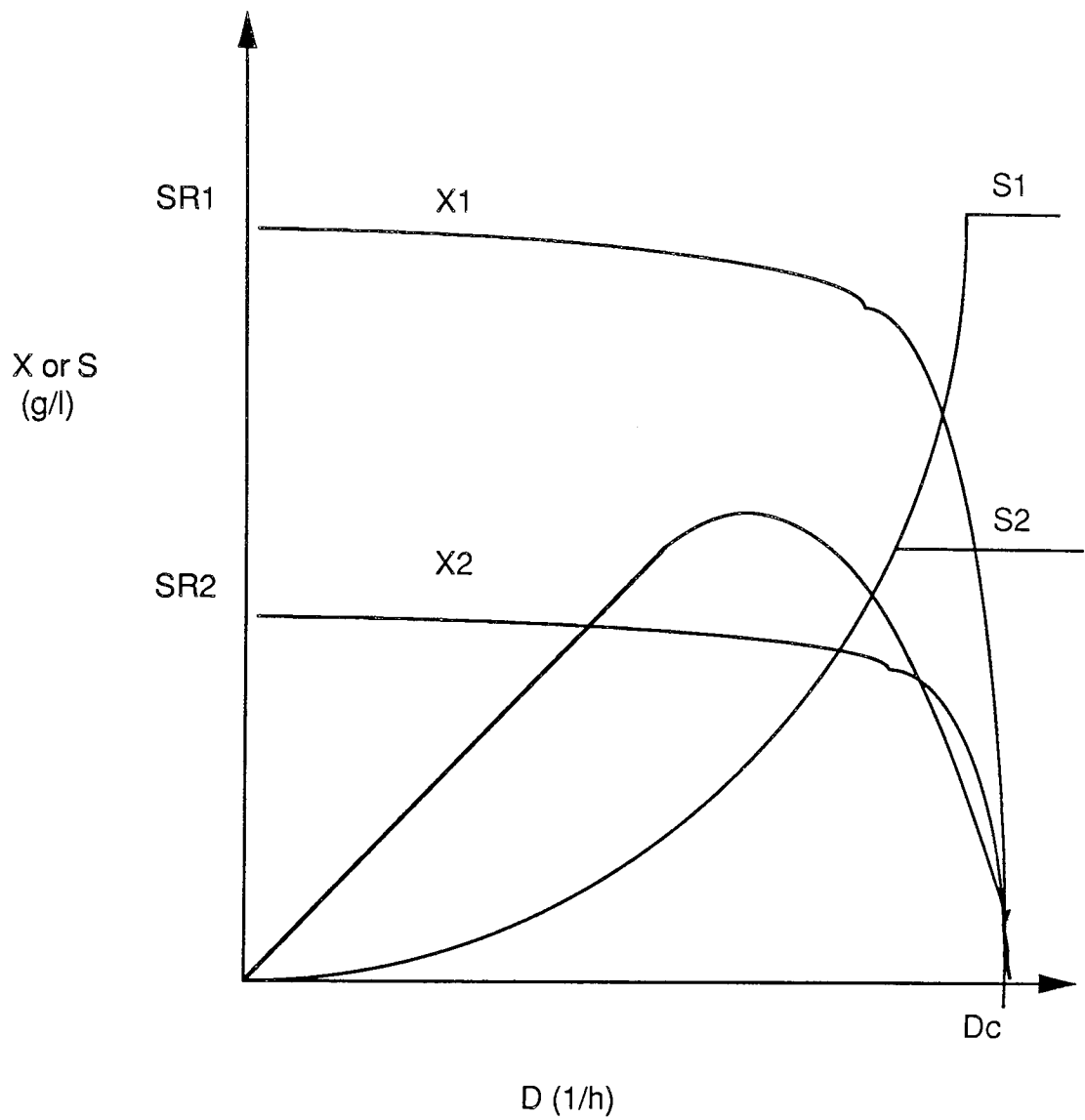


Figure 3.14

Relationship between steady state biomass, substrate, productivity and dilution rate

and involves smaller initial capital costs. It can also be used to maintain mixed cultures under steady-state growth conditions. In batch culture, one organism would usually outgrow the other, although in continuous culture a stronger organism will tend to washout a less dominant one, so care should be taken to avoid this.

3.8.4.2. Disadvantages of Continuous over Batch Culture.

The major disadvantages include the fact that the production of some non-growth related products cannot always be achieved and often require fed-batch culture techniques. Wall growth and cell aggregation can cause wash-out or prevent true steady-state growth. A faster growing strain of an organism can take over a system with continuous culture over long periods, resulting in loss of the original strain. The growth of filamentous organisms is often difficult due to the viscosity and heterogeneous nature of the culture, preventing steady-state growth. It is also important that aseptic techniques be carried out very efficiently to prevent any incidence of contamination, which becomes increasingly likely to occur with longer fermentations. The plant and auxiliary equipment must be of high quality and reliability to avoid mechanical failures during fermentations.

These are the major advantages and disadvantages of continuous over batch culture, and all factors must be taken into consideration when deciding whether it would be feasible to progress from the batch or fed-batch fermentation of a particular product into the continuous mode.

3.9. Bioreactor Hydrodynamics.

The purpose of a bioreactor (77) is simply to facilitate and promote microbiological growth and/or metabolism in order to produce a product. When designing a bioreactor, many factors should be considered along with the wide variation in process requirements for different microbial factors. The following sections will discuss some of the factors that are taken into consideration when designing a bioreactor and also some basic theory of fluid rheology, agitation and shear and how these factors affect the cell growth and the overall fermentation.

3.9.1. Bioreactor Design Considerations.

The major objectives that need to be taken into account when designing a bioreactor are listed in Table 3.2 below.

Table 3.2.
Bioreactor Design Objectives.

Exclude the unwanted/contain as required.

Allow the supply of nutrients (solids, liquids, gases) for growth and/or metabolism.

Facilitate the removal of such products as it is desirable, or possible to remove during the production cycle, selectively if appropriate.

Facilitate contact between phases as is necessary for transfer purposes.

Enhance contact between appropriate phases and heat transfer surfaces.

Allow for the bulk flow of nutrients in the reactor that is optimal with regard to the kinetic and other rate-limiting dynamics of the system and appropriate control loops.

It is important to be able to operate a bioreactor with the minimum possibility of contaminant organisms penetrating the vessel. A potential weakness in this respect are the shafts for the agitation drives and submerged seals for bottom-driven agitators. The use of magnetic stirrers would eliminate these problems, if it were possible for them to be operated reliably. The vessel must be capable of fulfilling three separate heat transfer processes, that of sterilization, cooling the bioreactor from sterilization to the operating temperature and maintaining the fermentation at a required temperature. The problem of stress corrosion due to inadequate design and manufacture can arise here, in internals such as heating/cooling coils. Penetration of contaminants can also occur due to a lack of thought in general bioreactor layout and construction.

Nutrient supply may be required to fulfil a steady-state demand or far more commonly an unsteady state demand. The former is very rare, occurring only in continuous operations, with truly batch processes also being uncommon. By far the most common mode of operation is fed-batch with the controlled addition of one or more nutrients over part(s) or all of the fermentation cycle, with requirements varying during different parts of the reaction cycle. It is important that the bioreactor is capable of maintaining nutrient concentrations

in the medium within the acceptable maximum (avoiding any toxic or inhibitory effects) and minimum (process-limiting) levels throughout the reaction volume, while the process demands for factors such as oxygen and heat transfer are changing.

It is also necessary to remove certain reaction products from the vessel over the course of the fermentation. Certain by-products of the microbial process may have limiting effects on the production of the required metabolite and so would have to be removed. Also many bio-products are transported away from the reaction in the gas phase, although this is mainly carbon dioxide. Whatever is done to alter oxygen transfer rates into the reaction invariably affects outward transfer rates and consequently the concentration of CO₂ in solution. Carbon dioxide is known to have some regulatory effects on metabolic activity and plays a role in regulating medium pH and mineral availability.

It is also important to increase the contact between various phases in the fermentation, as is necessary for mass transfer processes. An important factor here is the availability of oxygen and its utilization rate. The oxygen demand of an industrial fermentation process is usually satisfied by aerating and agitating the fermentation broth. Oxygen is normally supplied to microbial cultures in the form of air, this being the cheapest available source of the gas. The actual transfer of oxygen from air to the cell during a fermentation can be said to occur in a number of steps, with the first being the transfer of oxygen from an air bubble into solution, the second being the transfer of the dissolved oxygen through the fermentation medium to the microbial cell and the third being the uptake of the dissolved oxygen by the cell. It is thought that the limiting step in this process is the transfer of oxygen into solution. The rate of oxygen transfer from air bubble to the liquid phase can be described below :-

$$\frac{dC_L}{dt} = K_L a (C^* - C_L) \quad 3.50$$

where C_L is the concentration of dissolved oxygen in the fermentation broth (mmoles/l),
 t is the time (h),
 (dC_L/dt) is the oxygen transfer rate (mmoles/l.h),
 K_L is the mass transfer coefficient (cm/h),

a is the gas/liquid interface area per liquid volume (cm^2/cm^3), and C^* is the saturated dissolved oxygen concentration (mmoles/l).

K_L may be thought of as the reciprocal of the resistances to the transfer of oxygen from gas to liquid. It is extremely difficult to measure both ' K_L ' and ' a ' in a fermentation and so the two terms are usually combined in the term K_La , the volumetric transfer coefficient, the units of which are h^{-1} . The bioreactor must therefore be designed to allow for a K_La value such that the optimum oxygen concentration for product formation can be maintained in solution throughout the fermentation.

Problems of heat transfer are closely bound up with those of mass transfer, indeed, successfully improving mass transfer within the vessel causes more heat transfer problems to arise. Heat can be generated in a bioreaction in one of three ways. Heat is produced with the metabolic activity of the cells, by agitation and by aeration. The removal of this heat can be a problem, and is one that is solved during the design stage of the bioreactor, with the incorporation of internal coils and baffles.

Generally, fermentation processes involve a progression from one physiological state of the cell to another, over a time period that can be measured in hours, if not days. A culture may require different nutrients for growth phases than for the production of any metabolites. This is quite often the case with batch processes, with continuous fermentations usually applied to products based directly on cell growth.

There is another factor that is concerned both with the bulk flow of the reactants and transport processes within the medium, which is that the manner in which both are achieved will result in positional variations in the fluid shear rate, and perhaps local shear stress. It is important to determine whether such local variations may in turn lead to stressful and damaging effects on the cells themselves, or even if such effects can be used positively to improve performance.

3.9.2. Fluid Rheology.

Turbulence and rheological properties (78) are linked directly to flow patterns, power draw, mixing times, oxygen transfer rates and stresses on biological materials, amongst other factors (79).

For fluids undergoing laminar shear, as shown in Figure 3.15, the resistance to deformation depends on the dynamic viscosity. For a linear velocity gradient arising due to the movement of one parallel plate over another, the relationship between shear rate, $\dot{\gamma}$, and shear stress, τ , is given by :-

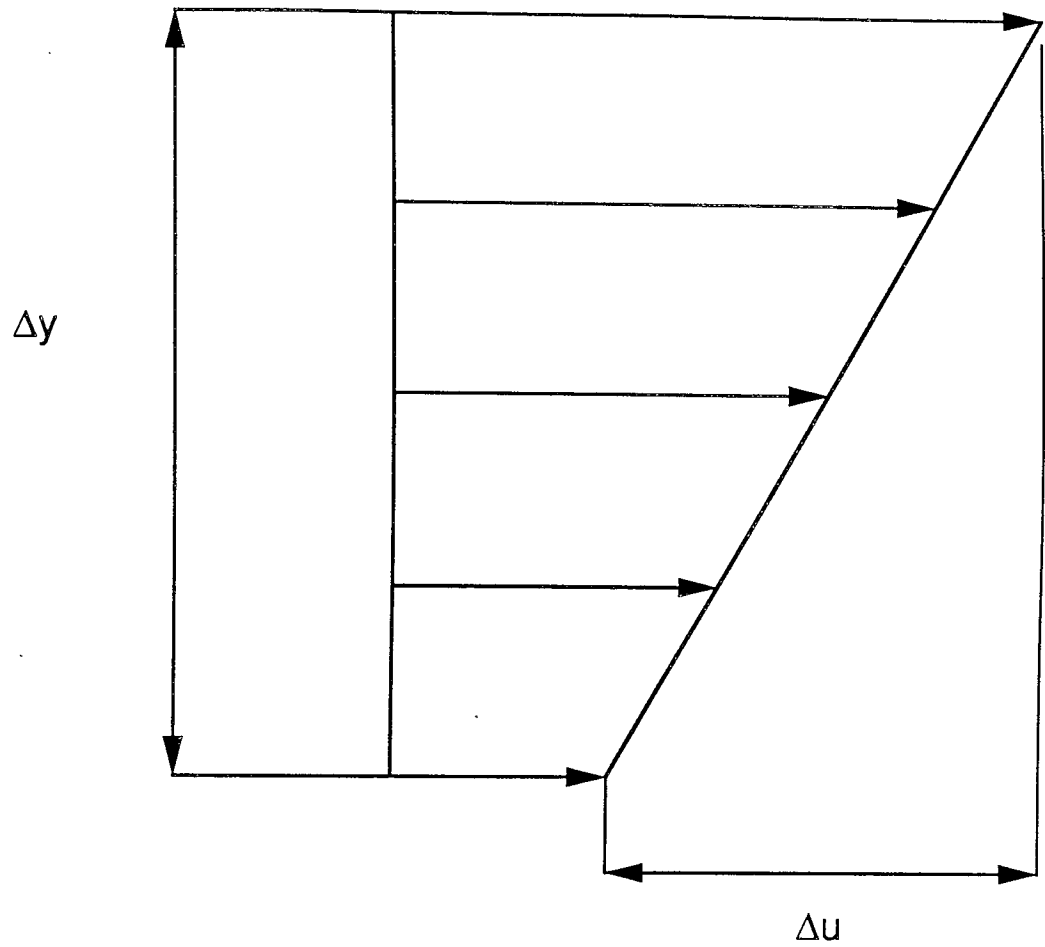
$$\tau = \mu du/dy = \mu \dot{\gamma} \quad 3.51.$$

where for Newtonian fluids, viscosity μ is constant and independent of shear rate, $\dot{\gamma}$. Fermentation media and broths containing simple cells in suspension will generally remain Newtonian with viscosities not much greater than water, as is the case with the broth in this project.

For many biological fluids, the viscosity is a function of shear rate, with this generally being the case for fluids containing large, complex molecules in solution, such as polymers (dextran). These are non-Newtonian fluids, and can be classified as either pseudoplastic or plastic. Many broths exhibit pseudoplastic properties, with apparent viscosity μ_a given by :-

$$\mu_a = \tau/\dot{\gamma} \quad 3.52.$$

With these fluids, viscosity decreases with increasing shear rate. There is usually good mixing in a bioreactor, near to the impeller, although there may be near-stagnant regions in the remainder of the vessel. Plastic materials are characterised by a yield stress, τ_y . This is found particularly with high viscosity polysaccharide broths (such as dextran and xanthan). In these cases, a minimum stress is required to break down the structure of the molecule. In this case, the well-mixed region close to the impeller, called a cavern, is often accompanied by a broth which elsewhere is only agitated by the sparged air.



$$\text{velocity gradient} = \frac{dy}{du}$$

$$= \text{shear rate} = \tau$$

Figure 3.15

Laminar shear flow and shear rate

Shear stress and shear rate can be related using the power law model, shown in Equation 3.53 :-

$$\tau = K\dot{\gamma}^n \quad 3.53.$$

where K is the consistency index and n is the flow behaviour index. If $n < 1$, the fluid is pseudoplastic; if $n = 1$, it is Newtonian, and $K = \mu$.

Figure 3.16 shows the way viscosity varies with shear rate for Newtonian, Plastic and Pseudoplastic fluids.

The rheological properties of the culture broth can affect the $K_L a$ value achieved in a fermentation vessel, along with other factors, such as the air flow rate, agitation rate and antifoam agents.

3.9.3. Mixing Fundamentals.

There are many purposes for mixing in a fermentation vessel (68, 80), including the need to suspend or disperse nutrients throughout the broth to all the cells, and to increase the uniform quality of the medium. The mixing of liquids is generally straight-forward, particularly if the liquids are of low viscosity and the material can be blended by a turbulent mixing operation. However, many mixing operations involve more than one phase, which almost always involves the inclusion of mass transfer as well. It is necessary in biological solutions that there be a homogeneous suspension of cells throughout the culture fluid. This process requires a high energy input to the agitator. There are many factors that can affect mixing in a bioreactor, one being the inclusion in the vessel of baffles. The primary circulation induced by a rotary mixer is a bulk rotation of fluid, with even axial flow agitators not producing much vertical motion unless the large swirl is hindered. This can be achieved with the use of wall baffles, four of which can usually provide enough braking action on the mixing swirl in low viscosity media. The profile of the bottom of the vessel also has a very big influence on the mixing capability of a system. A flat-bottomed tank will encourage the formation of "dead-zones" in the bottom of the vessel and a conical end would create a "dead-zone" in the base, which would be full of suspended solids. The preferred base is one with a dished end shape,

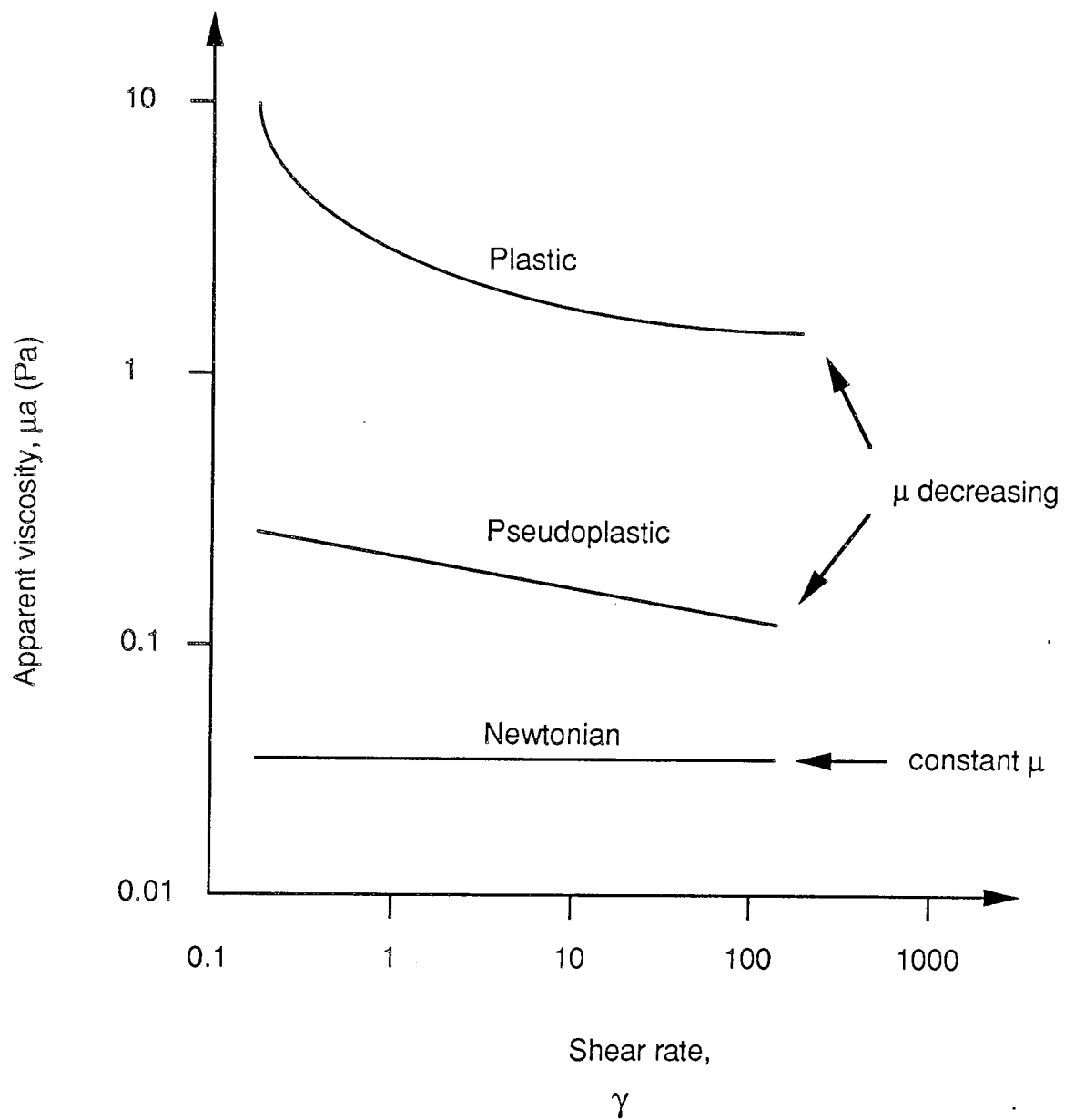


Figure 3.16

Examples of viscosity versus shear rate (log-log plot)

that will allow uniform mixing throughout the vessel, without the formation of any unwanted stagnant regions.

There are many difficulties in designing and operating mixing processes, with typical problem areas being in the prediction of mixing performances in the laminar and transitional flow regimes; in relating batch mixing time to residence time distribution and vessel homogeneity through the flow equipment; in the blending of liquids with very different viscosities; in the homogenization of highly non-Newtonian fluids, where local properties can be very variable; in dealing with a requirement for the rapid distribution of a component throughout a large volume of liquid; in predicting the effect of vessel internals on the mixing performance; and in using vessels of non-standard geometry. In these cases investigation of the particular problem is often the only course open, involving bench or pilot scale experiments.

Many bioreactor designs ⁽⁸¹⁾ are based upon tall vessels, and the reason for this will be discussed below. In general, biological processes are slow so that the rate of production of the desired product is usually limited. These slow kinetics lead to a need for very large reactor volumes and therefore there are difficulties in ensuring adequate homogenization throughout the whole vessel. Bioreactors are often tall as it is much easier to make and transport vessels which are tall for a given volume, than simply to increase the dimensions in all directions equally. The requirements of supplying nutrients over the whole of the reactor volume and maintaining a certain minimum oxygen level throughout, sets demands upon the large scale mixing performance. The preference for tall vessels coupled with the need to ensure good large scale circulation has led to the development of mixing vessels with multiple impellers. The impellers should not normally be mounted closer together than their diameter if interaction and consequent loss of mixing efficiency is to be avoided.

There has been a great deal of detailed research into this area in recent years, although it is not necessary to discuss more than basic mixing fundamentals here.

3.9.4. Shear Effects on Biological Materials in Bioreactors.

Many biological materials are delicate ⁽⁸²⁾ and can be adversely affected by processing. Shear is considered to be particularly important when taking into

account the effects it has on biological substances. Stirred vessels are used for many operations in the biological process industries and agitation is used here generally to ensure homogeneity of the vessel contents, to promote mass and heat transfer and to suspend particles in a fluid. Increased agitation will improve the performance of the system in these areas but will result in increased shear and other forces to which the vessel contents will be exposed.

Proteins are important products of industrial biotechnology either for their enzymatic or other biological activity. Proteins in solution are often subjected to shear and other forces during processes. Shear forces are often believed to be a possible cause of denaturation of proteins and of enzyme inactivation. There have been extensive studies in recent years on the effects of shear in proteins. Loss of enzyme activity has often been observed and attributed to shear, but Thomas and co-workers (1979) (82) showed that the effects of shear on proteins in solution were less severe than had been previously thought, provided that the proteins were subjected to shear alone. They showed that no significant losses in enzyme activity were observed provided air-liquid interfaces in the medium were avoided and reasonably high protein levels used. It would appear that a combination of high aeration and agitation is required for "shear" damage of enzymes to occur. They concluded that it should be possible to reduce or even eliminate "shear" damage to proteins in unaerated stirred tanks and even "attrition bioreactors" They realised that problems would still exist with aerobic fermentations where high degrees of mixing, in excess of those provided with aeration alone, were required.

In general, unicellular bacteria, such as *Leuconostroc mesenteroides*, are not considered to be shear-sensitive. Typical bacteria have sizes of approximately one micron, which is usually less than the microscale of turbulence in the fermenter. In addition, bacteria have strong cell walls. Despite some reports of changes of bacterial cell volumes due to agitation, it is reasonable to assume that shear of bacteria in fermentation processes is of no importance, although at certain very high tip speeds these problems may begin to occur.

4. BASIC BIOTECHNOLOGY.

4.1. Cell Structure.

Bacteria, yeast, animal and plant cells are all very different living organisms, with several factors in common with each other (83). They all contain within them proteins, ribonucleic acid (RNA) and deoxyribonucleic acid (DNA), which are the elements required for the flow of genetic information from DNA, via messenger RNA, to proteins. Also, all organisms perform certain common chemical activities, known collectively as metabolism, whereby they synthesize their cellular constituents from external chemicals. Finally, organisms share a common basic cellular structure. Cells can be divided into two groups ; eukaryotic and prokaryotic, depending principally on whether their genes are contained in a well defined nucleus or not. Prokaryotes are mainly bacteria and a few species of blue-green algae. They are simple cells with a chromosome number of one, and do not contain a nuclear membrane or take part in mitotic nuclear division. They contain relatively small quantities of DNA as compared to eukaryotes. Eukaryotic cells are mainly those of plants and animals and are much more complex in structure. They have chromosome numbers of greater than one and contain a nuclear membrane, with nuclear DNA bound to histones (a protein associated with nucleic acids in the chromatin of eukaryotic cells) and DNA present in organelles. Eukaryotes undergo mitotic nuclear division. Eukaryotes contain large quantities of DNA - in terms of number of molecules of DNA present in each cell, eukaryotes contain between 180 - 600, whereas prokaryotes contain only one.

The organism used in this research belongs to the prokaryote class (84-86), and so the cell structure of prokaryotes only will be discussed below. Prokaryotic bacteria contain a cell wall, which determines the shape of the cell and is required because bacteria do not possess an active osmoregulation system, whereby they can maintain the correct concentration of water, salts and other solutes. Bacterial cell walls can be split into two types, as shown in Figure 4.1, with the cell types being distinguished as Gram-positive or Gram-negative by means of the Gram Stain Test (See Section 5.4.6). External to the bacterial cell wall there is often found a characteristic mucilaginous material which, when firm, is often referred to as a capsule. This outer layer appears to have no well-defined function although in some cases it can be antigenic. Beneath the cell wall is the cytoplasmic membrane, about 80 Å thick, which represents the only real membrane structure in bacteria in

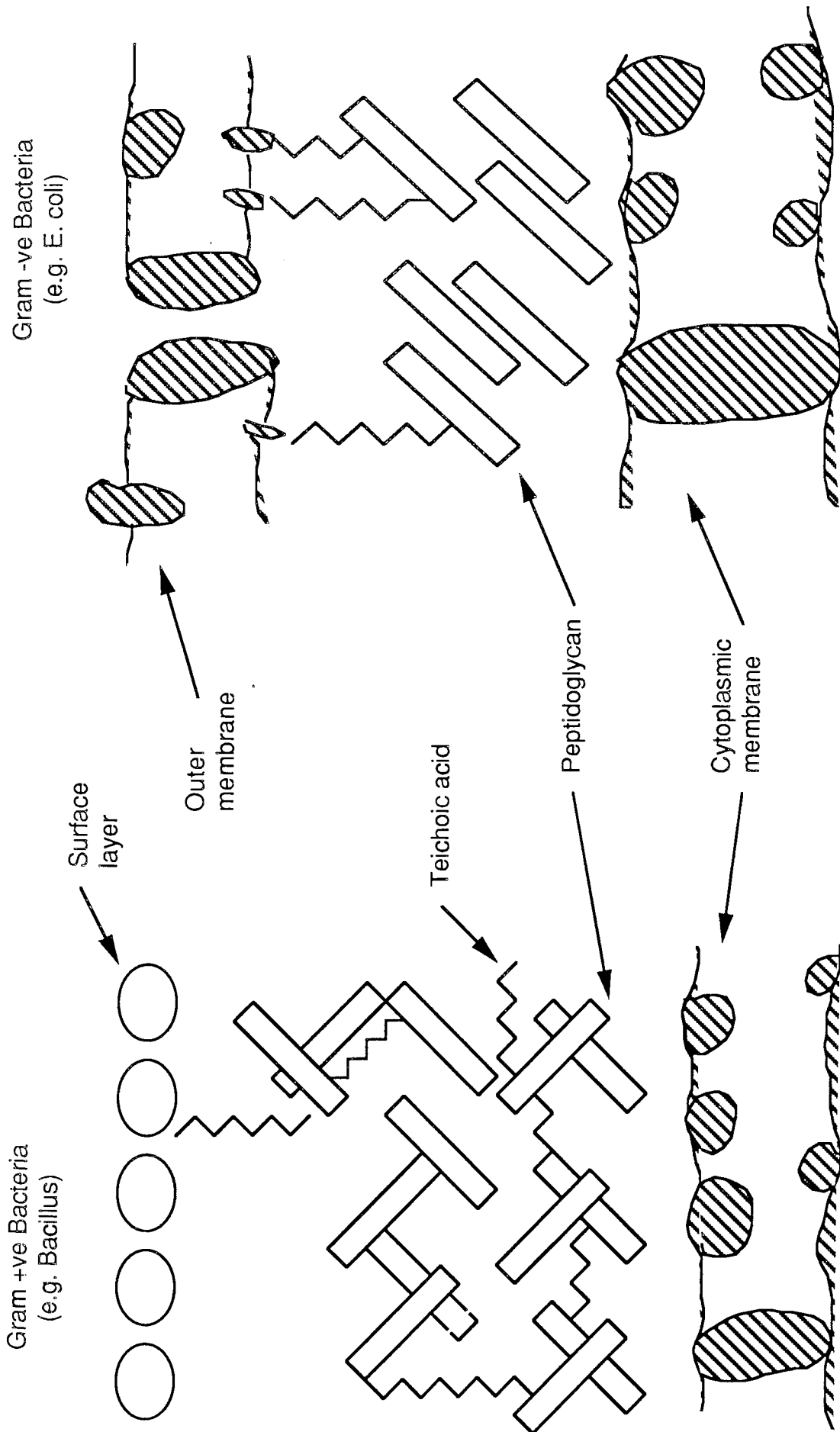


Figure 4.1
Bacterial Cell Wall Types

contrast to the eukaryotes. This membrane acts as a semi-permeable barrier which can determine the solutes that leave or enter the cell and it acts as a site of a number of enzymes. Certain bacteria are motile, propelled by flagella anchored in the cytoplasmic membrane. They are hollow structures with walls formed from helical rows of spherical protein subunits and are too fine to be seen through a light microscope. However, they do show up when coated with silver. The cytoplasm of bacteria contains very little as far as structure is concerned, apart from one or two aggregates of nuclear material. However, a structure that can be found in certain Gram-positive species is one known as Endospore. When conditions become difficult at some stage of the cell growth cycle, some bacteria form a refractile spore from an invagination (a tissue folding within another) of the cytoplasmic membrane and its thick wall infers a resistance to heat or drying. Spores can cause problems in fermentations, as small numbers can resist sterilization and so cause contamination to occur. The structure of a prokaryotic cell can be shown diagrammatically in Figure 4.2.

4.2. Cell Chemistry.

Microorganisms, like all other living cells, require certain nutrients for their growth and development (87). These nutrients must contain the chemical elements which are components of cellular materials, as well as those required for membrane transport, enzyme activity and for the generation of the energy required for biosynthetic processes. Water accounts for between 80 and 90% of the total cell dry weight. The remainder of the cell consists of hydrogen, carbon, oxygen, nitrogen, sulphur and phosphorus, in addition to about 18 other elements, frequently present in minute amounts. C, H, O, N, S and P form the bulk of the cell walls, proteins, lipids, membrane structures, nucleic acids and other cellular components. These molecular compounds are relatively stable and react with each other in the presence of enzymes (biological catalysts). Enzymes often require ions or trace elements as structural components in order to be catalytically active. Microorganisms exhibit some diversity as to the compounds that they are able to utilize as sources of C, H, O, N and S. Sulphur is usually taken up by the cells as sulphate and reduced to sulphide for biosynthetic purposes. However, certain bacteria are capable of growth on reduced sulphur compounds such as sulphide, elemental sulphur or thiosulphate, whilst methanogens only grow in the presence of H₂S. Nitrogen occurs naturally in many forms, including NH₄⁺, NO₃⁻, NO₂⁻, nitrogen containing organic compounds and molecular nitrogen. Most microorganisms can utilise NH₄⁺ which is the preferred

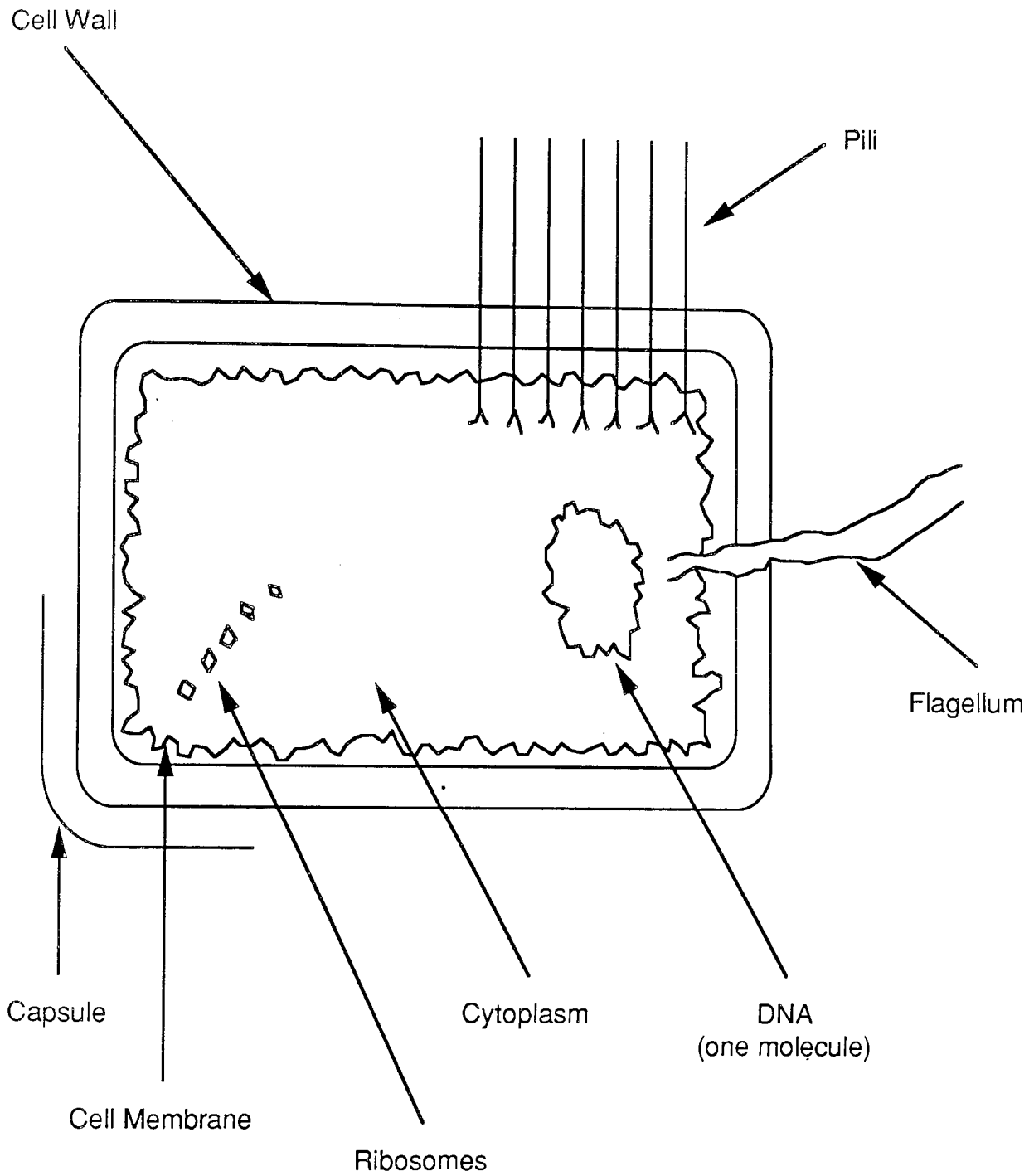
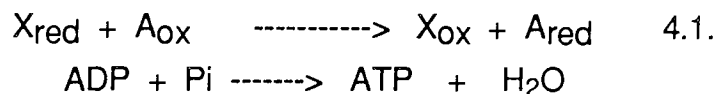


Figure 4.2
Generalised Bacterium (Prokaryote)

substrate. Some organisms can use NO_3^- , which must first be reduced to NH_4^+ , usually by some denitrifying bacteria. Certain 'nitrogen-fixing' bacteria are able to utilize molecular nitrogen, whilst many organisms can utilize complex, organic sources of nitrogen including amino acids, nucleic acids and methylated amines. C, H and O can be utilized by microorganisms in the form of either organic or inorganic compounds, including CO_2 , H_2 , H_2S , NH_4^+ , NO_3^- , SO_4^- , etc. With the possible exception of lignin and certain artificially-made organic polymers, almost all carbon compounds are potential growth substrates for microorganisms.

The nutrients required by a microorganism are not only for biosynthetic reactions for cell growth, but also serve as a source of energy to 'fuel' the biosynthetic, energy-requiring metabolic processes. Certain organisms use light as their energy source, whereas others derive their energy from chemical reactions. These are known as phototrophs and chemotrophs respectively. Phototrophs usually use CO_2 as their cellular source of carbon, in which case they should be termed photoautotrophs. During such growth the CO_2 must be reduced to the cellular level, usually using inorganic electron donors such as H_2 . When growing in this way they are termed photolithotrophs. Alternatively certain phototrophs use organic carbon sources, such as succinate or acetate, where the organic substrate supplies the electrons used in reduction reactions and the organisms grow as photoorganotrophs. Most microorganisms gain energy (ATP) for growth by oxidation-reduction reactions of the form :-



where one substrate is reduced at the expense of another. Most yeasts, fungi and higher organisms use organic electron donors (X_{red}) and O_2 as electron acceptor. Bacteria, however, may use NO_3^- , SO_4^{2-} , or CO_3^- as an electron acceptor in what is termed anaerobic respiration, organic compounds in anaerobic fermentations, or O_2 in respiration. The electron donor, X_{red} , may be organic or inorganic (eg. H_2S , H_2 , Fe^{2+} , NO_2^- or NH_4^+). Organisms which use an organic electron donor are called chemoorganotrophs, whilst those using inorganic donors are termed chemolithotrophs.

4.2.1. Requirements for Growth.

Most yeasts and algae are able to synthesize all the organic compounds needed for growth from the carbon source (88). Many bacteria are unable to do this and require specific growth factors to be present in the medium, in addition to a carbon and energy source. The most common requirement is for vitamins which are required in extremely small amounts as cofactors for various enzymes, cofactors being non-protein compounds that are essential for the catalytic activity of the enzyme. *Clostridium kluyveri*, for example, requires a medium supplemented with biotin and p-aminobenzoic acid, whereas *Leuconostoc mesenteroides* requires nicotinic acid, thiamine, pantothenic acid and biotin. Whilst it would seem logical to include high concentrations of the required compounds in the cell media, care should be taken to avoid any possible inhibitory effects. Table 4.1 lists some of the vitamins and related compounds which are frequently required for growth.

Table 4.1.
Vitamins and Related Compounds Required for Growth.

<u>Compound</u>	<u>Metabolic Function</u>
p-aminobenzoic acid	tetrahydrofolate precursor, involved in transfer of one-carbon units.
biotin	coenzyme involved in carboxylation reaction.
folic acid	tetrahydrofolate involved in transfer of one-carbon units.
nicotinic acid	precursor of NAD and NADP.
pantothenic acid	precursor of coenzyme A (carrier of acyl groups).
pyridoxine (Vit. B ₆)	pyridoxal phosphate involved in transamination and decarboxylation reactions.
riboflavin (Vit. B ₂)	component of FMN and FAD-prosthetic groups of flavoproteins.
thiamin (Vit. B ₁)	component of thiamine pyrophosphate in decarboxylases, transaminases and transketolases.
cyanocobalamine	(Vit. B ₁₂) a coenzyme involved in re-arrangement reactions (glutamate mutase).
vitamin K	precursor of menaquinone - an electron carrier (eg. fumarate reductase).
coenzyme M	involved in methanogenesis.

4.3. Microbial Physiology.

Physiology is the study of the functions of organisms. This section will concentrate on some basic microbial stoichiometry, along with product control and inhibition. Living organisms can be thought of as complex chemical reactors in which more than 1,000 independent enzyme-catalysed reactions occur, with the total of chemical reaction activities taking place within the cell being called metabolism. A cell maintains itself, and produces offspring, from its environment, with cell functions changing significantly in the presence, or absence of oxygen. The energy obtained from the environment is typically stored and transported around the cell in the form of high energy intermediates, such as ATP. The cell uses this energy to perform three types of work: chemical synthesis of large or complex molecules (growth), transport of ionic and neutral substances into or out of the cell or its internal organelles, and mechanical work required for cell division and motion. The reactions within the cell can be subdivided into three classes: degradation of nutrients, biosynthesis of small molecules and biosynthesis of large macromolecules. As already mentioned, the number of different chemical reactions necessary for sustenance of cell life is of the order of 1,000 or more, each being catalysed by an enzyme. The enzymes serve the essential function of determining which reactions occur and their relative rates. A further function of the cells is the release of metabolic end products. Many of these compounds are substances unnecessary or useless for the cell's function; others, eg. antibiotics and extracellular enzymes, serve a purpose. Industrially, these end products are often valuable, making it profitable to produce them by growing cells. Often, a species of organism is chosen, for a particular process, that is inefficient from a biological point of view, such that it produces much more of a commercially valuable biochemical.

4.3.1. Stoichiometry (89).

Cell growth obeys the laws of conservation of matter. Atoms of carbon, nitrogen, oxygen and other elements of life are rearranged in the metabolic processes of the cell, with the total amounts of each of these elements incorporated into cell material being equal to the amounts removed from the environment. Also the amount of some metabolic product formed or the amount of heat released by cell growth is often proportional to the amount of consumption of some substrate or the amount of formation of another product, such as CO₂. Cell growth involves the consumption of substrates which provide energy and raw materials required for the synthesis of additional cell mass. The free energy of substrates consumed should exceed the free

energy of cells and metabolic products formed. It is also necessary that the cellular enzymes are capable of utilizing the various nutrients supplied to the cell for growth and product synthesis. The elemental composition of different microbial cells is quite consistent, yet actual medium formulation can be more complicated for several reasons. Some substrate elements are released within the products and not drawn into the cells themselves. Rate limitations as well as stoichiometric limitations must be considered when designing the medium. Also specific nutrients may be limiting, or specific products may be inhibitory, due to the metabolic properties of a particular cell strain.

The stoichiometrically limiting nutrient in the growth medium can be identified on the basis of growth stoichiometry. If cell growth is viewed on an overall basis, with cells consuming substrates, and converting them into more cells and metabolic products, the stoichiometrically limiting nutrient is the one that will be completely exhausted first. This is different from the growth limiting component, whose concentration in a specific medium can suddenly increase, resulting in an increase in cell growth rate. The growth limiting component is not necessarily the same as the stoichiometrically limiting component. This is because one compound may limit the growth rate of the cells under certain environmental conditions, but it may be the exhaustion of a different compound that causes the growth of the cells to stop in a batch system.

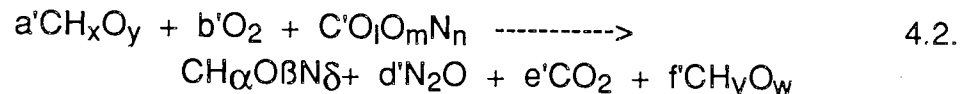
During cell growth there are a variety of metabolic end products that are released into the growth medium, or accumulated intracellularly. The stoichiometries for product formation can usually be classified into the following four groups :-

- (i) The main product appears as a result of primary energy metabolism, an example being ethanol production during anaerobic growth of yeast.
- (ii) The main product is formed indirectly from energy metabolism, for example citric acid formation during aerobic mould cultivation.
- (iii) The product is a secondary metabolite with an example of penicillin production in aerated mould culture.

(iv) Biotransformation. The product is obtained from the substrate through one or more reactions catalysed by enzymes in the cells. An example of this process is steroid hydroxylation.

Dextranase formation falls under the third of the categories mentioned above.

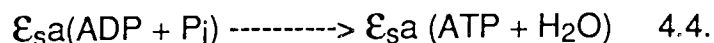
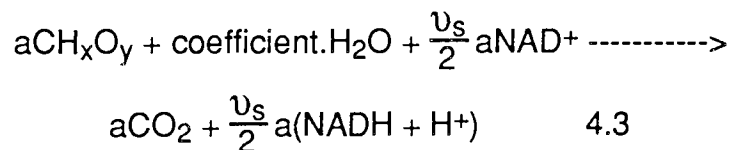
Class (i) processes have a relatively simple stoichiometric description. Product appears in relatively constant proportions as cell mass accumulates and substrate is consumed. Here the processes of substrate utilization, cell mass synthesis, and product formation can be lined in a simple chemical reaction, as shown in Equation 4.2 below :-



where CH_xO_y is the carbon source,
 $H_eO_mN_n$ is the nitrogen source,
 $CH_\alpha O_\beta N_\delta$ is the cell, and
 CH_vO_w is the product.

a' , b' , c' , d' and e' are unknown stoichiometric coefficients.

In class (ii) cases, the simple stoichiometry of Equation 4.2. does not apply. Product formation is not necessarily proportional to substrate utilization or cell mass increase. Representation of the stoichiometry in such a case requires an independent reaction Equation for product formation. Energy source dissimilation is shown in Equations 4.3 and 4.4 :-

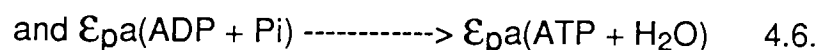
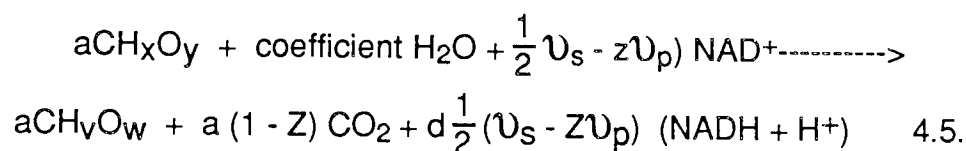


The parameter s denotes the degree of reductance of carbon source (substrate). The reductance degree is the number of equivalents of available electrons per g atomic carbon, based on the following values :-

Carbon = +4 ; Hydrogen = +1 ; Oxygen = -2 ; Nitrogen = -3 (i.e. the ionic charge of the atom after the required number of electrons have been added or removed to provide a full outer shell of electrons).

The stoichiometric coefficient of water has been written simply as "coefficient" as water consumption or production is usually not significant relative to the amount present. Also ϵ_s denotes the number of substrate-level phosphorylations per carbon mole passing through a dissimilation metabolism.

The addition of a product formation step to Equation 4.3. gives :-



where p is the degree of reductance of product,
 Z is the carbon fraction of a substrate used for product formation which is found in the product and
 ϵ_p is the number of ATPs generated by product formation.

The stoichiometric descriptions for classes (iii) and (iv) depend on the particular substrates and products involved. Product formation in these cases is usually independent of cell growth. Secondary metabolite accumulation is dictated by kinetic regulation and activity of the cells.

As mentioned previously, there are very many extremely complicated reaction processes that occur as a cell grows. However, a few central features occur quite often and provide a helpful unifying link between all aspects of cell metabolism. The most important common aspects of cell biochemistry are probably ATP's role as an energy carrier, and the reducing power shuttle, NAD. Stoichiometric relationships can be used to interrelate the chemical changes which result from cell metabolism (see Equations 4.2 - 4.6).

4.3.2. Microbial Control of Product Biosynthesis. (57)

As discussed in the previous section, an organism grows and forms products using a large number of enzyme-linked metabolic pathways. It is through these pathways that microbial control is achieved. The control of these enzymes can take two basic forms; the alteration of enzyme activity or alteration of the number of enzyme molecules. There are a number of methods of control within this classification, and they are shown in Table 4.2.

Table 4.2.
Biochemical Controls. (83)

- A. Alteration of Enzyme Activity.
 - 1. Substrate Control.
 - 2. Allosteric Control - feed-back control.
 - a) Simple
 - b) Sequential
 - c) Multiple Enzyme
 - d) Concerted
 - e) Cumulative
 - 3. Integrated Control via energy charge.
 - 4. Enzyme Modification.
 - a) Irreversible covalent modification
 - b) Reversible covalent modification
- B. Alteration of the Number of Enzyme Molecules.
 - 1. Transcriptional control.
 - 2. Translational control.
 - 3. Enzyme degradation.

The simplest level of control is that which is exerted by the substrate level itself. If the substrate level is about or below the K_m of the enzyme, the velocity of the enzyme reaction will be related to the substrate concentration according to the Michaelis-Menton relationship.

$$V = \frac{V_{\max}S}{K_m + S} \quad 4.7$$

Allosteric control usually leads to enzyme inhibition, and as such will be discussed in Section 4.3.3.

The use and production of the high energy intermediate ATP is usually balanced within the cell. Over short periods, the cell pool of adenylate compounds, AMP, ADP and ATP (mono-, di- and tri-phosphate) can be regarded as constant. The total amount of energy present in the cell can be viewed as the proportion of ATP and ADP to the total energy in the cell, with the higher energy ATP counting twice that of ADP. This sum is known as the energy charge.

$$\text{Energy charge} = \frac{[\text{ATP}] + \frac{1}{2}[\text{ADP}]}{[\text{ATP}] + [\text{ADP}] + [\text{AMP}]}$$

The relationship between the energy charge and the adenylate compounds is shown in Figure 4.3. The effect of energy charge on several enzymes has been investigated and the control follows the enzymes' function in the use or generation of ATP. Certain enzymes can be inhibited by ATP, and activated by AMP, or vice-versa.

Enzymes can be activated or de-activated by either reversible or irreversible covalent modification, often carried out by a second enzyme. Some enzymes are synthesized in an inactive form which can be activated later, by releasing the active site of the enzyme by the hydrolysis of specific peptide bonds. Increasing or decreasing the number of enzyme molecules present by altering the rate of synthesis of the enzyme involves control at the transcription or translation level. In bacteria the expression of a gene is principally controlled at the transcription level by inducing or repressing synthesis. A good example here is the enzyme β -galactosidase in *E.coli* which is required to utilize lactose. The enzyme is induced when *E.coli* cells are exposed to lactose, with β -galactosidase being part of a series of genes termed an operon (the genetic unit that regulates the expression of inducible enzymes in bacteria). Initially the process was thought to be relatively simple with the inducer, lactose, binding to the repressor molecule and allowing expression of the genes. However, the observation that glucose blocked the lac operon and was involved in levels of cyclic AMP in *E.coli* suggested a more complicated mechanism. This will not be discussed further here, except to comment on the fact that dextransucrase is also an induced enzyme, with sucrose being its

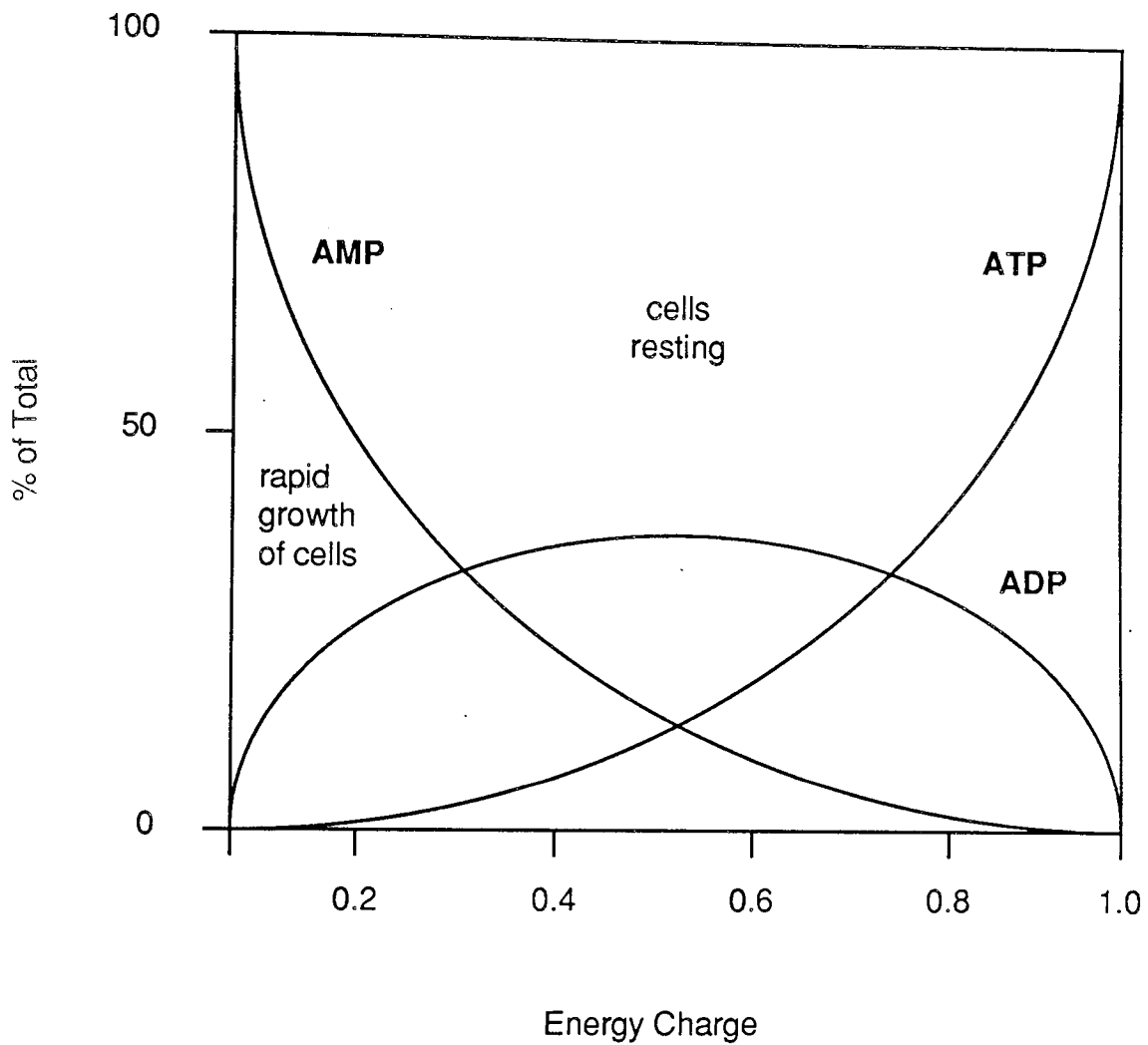


Figure 4.3

Relationship between the Energy Charge and the Adenylate Compounds

only known inducer. The brief explanation above, of lactose inducing β -galactosidase production in *E-coli*, could be a similar mechanism to the sucrose induction of dextransucrase.

4.3.3. Inhibition. (83)

The inhibition of enzymatic activity by specific small molecules and ions can serve as a major control mechanism. In addition, many drugs and toxic agents can inhibit enzyme activity. This enzyme inhibition can be either a reversible or an irreversible process. Irreversible inhibition can be carried out by agents such as alkylating reagents (iodoacetamide) which modify cysteine and other side chains. Reversible inhibition can be both competitive or non-competitive in terms of their inter-action with the active site of the enzyme (see Figure 4.4).

Allosteric enzymes (see Table 4.2) show unusual kinetic properties in response to changes in substrate concentration and/or in response to the action of allosteric effectors that are not substrates. In allosteric enzymes one active site in the molecule can affect another site in the same molecule. Two *principal models* have been proposed for the allosteric reaction; concerted and sequential. The concerted model predicts that binding of a substrate molecule switches the enzyme to a high affinity form which can bind further substrate. The effect of inhibitors or activators can also be readily accounted for by such a model. The sequential model makes three assumptions; the first that there are only two conformational states possible for any one subunit; the second that the binding of substrate changes the shape of the subunit to which it is bound, with the other subunits not affected; and the third that the change caused by the substrate binding can increase or decrease the substrate binding affinity (see Figure 4.5 for concerted and sequential models). Both models are applicable for certain enzymes.

Various types of allosteric inhibition were listed in Table 4.2, the first of which was simple feedback inhibition. The first irreversible reaction in a pathway is often an important regulatory site and the final product of the pathway is often the regulator, a process termed feedback or end-product inhibition. This type of control is useful in the conservation of energy and to stop over-production. An example is the synthesis of isoleucine in *E-coli*, where isoleucine acts as an allosteric inhibitor of threonine deaminase (see Figure 4.6). This is the simplest example, but metabolic pathways are often branched and therefore a number of examples of control have been found; sequential, multiple enzyme,

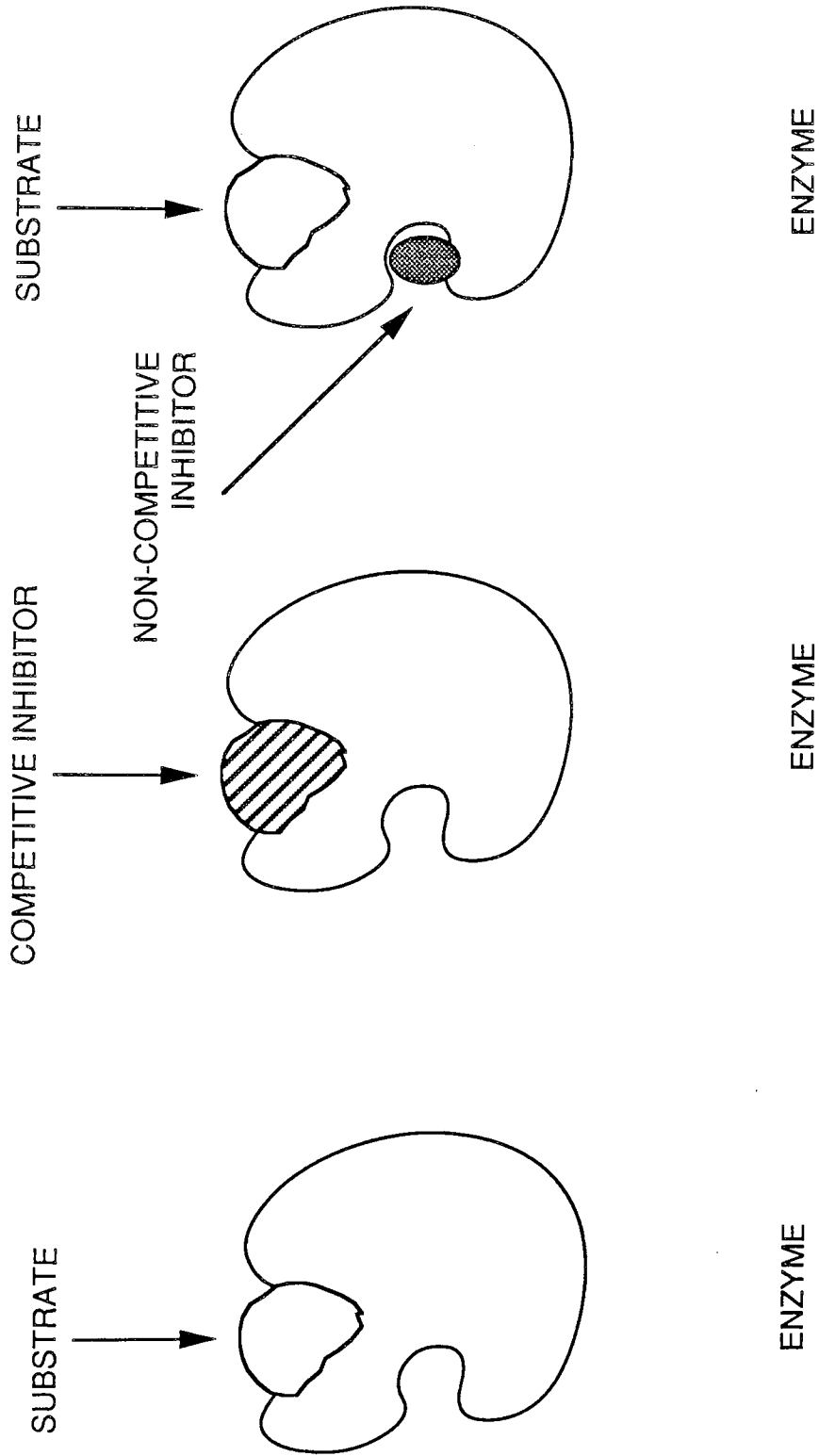


Figure 4.4

Distinction between Competitive and Non-Competitive Inhibitors

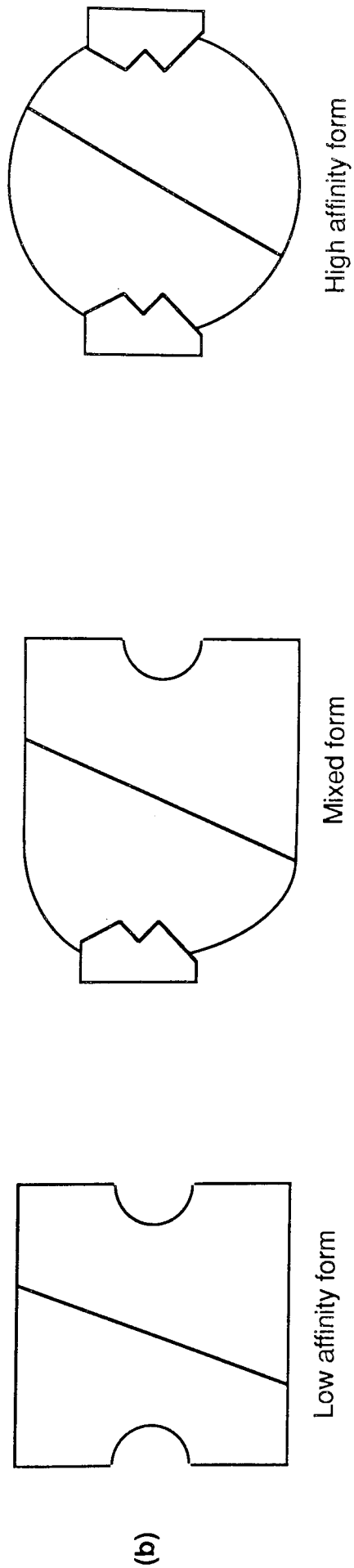
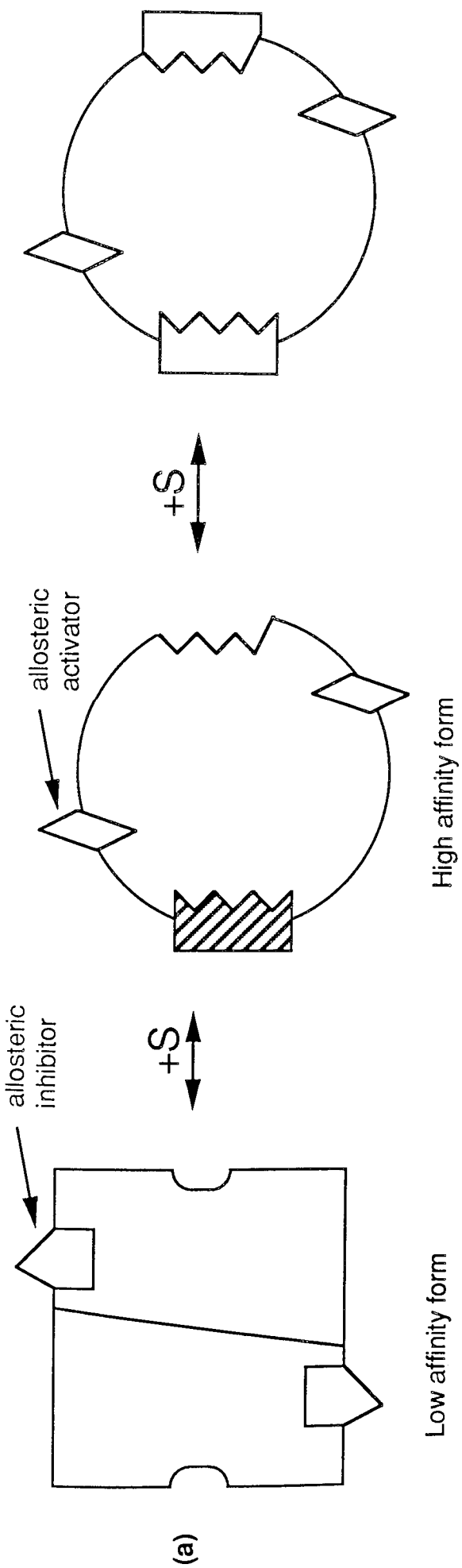


Figure 4.5
Concerted Model (a) and Sequential Model (b) for allosteric interactions

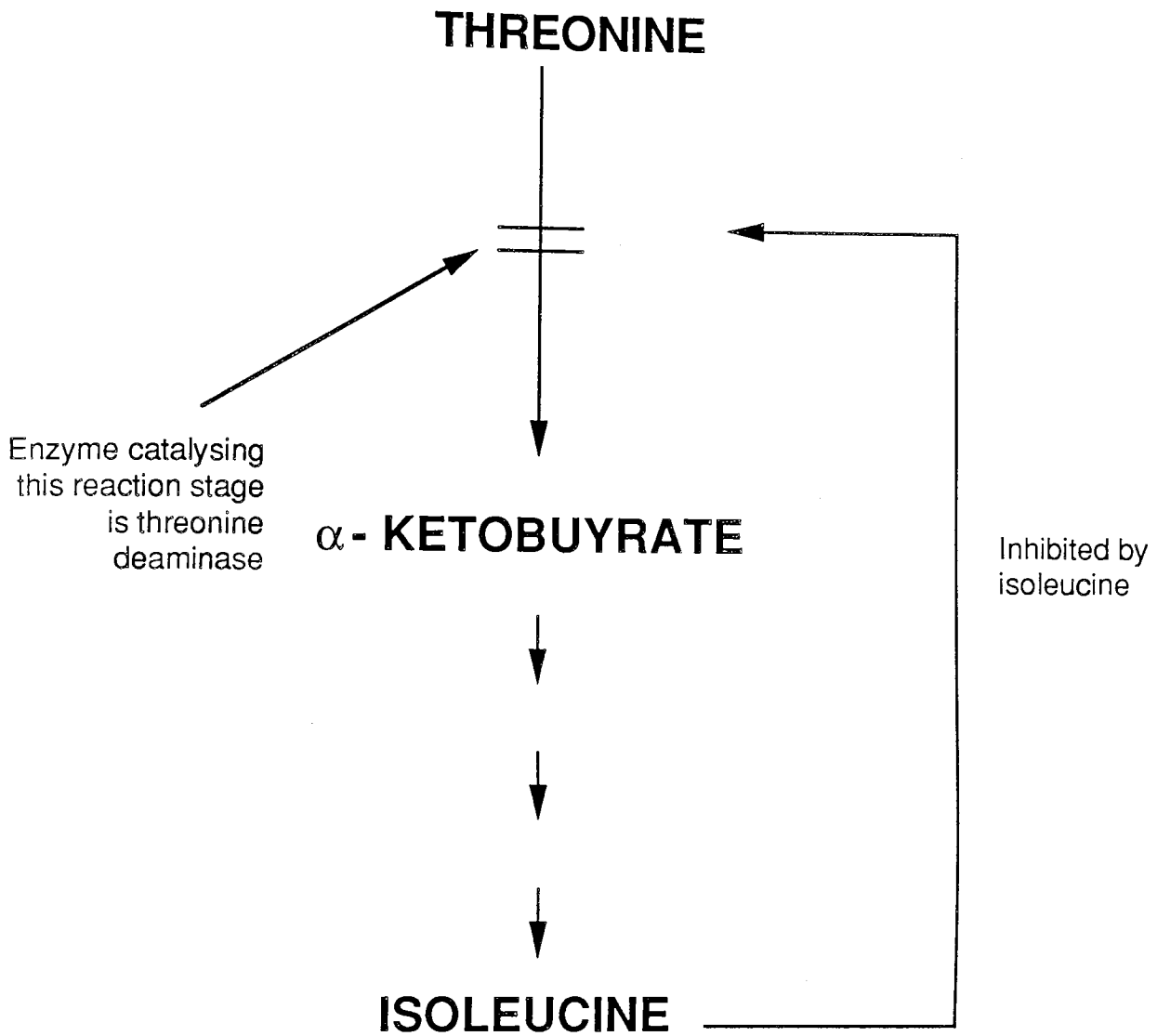


Figure 4.6

Example of Feedback Inhibition

concerted and cumulative (Table 4.2). In sequential inhibition, the final products of a branched pathway only inhibit the reaction leading from the branch point and the branch point intermediate inhibits the common pathway (see Figure 4.7). With multiple enzyme control, the initial sequence of a branched pathway is catalysed by a number of enzymes, each of which is inhibited by one end-product or another. An example is shown in Figure 4.8. With concerted inhibition, the key enzyme can only be completely inhibited by the presence of a number of factors together. For example, aspartokinase from *Rhizopus capsulatus* is inhibited by excess lysine and threonine, but only when together. In the case of cumulative inhibition, the end products are separate, and inhibition is achieved with all the end-products having a separate controlling effect on the key enzyme. An example is shown in Figure 4.9, where the enzyme glutamine synthetase produces glutamine, which is in turn a precursor of a large number of products, all of which would separately affect the enzyme. However, action by all the necessary components results in complete inhibition of the enzyme.

These are some of the basic ways in which enzyme inhibition can occur. In a natural environment, the cell growth is regulated to produce a minimum amount of unnecessary metabolites, but in an industrial process, it may be necessary to regulate the cells' metabolic activity in such a way as to encourage high levels of formation of particular unwanted by-products which may have high commercial value.

4.4. Basic Enzyme Theory.

Enzymes are the catalysts of the living world (90), without which the chemical reactions necessary for life would proceed at a rate too slow to allow any life! Enzymes are also proteins, the direct products of gene expression and they make possible the vast array of metabolic pathways that lie at the heart of biotechnology. Enzymes have very high catalytic power, giving up to 10^9 - 10^{12} fold increase in the rate of reaction over non-enzymic processes. Individual enzymes have a high substrate specificity, whilst enzymes as a whole have a broad range of activity. Enzymic reactions can also be run under mild conditions of pH, temperature and pressure. The following sections will give a brief description of the method used to classify enzymes, along with some basic enzyme kinetics.

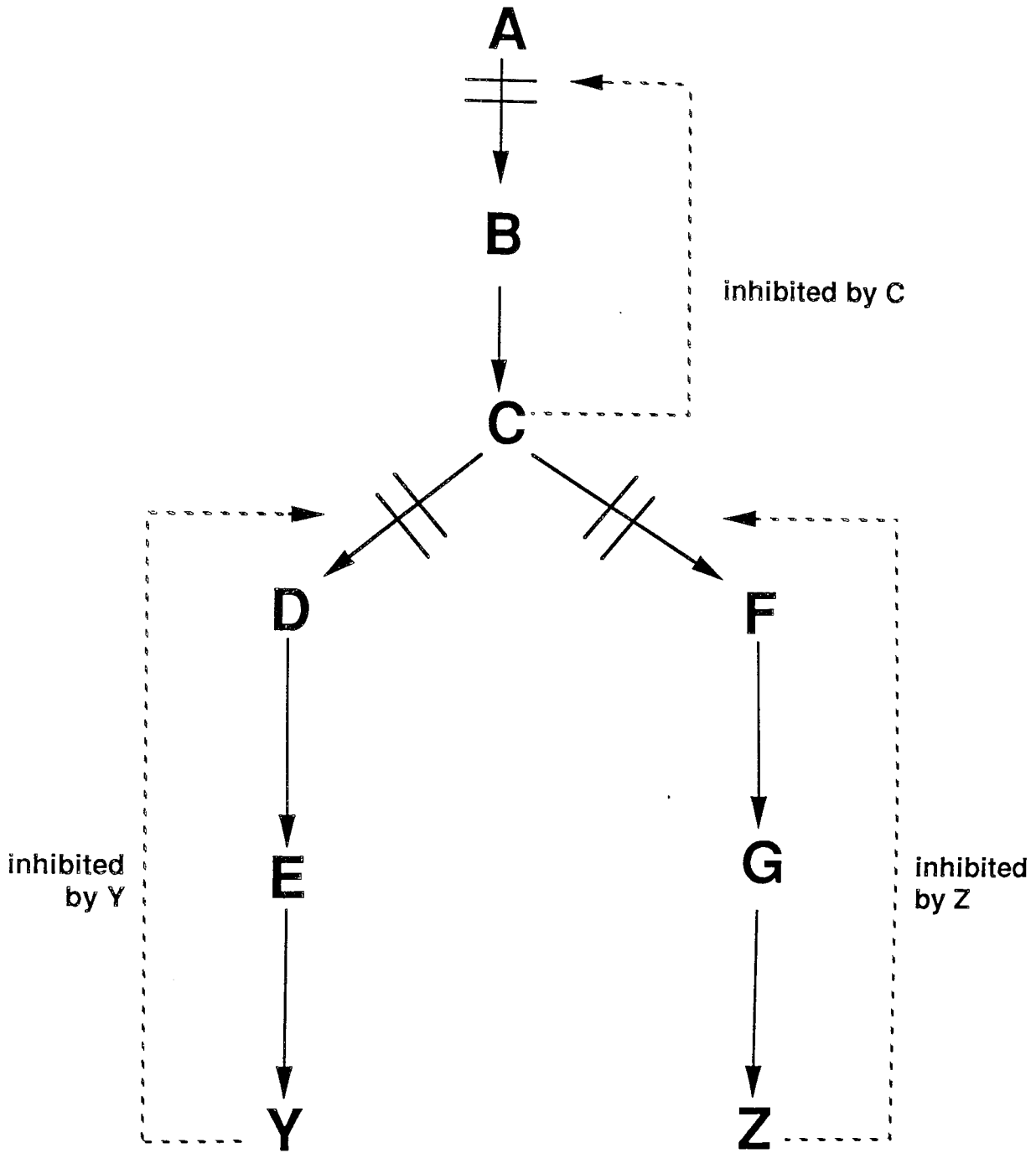


Figure 4.7

Sequential Feedback Control

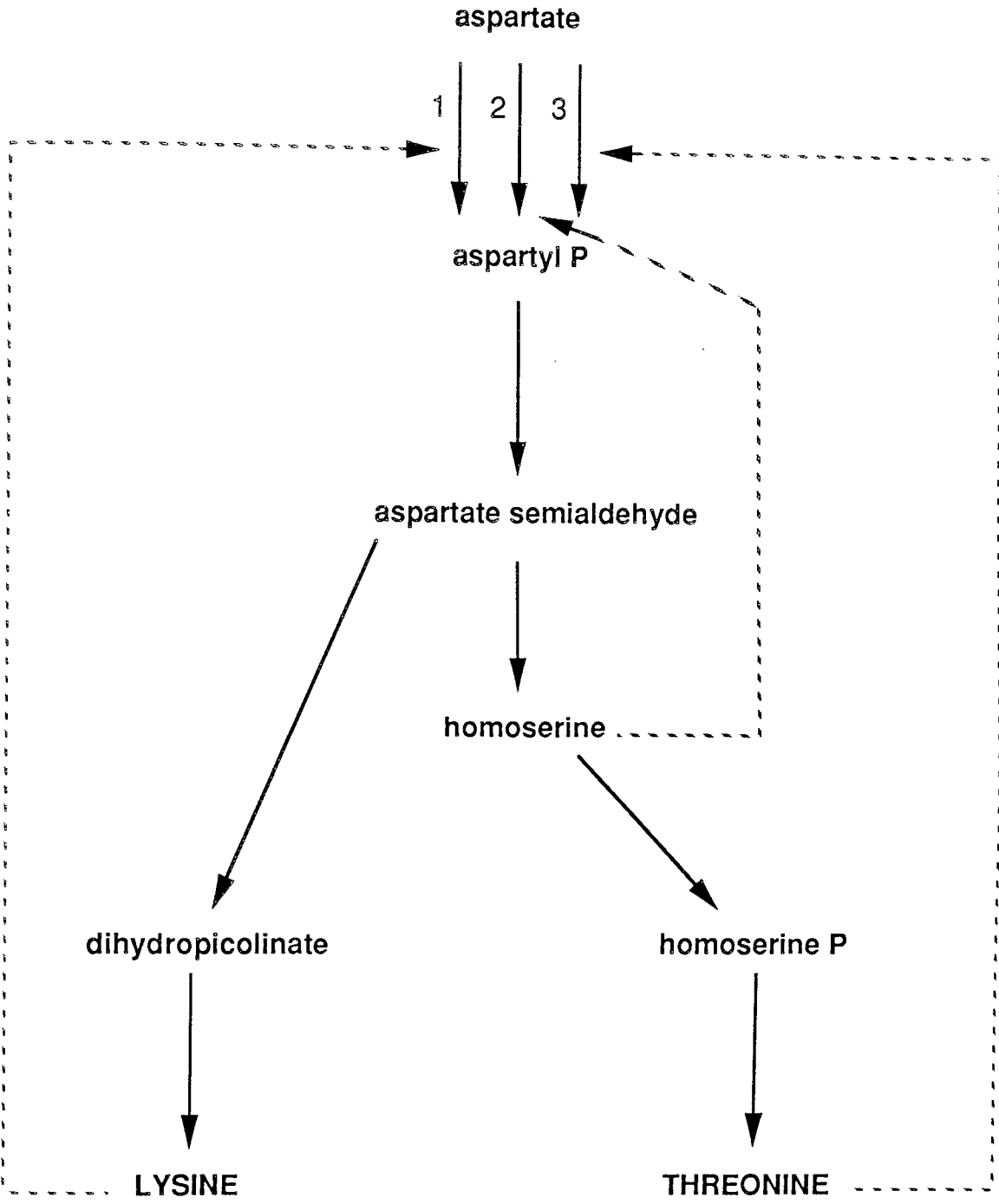
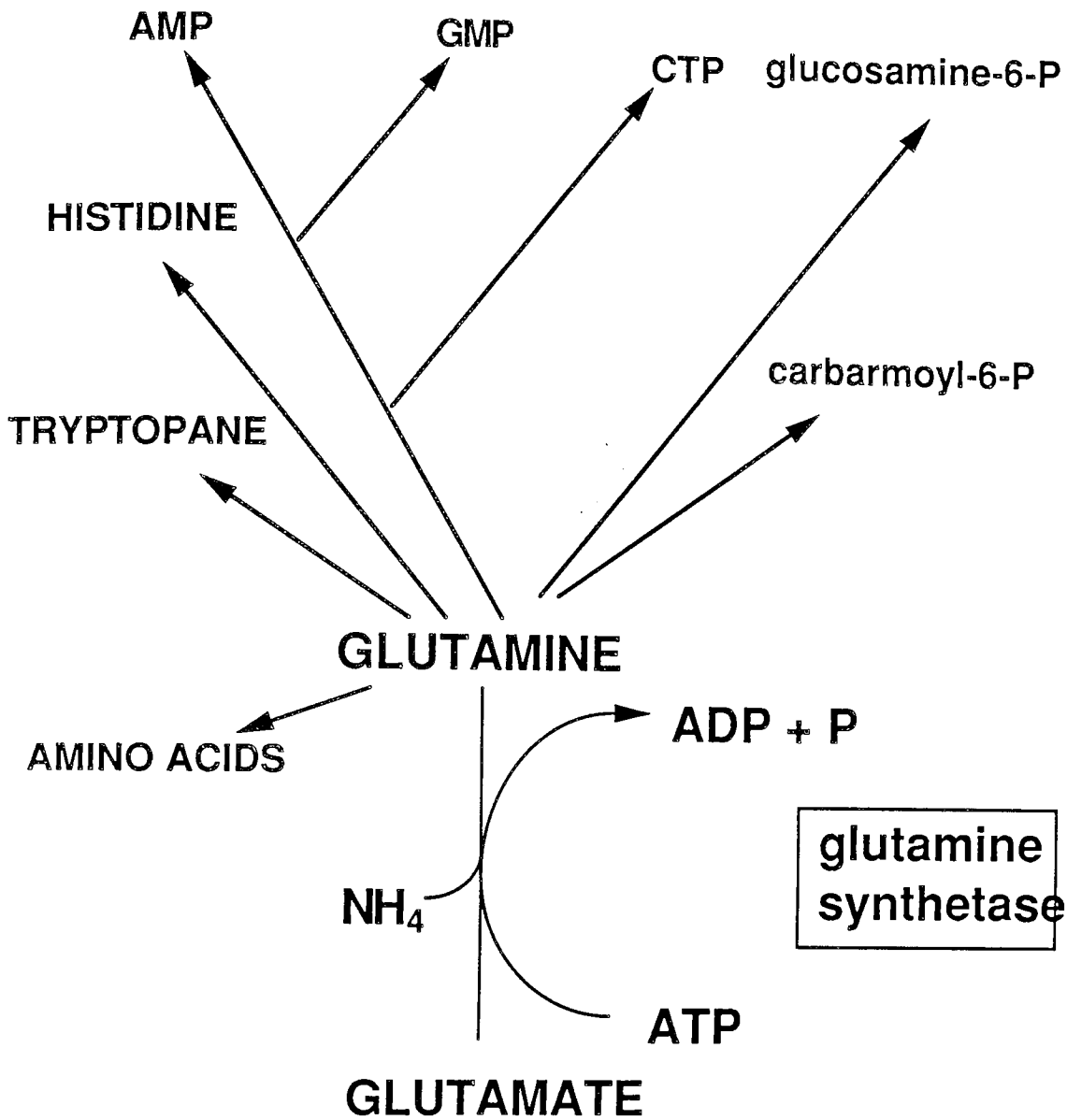


Figure 4.8

Simplified Example of Multiple Enzyme Control



All these components affect glutamine synthetase.
 If they were all present they would completely inhibit the enzyme.

Figure 4.9

An Example of Cumulative Feedback Control

4.4.1. Enzyme Classification.

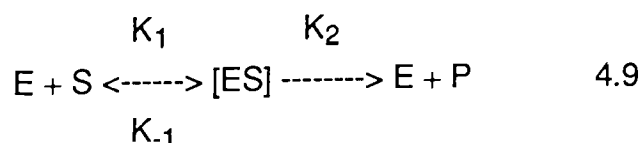
Enzymes are often given trivial names which may describe the reactions which they catalyse, eg. dextransucrase synthesizes dextran from sucrose, and sucrose phosphorylase phosphorylates sucrose. Frequently, however, the trivial names do not reflect the reaction in which the enzyme is involved, eg. renin, trypsin, malic enzyme, papain. As a result, an internationally accepted nomenclature of enzymes has been produced by the European Commission. There are six major types of enzyme catalysed reaction (91):-

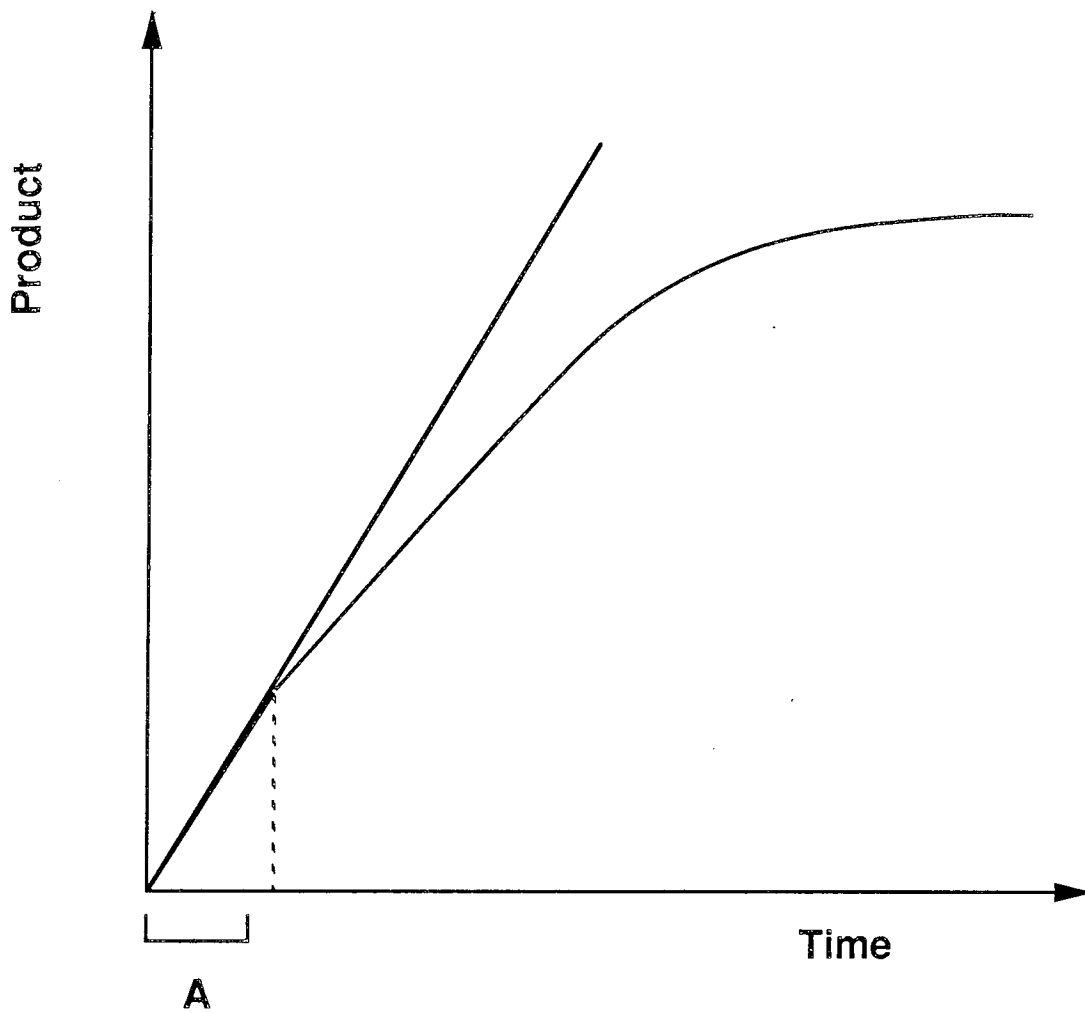
1. Oxidation-reduction reactions catalysed by oxidoreductases.
2. Group transfer reactions catalysed by transferases.
3. Hydrolytic reactions catalysed by hydrolases.
4. Elimination reactions with the formation of a double bond, catalysed by lyases.
5. Isomerization reactions catalysed by isomerases.
6. Reactions involving the joining of two molecules at the expense of an energy source, usually ATP, catalysed by ligases (or synthetases).

The European Commission describes a full systematic name which gives details of the type of reaction and the type of substrate(s) acted upon. In addition, each enzyme is given an E.C. number, an example of this being, in the case of dextransucrase, a systematic name of sucrose 1,6 - α - D glucan-6 α glucosyltransferase E.C. 2.4.1.5.

4.4.2. Enzyme Kinetics. (92, 93)

Simple enzyme kinetics are based on three experimental principles, the first being that the substrate, S, forms an intermediate enzyme-substrate complex, ES, with the enzyme, E. Secondly, that the rate of reaction at time, t (either -ds/dt, or dp/dt) is represented by the slope of the curve P or S = f(t)). These slopes vary with time during the course of the reaction, due to the disappearance of substrate (see Figure 4.10). Kinetic measurements are usually based on the linear part of the curve, ie. initial velocity or initial reaction rate. In this region, the concentration of product is extremely small, and consequently the breakdown of product to substrate (determined by the rate constant, K₂) can be ignored and the Equation below can be written :-





A: time interval for which dP/dt remains constant

Figure 4.10

The Change in Product Concentration as a Function of Time

Thirdly, for a given substrate concentration, a study of the variation in reaction rate as a function of enzyme concentration is not linear, but hyperbolic, due to all the substrate being in the ES complex at high enzyme concentrations. Consequently, the initial reaction rate as a function of enzyme concentration remains constant under these conditions. For kinetic measurements it is necessary to work over the linear region of the curve (Figure 4.11) at low [E] so that the reaction rates are dependent on enzyme concentration.

Enzyme catalysed reactions exhibit saturation kinetics. At low substrate concentrations, the reaction rate is proportional to the substrate concentration and the reaction is first order with respect to substrate. As the substrate concentration is increased, the reaction rate slows and is no longer proportional to substrate concentration with the reaction becoming mixed order. At still higher concentrations the reaction rate becomes constant and independent of substrate concentration, with the reaction becoming zero order.

From Equation 4.9, the following Equation can be written :-

$$K_1 [E] [S] = K_{-1} [ES] \text{ since } K_2 \ll K_1 \quad 4.10.$$

and

$$[E] = \frac{[ES] K_{-1}}{K_1 [S]} \quad 4.11$$

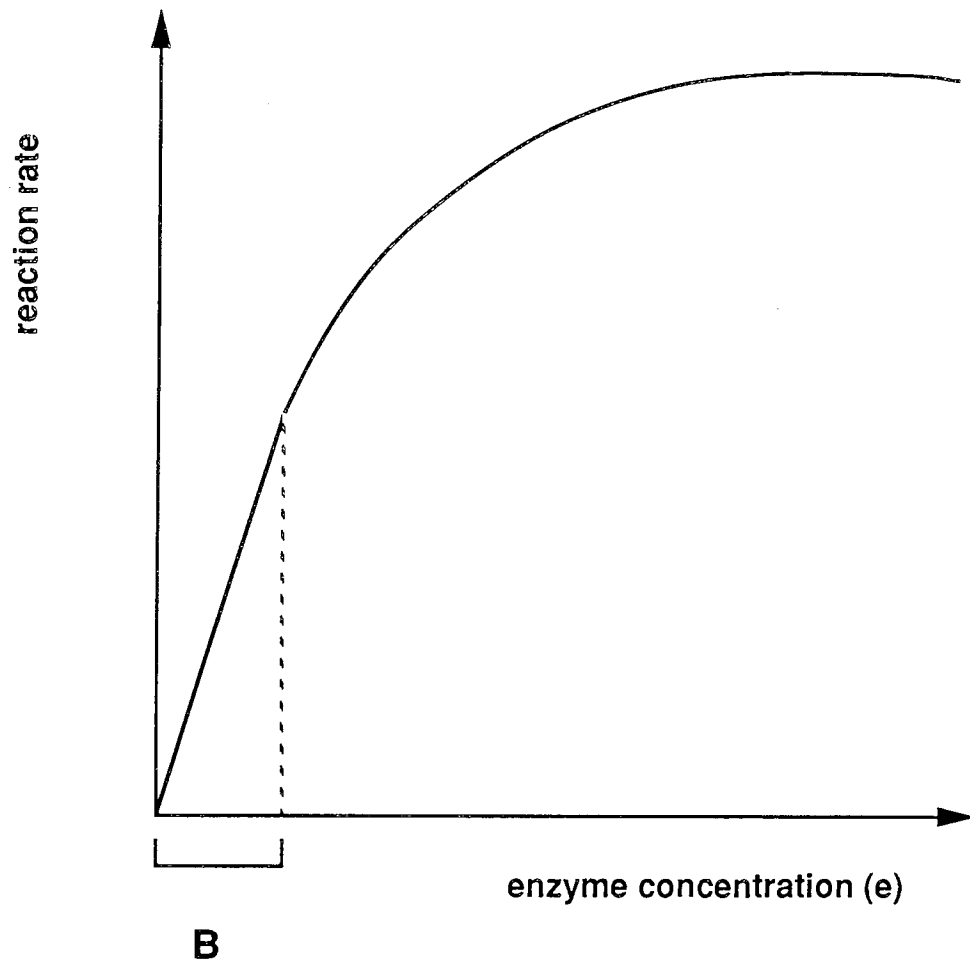
The law of conservation of mass requires that the total enzyme $[E_T]$ does not change. Therefore :-

$$[E_T] = [E] + [ES] \quad 4.12.$$

and substituting this result into Equation 4.11 gives :-

$$[E_T] = [ES] \left\{ 1 + \frac{K_{-1}}{K_1 [S]} \right\} \quad 4.13$$

The overall rate of reaction is $V = K_2 [ES]$ since reaction K_{-2} can be ignored.



B: concentration range over which rate equations apply

Figure 4.11

Reaction Rate as a Function of Enzyme Concentration

Therefore :-

$$V = K_2 [E_T] = K_2 [E_T] [S] \quad 4.14.$$

This expression relates the reaction rate to the total enzyme concentration $[E_T]$ and the substrate concentration $[S]$. The rate terms K_{-1} , K_1 and K_2 are constants, and so the constant K_{-1}/K_1 is also a constant, and can be represented by the term K_m , the Michaelis Constant. Also, $K_2 [E_T]$ is the maximum reaction rate, so that Equation 4.14 can be rewritten as :-

$$V = \frac{V_{\max} [S]}{K_m + [S]} \quad 4.15$$

when the substrate concentration is high compared to K_m , V approaches V_{\max} , since under these conditions:-

$$V = \frac{V_{\max} [S]}{[S]} = V_{\max} \quad 4.16$$

However, when $[S]$ is small compared to K_m :-

$$V = \frac{V_{\max} [S]}{K_m} \quad 4.17$$

When $S = K_m$:-

$$V = \frac{V_{\max} [S]}{[S] + [S]} = \frac{V_{\max}}{2} \quad 4.18$$

As a result K_m is the substrate concentration which gives half the maximum reaction rate. This is the correct definition of K_m . However, this only holds when $K_m = K_{-1}/K_1$, that is when the Michaelis Menton assumption holds. If $K_2 \gg K_{-1}$, then K_m would be much larger than the dissociation constant and would wrongly suggest a low affinity for substrate, or high value for K_m .

4.4.3. Inducible Enzymes. (94)

There are two identifiable classes of enzymes. The first group comprises the constitutive enzymes that are produced continuously, regardless of whether or not substrate is present (eg. sucrose phosphorylase in *L. mesenteroides*). The second, much larger group, comprises the inducible enzymes. These enzymes are produced at very low, but significant levels until a suitable

substrate becomes available to the organism. The substrate itself, or degradation products derived from it, may then induce a greatly increased synthesis of the enzyme. It is quite common for synthesis of an induced enzyme to increase by a thousand-fold or more under these circumstances. An example of an induced enzyme is dextransucrase from *L. mesenteroides*. Induction can be a difficult phenomenon to follow experimentally, as the inducer is often metabolized by the induced enzyme.

Regardless of whether an enzyme is constitutive or inducible, there is a further control mechanism that can operate. In the presence of a readily assimilated carbon source, eg. D-glucose, the synthesis of many enzymes is repressed. This is due to the fact that an organism can utilize glucose without the need to expend energy synthesizing enzymes for the degradation of more complex carbon sources. Carbon-catabolic repression is a commonly occurring feature of micro-organisms and many constitutive and inducible enzymes are affected in this way.

5. EXPERIMENTAL TECHNIQUES.

5.1. Fed-Batch Fermentations.

A large number of fed-batch fermentations were carried out over the course of the project in one of three vessels. The majority were carried out in the laboratory, using a 16-litre "Microgen" fermenter supplied by New Brunswick Scientific (N.B.S.) Co. Inc. A small number of large scale fermentations were carried out in either a 1,000 litre vessel at the Fisons site in Holmes Chapel, or in an 800 litre fermenter at the Centre for Biochemical Engineering, University of Birmingham. Both of these vessels will be described in the section concerning large scale fermentations (5.2). The following sections will include a description of apparatus used for the laboratory-scale fed-batch fermentations, and the experimental techniques employed.

5.1.1. Description of Apparatus.

The fermenter used for fed-batch culture was a 16-litre stainless steel SF116 "Microgen" fermenter, manufactured by the New Brunswick Scientific (N.B.S.) Co. Inc., New Jersey, U.S.A., and supplied by N.B.S. (U.K.) Ltd., Watford (95). The vessel is a self-contained fermenter, with integral piping systems and panel-mounted controls designed for the growth of microbial cells under controlled environmental conditions. The unit is suitable for batch or fed-batch culture of aerobic and anaerobic bacteria, moulds, yeasts and streptomycetes.

The fermenter is self-sterilizable using an external steam supply, with only a small autoclave being required for the sterilization of additional equipment, including tubing, filters and inoculum broth. The services connected to the fermenter include electricity, water, steam and air in one integrated unit. The fermenter vessel contains a hollow baffle heat exchanger which is immersed in the culture fluid. The heat exchanger carries steam (during sterilization), cold water (during crash cooling), and cooling water (during fermentation). The fermentation temperature is measured by a resistance thermometer.

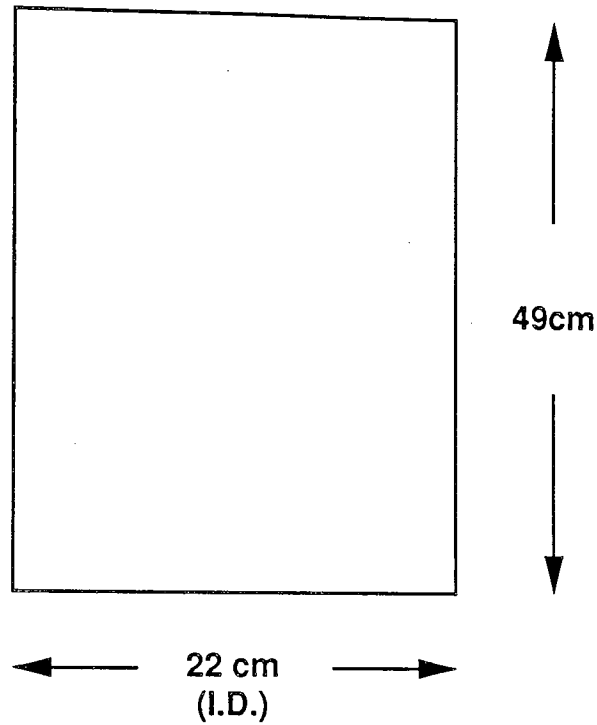
The fermenter vessel has a total volume of 16 litres, with a working volume of approximate 2 to 12 litres, and an operating pressure of 0 - 25 p.s.i.g. (0 - 1.7 bar). The drive motor runs on a 0.25 HP (0.186 kW) motor, and turns an

optical tachometer which feeds back to the motor speed control unit to provide accurate agitation control, which can be altered between 120 and 1,200 rpm. The air flowmeter has the capacity of passing between 1.6 and 16 litres of sterile air through the fermenter per minute, when the fermenter is run aerobically. Figure 5.1 shows the overall dimensions of the fermenter.

The pH of the fermentation broth is controlled and monitored by a pH 19 pH control module (range 0 - 14), with control of the pH being achieved via addition of acid or alkali from the pump in the peristaltic pump module (pM 10), each pump being incorporated with a dose and delay timer. The dissolved oxygen is controlled and monitored by a PO 217 oxygen control module in the ranges 0 - 10% and 0 - 100%. Control of dissolved oxygen concentration is achieved by automatic variation of agitation speed.

The pH probe ⁽⁹⁶⁾ used in the fermenter is an Ingold type 465 steam sterilizable combined electrode, which is inserted into a special pH port in the front of the fermenter. The probe is sterilized in situ with the fermentation medium.

The probe used for monitoring dissolved oxygen concentration ⁽⁹⁶⁾ within the fermenter is a steam sterilizable dissolved oxygen electrode supplied by N.B.S. Inc. The probe has been designed to withstand the severe conditions of temperature, pressure and moisture that occur during sterilization. The probe itself is a galvanic cell containing a lead anode and a platinum cathode, covered by a stretched, replaceable membrane (fluorinated ethylene propylene : F.E.P.). The probe has a response time of 60 seconds for 90% response, and 1% linearity and reproducibility at constant temperature. The unit also contains an antifoam control module, which has two modes of operation. It can be operated by a timer with variable interval and dose settings, or it can be operated by activation of a peristaltic pump from contact made between the conductivity probe and foam bubbles rising from the culture medium. This facility is rarely used during dextransucrase fermentations, as the addition of a small amount of antifoam into the medium before sterilization is sufficient to prevent foam formation during the fermentation.



Vessel Dimensions

Capacity: 16 litres

Maximum Working Capacity: 12 litres

Minimum Working Capacity: 3 litres

L/T = 2.2

Figure 5.1

Diagram showing Dimensions of New Brunswick Fermenter

5.1.2. Inoculum Preparation.

The inoculum was prepared for use in a 6 litre working volume fed-batch fermenter as described below. A working volume of 6 litres was used for all fed-batch fermentations carried out in the New Brunswick Vessel. The culture - *Leuconostoc mesenteroides* N.R.R.L. B 512 (F) : C31 - was supplied by Fisons plc Pharmaceutical Division, in the form of freeze dried vials. These vials were stored in a frozen state, at - 10°C, until required. In this form, the culture can remain stable for a number of years. The organisms in this vial were reconstituted with sterile MRS (de-Man Rogosa Sharpe) nutrient broth. A few drops of this suspended culture were inoculated onto sterile slopes of MRS Agar, which were then incubated at 25°C for approximately 48 hours, or until the growth of the organism was clearly visible on the slopes. The slopes were then stored in a refrigerator at 5°C until required. This preparation of the slopes of *Leuconostoc mesenteroides* was standard for all the fermentations carried out during the course of this project.

The culture was then prepared for use in a fermentation. The inoculum had to be developed in stages to a point where it was approximately 3 - 10% of the working volume of the fermentation. For a 6 litre experiment, two stages were required. Firstly, a 10 cm³ inoculum was prepared using the same sterile MRS nutrient broth as previously mentioned, and one slope of culture. The cells from the slope were transferred to the broth with the use of an inoculation loop. This process was carried out in a totally sterile environment, in a Laminar Flow Cabinet (LFC) cleaned with ethanol. The loop itself was sterilized in a bunsen flame, and all work was carried out at the rear of the LFC. It was possible for this method of inoculum transfer to be somewhat imprecise, due to the amount of grown culture on the slope, and the amount transferred varying for each fermentation. It was important, therefore, for great care to be taken when carrying out this preliminary step of the fermentation. The 10 cm³ inoculum was incubated at 25°C for 12 - 15 hours. At the end of this period, the cells should have grown such that the turbidity of the medium gave an OD₅₉₀ value of approximately 0.1. The inoculum was then transferred to a second stage, i.e. a flask containing approximately 300 cm³ of sterile MRS broth. This process was again carried out in the LFC. For a 6 litre fermentation, this would provide a 5% by volume inoculum which was

quite satisfactory. This stage was again incubated at 25°C for 12 - 15 hours, before transfer to the main fermenter. The time period allowed for the inoculum to grow has been carefully optimized, as it is important that the cells are still metabolically active, i.e. in exponential phase of growth, on transfer to the next stage. Once transfer has taken place, the cells require a certain period of time to adapt to their new environment, this time being determined by the active state of the cells and the similarities of the inocula development media in the various stages. It is important that, on transfer to the main fermenter, the inoculum is in a sufficiently active state so as to give as short a lag phase as possible during the fermentation cycle.

Following the incubation period, the 300 cm³ inoculum was transferred to the main fermenter using sterile silicone rubber tubing, passing through a peristaltic pump, connected to a port on the top plate of the fermenter. As it is the exponential phase, not the lag phase, that needs to be monitored over the course of the fermentation, the inoculum development procedure described above is carried out such that the transfer of the inoculum to the fermenter occurs automatically during the night, and so the lag phase (6 - 8 hours) has been completed by the following morning, leading to the exponential phase - timing that is convenient for the operator who will be monitoring the enzyme production of the fermentation. Figure 5.2. shows the inoculum development procedure for 6-litre fed-batch fermentations.

5.1.3. Fermentation Medium.

As mentioned in Section 2.2 *Leuconostoc mesenteroides* can grow on a large number of carbohydrates, but the only substrate that will induce the production of dextransucrase is sucrose. Schneider *et al*, working on behalf of Fisons plc Pharmaceutical Division, showed that it was necessary to maintain a low sucrose level of between 5.0 and 10.0 g/l in the fermenter, for the economic production of dextransucrase. For fed-batch fermentations, the medium composition used was the same as that employed by Schneider *et al*, and is shown in Table 5.1.

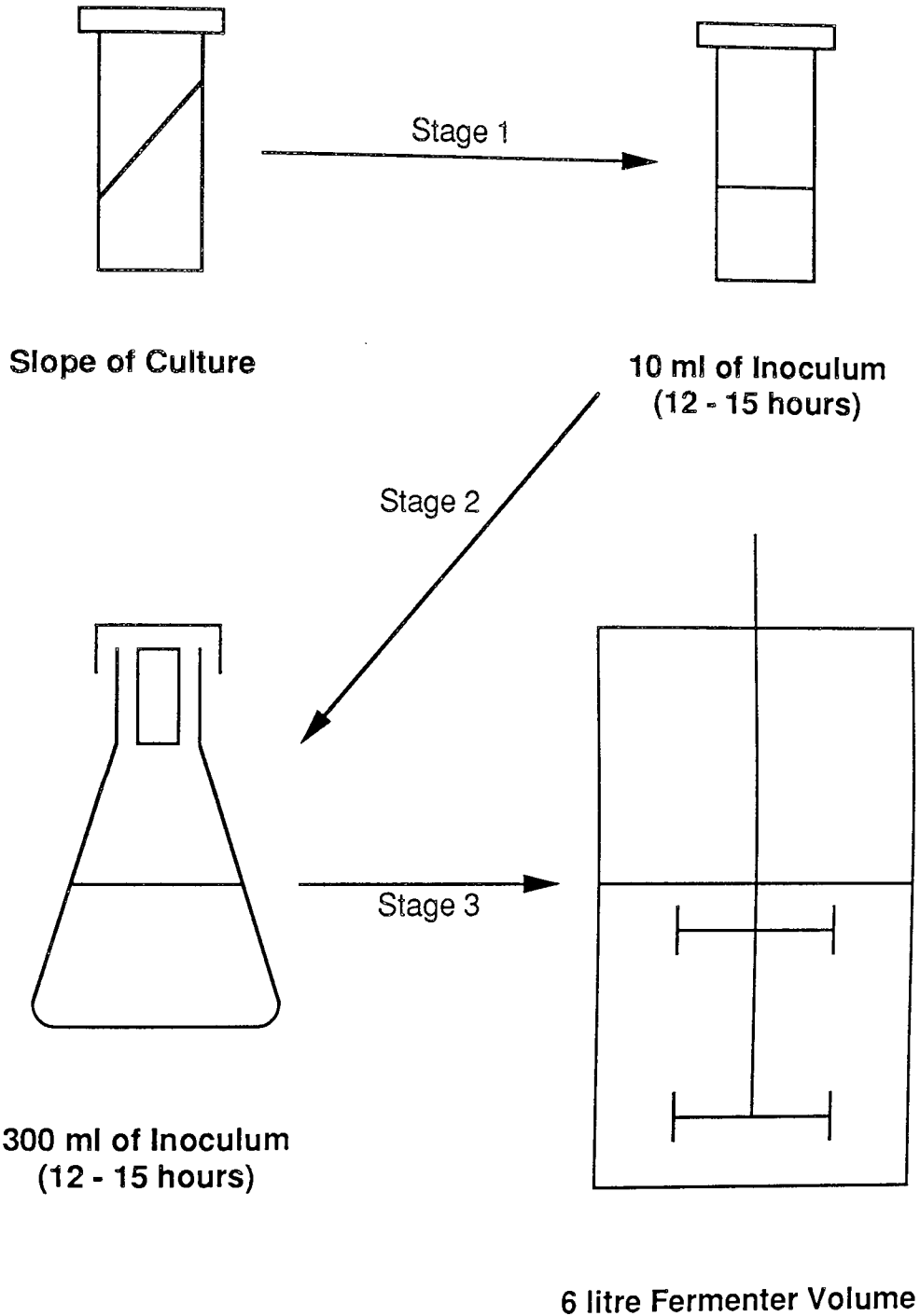


Figure 5.2

Inoculum Development Procedure for 6 litre Fed-Batch Fermentations

Table 5.1
Composition of Fermentation Medium.

Sucrose	10 g/l
Yeast Extract	40 g/l
K ₂ HPO ₄	20 g/l
R* Salts	0.5% v/v.
Antifoam (*)	0.1% v/v.

R* Salts.

MgSO ₄ . 7H ₂ O	4.0 g
NaCl	0.2 g
FeSO ₄ .7H ₂ O	0.2 g
MnSO ₄ .H ₂ O	0.2 g
Water	to 100 cm ³

(*) Optional.

The above medium components were dissolved in town or deionised water, and made up to the required volume. The pH was then adjusted to a value of 7.0 - 7.1, before the sterilization cycle.

5.1.4. Additional Media.

For fed-batch processes, the pH of the culture was controlled with the addition of a sucrose/NaOH solution. In other words, the sucrose was fed to the fermentation on alkali demand. The composition of the sucrose/alkali mixture used is shown in Table 5.2.

Table 5.2
Composition of Sucrose/Alkali Mixture.

Sucrose	900 g
NaOH	200 g
Water	to 1,500 cm ³

This solution was prepared as follows :-

The NaOH was dissolved in a small amount of water, to make an extremely strong alkaline solution. The sucrose was dissolved in hot water - to a total volume of 1,500 cm³, or any volume required - and both solutions were allowed to cool down. Once they were sufficiently cool, the two solutions were mixed by slowly adding the caustic to the sucrose solution, whilst constantly stirring the resulting mixture. This process is extremely exothermic and it is vital that the two separate solutions are cool before mixing takes place, otherwise the solution turns a dark red colour. The solution will also become this colour if a low grade NaOH is used, although this does not affect the fermentation. The preferred colour for the sucrose/NaOH solution is golden yellow. Once the mixture had been prepared, it was cooled quickly before use. It was not necessary to sterilize this mixture as it was sufficiently strongly alkaline to prevent the growth of any micro-organisms. This solution was now ready for use as the feed in a fed-batch fermentation.

5.1.5. Sterilization and Aseptic Techniques.

The fermenter vessel, together with the pH and DOC probes, were sterilized *in situ* using an external steam supply, with steam being passed through the fermenter heat exchanger. The vessel and contents were heated, with agitation, to a temperature of $116 \pm 0.5^{\circ}\text{C}$ and maintained at this high value for 20 minutes. The fermenter was then rapidly cooled by passing cold water through the heat exchanger, until the fermentation temperature was reached.

Additional equipment, including tubing, MRS Agar slopes, and MRS broth bottles and flasks, were sterilized in a small bench-top Adelphi Autoclave, at $121 \pm 1^{\circ}\text{C}$ for 20 minutes.

It was also important that all the procedures carried out for the fermentation were done so in a totally aseptic manner. All the inoculum preparation stages were carried out in a laminar flow cabinet, which had previously been swabbed with a 70% v/v alcohol solution. All fermenter addition ports, and the ends of all sterile tubings were also swabbed with ethanol before use.

A positive pressure of 0.3 - 1.0 bar was maintained in the fermenter throughout the process, to prevent a vacuum forming in the vessel, and sucking in any contaminant organisms. Any contamination could be detected by visual examination of the fermenter, or by a Gram Stain Analysis (Section 5.4.6).

5.2. Large-Scale Industrial Fermentations.

During the course of the project, it was possible to perform scale-up experiments to determine whether the fed-batch fermentation of dextransucrase could be performed on the Industrial Scale. Two separate large fermenters were used for these experiments, one being a 1,000 litre anaerobic seed fermenter located on the Fisons site at Holmes Chapel; the other, a modern 800 litre vessel belonging to the University of Birmingham. The objective of these fermentations was to determine whether it was possible to successfully scale-up the process to consistently produce maximum yields of dextransucrase.

5.2.1. Description of Apparatus.

The two fermenters, along with their respective procedures, inoculum development, medium preparation, and sterilization cycles, will be described separately in the following sections.

5.2.1.1. 1,000 Litre Vessel - Holmes Chapel.⁽⁹⁷⁾

The seed fermenter (volume 1,000 litres) at Holmes Chapel was usually used as the final stage for the development of the inoculum which was to be transferred to the main 50,000 litre fermenters that were used for the production of the clinical dextran being manufactured industrially by Fisons plc. This vessel was anaerobic, and could be agitated with a constant stirrer speed of 140 rpm. The fermenter had no pH control system, before it was modified in preparation for its use in the scale-up studies in dextransucrase production. Figure 5.3. shows the modification to the fermenter. The major alteration was the incorporation of a pH control module, with the pH probe being inserted into the recirculation line. The temperature of the fermentation was monitored by one probe inserted into the vessel, and was controlled by manually opening a series of valves to allow steam or cooling water to pass

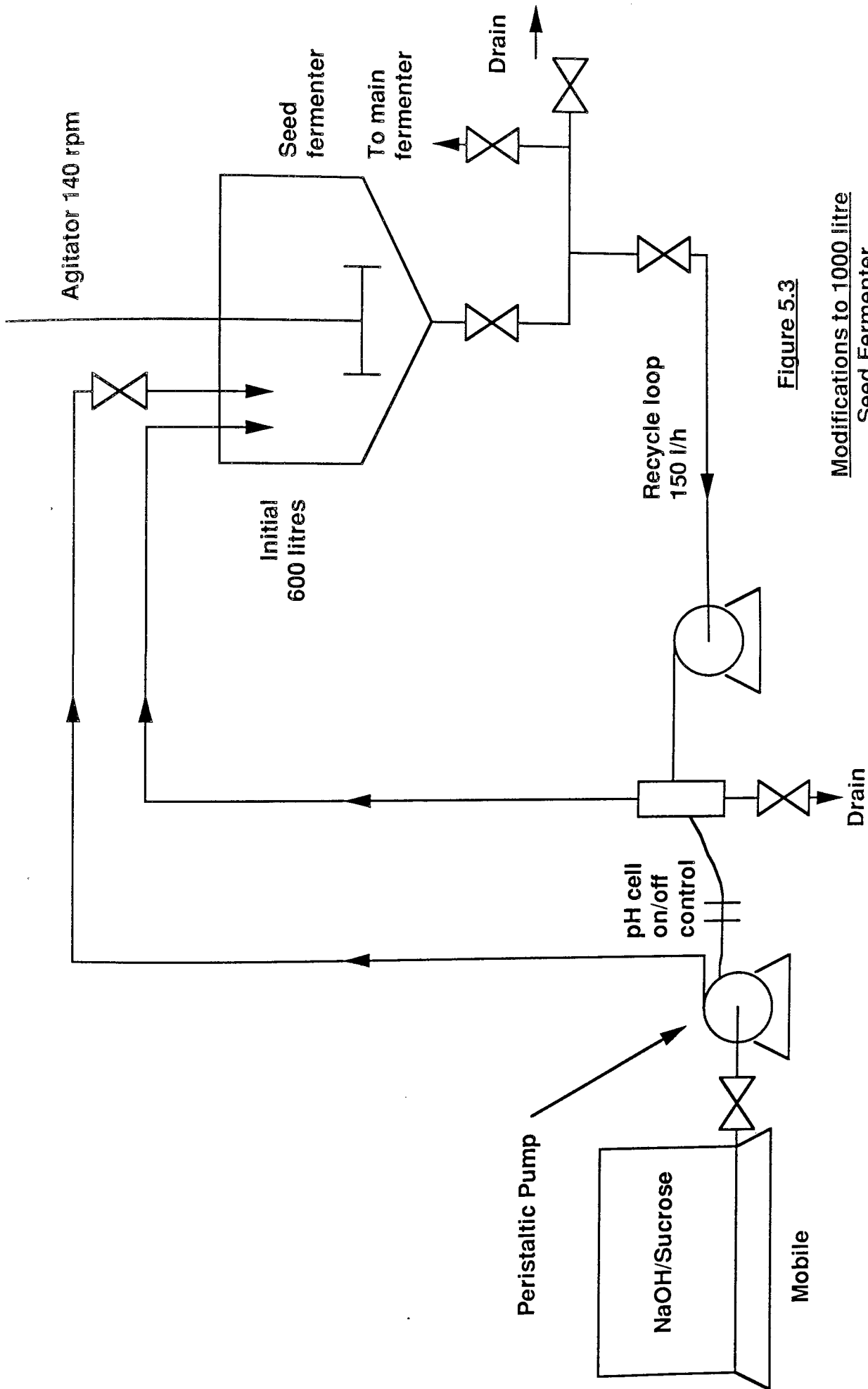


Figure 5.3

Modifications to 1000 litre
Seed Fermenter
(Holmes Chapel)

through the jacket on the vessel. This lack of an automatic control system necessitated the presence of a plant operator to maintain the correct temperature.

5.2.1.2. 800 Litre Vessel - Birmingham University.

The working volume of this fermenter was between 400 and 600 litres, with a height of 1,770 mm and a diameter of 800 mm, leading to a L/T ratio of approximately 2.2:1. The vessel contained 3 Rushton turbines and 4 baffles, each being 0.1 of the diameter of the vessel. The agitator was driven by a 7.5 kW motor, between agitation rates of 50 - 500 rpm. The fermenter design included a mechanical graphite and plasma steel, self-lubricating double seal, with a sterile steam condensate barrier and automatic adjustment for wear. This seal was steam sterilizable along with the vessel and accessories. Aeration was provided to the vessel via a ring sparger unit, which included a detachable air distributor. The air inlet and outlet circuitry was designed to provide up to 900 l/min of sterile air into the vessel, with 3 bar gauge inlet pressure and 1 bar g head-space pressure. The pressure ratings were identical for both the vessel and the jacket, with both having withstood 6 bar g under test conditions, although values of only up to half of this value were preferred during actual fermenter operation. The vessel had a working temperature of up to 140°C. The vessel, jacket and pipework were constructed of 316 L grade stainless steel, with the skid and stairway constructed of mild steel. All the welds were continuous and free from pin-holes, crevices and inclusions, with all internal welds having been ground smooth to limit any contamination problems.

There were various fittings on the vessel. The head plate contained a pressure gauge of range 0 - 4.0 bar g, with a membrane separator, along with a viewing and input port with a diameter of approximately 170 mm fitted with sight glass, hinged lid and steam condensate cleaning system. The air outlet condenser and the capacitance type foam probe were fitted on the head-plate, along with a welded port for the connection of the septum assembly unit. The upper side-wall contained three ports for the connection of acid, base and anti-foam feeds, and a port for a viewing lamp assembly for the illumination of the head-space. There was also a re-sterilizable inoculation port and valve for rapid transfer of inoculum and feeds to the vessel, along

with ports to allow air to pass through to the head-space and for the ring sparger unit. The mid-section side-wall contained three DN25 ports fitted with blank plugs, which could be used for the insertion of probes. The lower side-wall contained five DN25 ports for the connection of temperature, pH and DOC probes, with any spare ports being blanked off, as with those in the mid-section side-wall. There was also a DN25 port housing a lever action sampling valve with steam and condensate connections for repeated sampling and re-sterilization during the fermentation. In addition there was an 80 mm viewing window. The dished base contained a flange for the connection of the bottom drive agitation system and a flange for the connection of the pneumatically operated mushroom type flush mounted harvest valve, which was fitted with an integral steam system for the sterilization of down stream pipework and equipment.

The skid had dimensions of 1,900 x 2,000 mm, including the stairway, and was fitted with a temperature control circuit consisting of a steam heat exchanger, cooling valve and overflow and a pump for the circulation of thermostatted water around the vessel jacket. There was also a sterilization control circuit, which allowed steam injection into the jacket vessel and through the agitation seal, inoculum ports, air inlet filter and gas outlet circuit for continuous pressurization of the agitation seal to provide a sterile barrier, and a parallel air inlet circuit via a rotameter, with a manual adjustment valve and a mass flow meter with an automatic control valve.

All the interface connections for the transmissions of signals to and from the control cabinet were centralised in an enclosure, mounted to the rear of the fermenter. The control cabinet housed the bank of electropneumatic relays for the operation of all the automatic valves, the power supply relays and connector block for the operation of the proportional valves, the connections for temperature, pH and DOC sensors, the power supply for the exhaust air heater element, the power supply and amplifier for the pressure sensor, the power supply and timer for the operation of the viewing lamp, connections for the mass flow sensor and the electrical isolator and overload protection trips.

The temperature was monitored by a Pt 100 sensor in a stainless steel housing, with a plug connection, whilst the pH probe was an Ingold combined

pH electrode mounted in a 764-50 housing, with the DOC being measured by an Ingold polarographic probe. The pressure sensor was a Wilta combined transducer and gauge and the air flow was monitored by a Hastings mass flow sensor.

The various fermentation parameters were controlled very accurately with a series of sophisticated control loops. The agitator unit consisted of a drive analog input to an 8-loop controller, with the output via the analog driver setting the stirrer motor speed. The signals from the temperature probe were transferred to a pre-amplifier with analog input to the 8-loop controller. The output from the controller, via twin digital drivers, controlled the heating, cooling and steam valves for various fermentation processes. The operating range of the temperature controller, excluding the sterilization cycle, was $20 - 55 \pm 0.2^{\circ}\text{C}$. The pH probe transmitted signals to a pre-amplifier with analog input to the 8-loop controller, with the output, via a digital driver, controlling the acid/base feed valves, the range being $2 - 12 \pm 0.1$ pH units. The DOC probe transferred signals in a similar manner as the pH probe to the controller. The output operated in a cascade mode within the system to modify agitation speed or airflow, with a DOC range of 0 to 100%. The airflow meter had an analog input to the 8-loop controller with the output, via a digital driver, providing contacts for the adjustment of the degree of opening of the proportional valve. The foam level in the vessel was monitored by a sensor inserted through the vessel head plate. The signal passed to the 8-loop controller, with the output, via a digital driver, controlling the valve on the antifoam addition tank.

Three addition vessels were included with the main fermenter for the sterile addition of acid, base and antifoam to the broth. They were each of 13 litre total volume - 10 litre working volume - and were fitted with manual valves, heat exchangers and fixed speed drive motors for independent full or empty sterilization. These vessels were each fitted with a longitudinal viewing window, with a three valve set being fitted to the base of each vessel to allow separate sterilization of the feed line. The Birmingham University fermenter

was the most sophisticated and accurately controlled vessel used during the course of this project and a flow diagram of the entire unit is shown in Figure 5.4.

5.2.2. Inoculum Preparation.

The inoculum development procedures used for the two fermenters are summarised in the following sections.

5.2.2.1. 1,000 Litre Vessel.

The three-stage inoculation procedure can be seen diagrammatically in Figure 5.5. In all the inoculum development stages, MRS broth was used as the medium as with the inoculum preparation steps described in Section 5.1.2. From Agar slopes of the culture, the inoculum size was increased by transfer firstly to 9 cm³ MRS broth bottles, followed by transfer of 5 cm³ of inoculum to 500 cm³ MRS broth bottles. The final stage was the addition of 150 - 500 cm³ of culture to 10 litre MRS broth bottles. At all stages, incubation was maintained at between 23 to 25°C, with the first stage being incubated for approximately 24 hours, the second stage between 12 and 17 hours, and the third stage between 14 and 19 hours, until the OD₅₉₀ values of the culture reached approximately 0.1. These inoculum preparation stages were carried out by the Microbiology Department at Fisons, Holmes Chapel, each stage being duplicated between 4 and 6 times, to allow for any problems that might occur with any batch of culture - thus providing a maximum of 60 litres of inoculum to be transferred to the main fermenter.

5.2.2.2. 800 Litre Vessel.

The three-stage inoculation procedure for this fermentation is shown diagrammatically in Figure 5.6. MRS broth was used as the medium in each stage of inoculum development. Initially, six McCartney bottles, with a volume of 10 cm³ broth, were inoculated with cells from two slopes of culture. These cells were incubated at a temperature of 25°C for approximately 12 hours, after which time the cells were transferred to six flasks, each containing 250 cm³ broth. This inoculum was then transferred after 13 hours to three flasks, each containing approximately 16 litres of MRS broth, with the culture from two 250 cm³ flasks being added to each 16 litres of sterile growth medium.

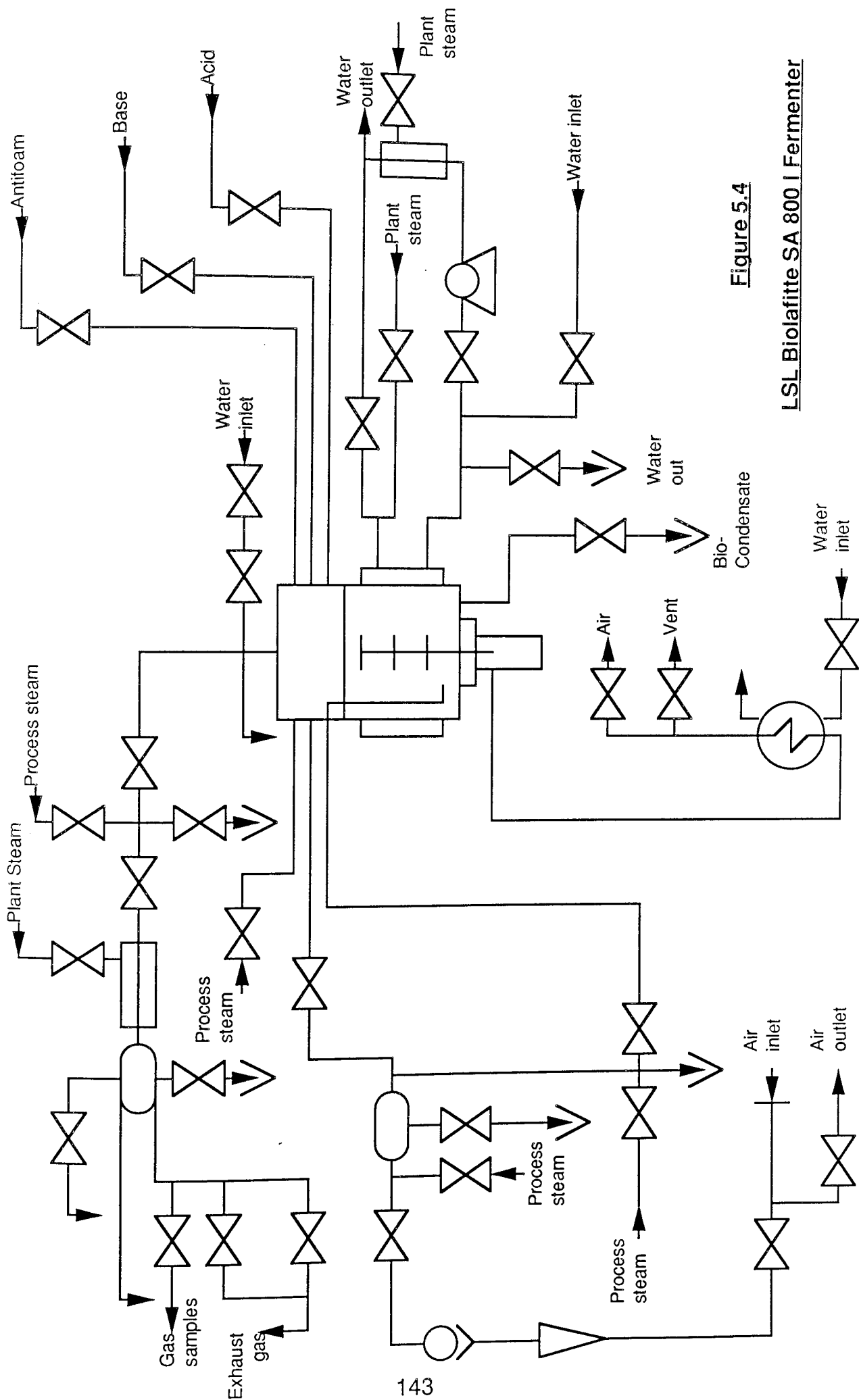


Figure 5.4
LSL Biolafitte SA 800 I Fermenter

**C31 Culture available from Microbiology
Department, Fisons plc**



Inoculate 2 x 500 ml MRS broth bottles (5 ml each) (23 deg. C)



12 - 16 hours

Inoculate 6 x 10 litre MRS broth bottles (100 ml) each (23 deg. C)



12 - 16 hours

**ENZYME
PRODUCTION**

Inoculate Seed Fermenter with 6 x 10 litre bottles (23 deg. C)



16 - 20 hours

**DEXTRAN
SYNTHESIS**

Inoculate Main Fermenter with seed (HZ39T Only) (23 deg. C)



24 - 48 hours

**Process Dextran as usual
(use 85% alcohol)**

Figure 5.5

**Inoculum Development (and subsequent Process Outline)
for 1000 litre Fermentations**

Culture from 2 x slopes of
Leuconostoc mesenteroides



Inoculate 6 X 10 ml MRS broth bottles (25 deg. C)



12 hours

Inoculate 6 x 250 ml MRS broth bottles (10 ml each) (25 deg. C)



13 hours

Inoculate 3 x 16 litre MRS broth bottles (500 ml each) (25 deg. C)



15 hours

ENZYME
PRODUCTION

Inoculate main fermenter with (23.5 deg. C)
3 x 16 litre bottles

Figure 5.6

Inoculum Development Process for 800 litre Fermentation

This final inoculum was allowed to grow for at least 15 hours, before it was transferred to the main fermenter. The growth times selected for each stage of the inoculum development procedure were such as to allow sufficient cell growth as would be denoted by an OD₅₉₀ value of at least 0.1. The inoculum was transferred to the main fermenter using a simple gravity feed technique.

5.2.3. Fermentation and Additional Media.

5.2.3.1. 1,000 Litre Vessel.

The initial working volume of the fermenter was 600 litres, and medium with the same composition as that used for maximum enzyme production in the 6 litre fermenter was prepared. The initial quantities of the various medium components are shown in Table 5.3.

Table 5.3
Medium for 1,000 litre Fermentation.

Sucrose	6 kg
Gistex Yeast Extract	24 kg
K ₂ HPO ₄	12 kg
R* Salts	3 l
(Laboratory AR Grade)	
Water	to 600 l
HCl (~11 M)	~0.7 l
	(to pH 7.1)

R* Salts.

MgSO ₄ · 7H ₂ O	120 g
NaCl	6 g
FeSO ₄ · 7H ₂ O	6g
MnSO ₄ · 4H ₂ O	7.8g

The sucrose/NaOH solution was prepared by dissolving 180 kg of sucrose in water - to a volume of 200 litres, mixing with 57 litres of 17 M NaOH, and adjusting the final volume to 300 litres with water. This mixing process was carried out slowly and carefully as in Section 5.1.4. Again this mixture was

not sterilized, and was transferred to a mobile which was attached to the pH control unit of the fermenter. Whenever the pH dropped below a value of 6.6, this unit triggered a peristaltic pump, which passed the sucrose/NaOH solution into the fermenter.

5.2.3.2. 800 Litre Vessel.

The initial working volume of the fermenter was 450 litres, and the same composition was used for the medium that was prepared in the laboratory for the optimum production of dextransucrase from a fed-batch fermentation. The initial quantities of the various medium components are shown in Table 5.4.

Table 5.4.
Medium for 800 litre Fermentation.

Sucrose	4.5 kg
Gistex yeast extract	18.5 kg
K ₂ HPO ₄ (Laboratory Grade)	8.9 kg
R* Salts (Analytical Reagents)	2.25 l
Water	to 450 l
H ₂ SO ₄ (98%)	~ 0.25 l
	(to pH 7.10)
<u>R* Salts.</u>	
MgSO ₄ . 7H ₂ O	90 g
NaCl	4.5 g
FeSO ₄ . 7H ₂ O	4.5 g
MnSO ₄ . 4H ₂ O	4.5 g

The sucrose/NaOH solution was prepared by dissolving 90 kg of sucrose in water - to a volume of approximately 100 litres, followed by mixing with 28.5 litres of 17 M NaOH. This medium was then adjusted to a volume of 150 litres with the addition of extra water. This mixing process was carried out with great care, as described in Section 5.1.4, to prevent an excessively exothermic reaction from occurring. This medium was not sterilized. The alkali addition vessel for this fermenter had a working volume of

only 10 litres and so it was necessary to continuously add the sucrose/NaOH control fluid to this vessel in order to provide sufficient medium for the cells' sucrose demand.

5.2.4. Preparation of Fermenter and Sterilisation.

5.2.4.1. 1,000 Litre Vessel.

The vessel was initially cleaned by filling it with water and autoclaving, by passing steam through the vessel jacket, to a temperature of 115°C for 20 minutes. The feed input and recirculation lines were disconnected from the vessel, steamed out for 15 minutes, and then sealed with alcohol swabs. The pump body was flushed with alcohol and also sealed with alcohol swabs. The medium was sterilized in the fermenter at 115°C for 20 - 30 minutes, and then controlled at $23 \pm 1^\circ\text{C}$ under a positive sterile air pressure of 0.4 bar.

5.2.4.2. 800 Litre Vessel.

Prior to the commencement of this fermentation, the vessel had been thoroughly cleaned by filling it with water and sterilizing it. This water was then cooled and removed from the fermenter before the insertion of the probes. The pH probe was cleaned and reactivated for 1 minute in a HCl/HF solution and then recalibrated using pH 4.0, 7.0 and 10.0 buffers. The medium components, dissolved in a known volume of water, were then added to the vessel, which was then filled to a level of 450 litres with the use of a hosepipe. Before the sterilization cycle was commenced, the pH was checked using a manual probe, and found to be at a level of 7.53, whilst the fermenter probe was reading a value of 7.39. The probe was subsequently recalibrated to the correct value, and the medium pH was then adjusted to a value of 7.10 with the addition of 98% H₂SO₄. The fermenter was then sealed and the computer, supplied as an integral part of the unit, was programmed with the details of the sterilization cycle required. The fermenter was sterilized at 121°C for 21 minutes, and then cooled to a temperature of 23.5°C. This entire sterilization process was controlled and monitored by the fermenter computer, with no necessity for any manual adjustments to be carried out. The sterilization cycle was recorded graphically by the computer and Appendix A.3 shows the vessel pressure, temperature and sterilization setpoint over the sterilization cycle period.

5.3. Continuous Fermentations.

Although the majority of continuous fermentations carried out during the course of this project were with a 2.5 litre Minibioreactor (MBR) fermenter, supplied by Sulzer (U.K.) Ltd., a small number of experiments had previously been carried out using a New Brunswick BioFlo C30 Chemostat. Therefore, a small part of this section will concern the New Brunswick Fermenter, whilst the larger part will be concerned with a discussion of the MBR Fermenter.

5.3.1. New Brunswick Model.

The New Brunswick BioFlo C30 Fermenter was a bench top Chemostat which was loaned to the project by Dr. P. Lambert of the Department of Pharmaceutical Sciences, Aston University. The fermenter was equipped with a fixed overflow tube, so that the working volume of the fermenter was approximately 338 cm³. The fermenter was fitted with a temperature and pH control unit, and agitation was by means of a magnetic stirrer (200 - 1,000 rpm). The vessel was not self-sterilizable, so it was necessary to sterilize it, together with medium and probes, in the Adelphi Autoclave at 116 - 121°C for 20 minutes. After the sterilization cycle, the fermenter was fitted into the BioFlo Module, and the temperature controlled at 23 ± 0.1°C, by means of a cooling finger inserted into the vessel. The small number of runs carried out using this fermenter will be discussed in Section 8.1.

5.3.2. MBR Model.

The MBR BioReactor was purchased as an ex-demonstration model from Sulzer (U.K.) Ltd. in July 1988 ⁽⁹⁸⁾. The unit consisted of a glass cylinder vessel with stainless steel head and bottom plates. The total volume of the fermenter was 2.5 litres. The fermenter was supplied with an adjustable overflow weir, such that the fermenter could be operated with a variety of working volumes. The bottom plate contained eight process ports, of 12 mm. diameter, into which were inserted two 400W heaters, a cooling finger, a temperature probe (PE 100), and a safety thermostat. The remaining ports were used for the overflow tube and the sampling port. Any outstanding ports were blanked off with a blind plug. The head plate contained eight ports,

seven of which were 12 mm in diameter, and were used for the pH probe, the air inlet valves and any hypodermic needle connectors, which were used for the inoculum addition, pH control fluid addition and medium feed. The eighth port had a 19 mm diameter and was used for the insertion of the dissolved oxygen probe.

The temperature of the fermentation was controlled with a PID Controller, with either one of the heaters being switched on for a short period of time, or by cooling water passing through the cooling finger inserted within the fermenter. The cooling water inlet was connected to a solenoid valve located at the base of the fermenter, with the output from the cooling finger being connected to a drain.

The compressed air was connected to a Serto connector on the cabinet on which the fermenter was mounted. The allowed air pressure was 2 to 7 bar.

The fermenter was agitated by a stirrer with two multi-bladed impellers, with the stirrer shaft entering the vessel through the bottom plate, being driven by a DC motor located in the cabinet on which the fermenter was mounted. Generally, bottom entry stirrers tend not to be used as the bearings are submerged, although improved design has reduced this problem. Bottom entry is chosen when the top plate of a fermenter is required for a large number of entry ports, as in this case. The fermenter was fitted with an hour counter which indicated the total amount of working hours of the stirrer. The agitation rate was controlled by a PI characteristic controller.

The dissolved oxygen of the broth was monitored by an amperometric DO electrode, with measurement being based on a current measurement. The controller had a PI characteristic and a continuous output, and when controlling the DO concentration, it did so by altering either the stirrer speed or the air flow rate into the fermenter.

The pH of the broth was monitored by an Ingold type 465 glass electrode, with a range of 0 - 14 (± 0.01). The controller had a PI characteristic and two semi-continuous outputs (acid and base).

5.3.3. Inoculum Preparation.

From MRS Agar slopes of culture, a one stage inoculum was prepared by transferring cells as in Section 5.1.2 to a 100 cm³ MRS broth container. A one-stage inoculum procedure was sufficient due to the low working volume of the continuous fermentations (approximately 1 litre). The inoculum was incubated at 23 - 25°C for 20 - 24 hours, until there was sufficient cell growth for transfer to the main fermenter. For the New Brunswick Fermenter, an inoculum size of 15 cm³ was required, as the working volume of the fermentation was only 338 cm³ with a stirrer speed of 200 rpm. The inoculum was incubated at 23-25°C for approximately 15 hours (overnight).

5.3.4. Fermentation Medium.

The medium used for the continuous fermentations was similar to that used for fed-batch fermentations, except for the variation in sucrose concentration. Sucrose levels in the medium varied between 1 and 5% w/v during the course of the project. It was also necessary to substitute K₂HPO₄ with the more acidic KH₂PO₄ as the dilution rate of the fermentation was increased, since at the higher dilution rate, the more alkaline medium (i.e. that prepared with the K₂HPO₄ tended to increase the overall pH of the medium, as the acid produced by the growing cells could not compete with the incoming basic medium. The medium composition (both initial and feed) is shown in Table 5.5

Table 5.5.

Medium for Continuous Fermentations.

Sucrose	10 - 50 g/l
K ₂ HPO ₄ or KH ₂ PO ₄	20 g/l
Gistex Yeast Extract	40 g/l
R* Salts (+)	0.5% v/v
(+) (as Table 5.1).	

5.3.5. Additional Media.

Unlike with the fed-batch fermentations, it was not necessary to control the pH with a sucrose/alkali solution, as the continuous feed to the fermenter contained all the nutrients required for growth and enzyme production.

Instead, the pH was controlled with the addition of 5M NaOH solution. It was not found necessary to sterilize this solution.

5.3.6. Sterilization and Aseptic Techniques.

All inoculation preparation techniques were carried out in the Laminar Flow Cabinet, as described in Section 5.1.5. All connections made during the course of the fermentation, including connections and disconnections of bottles of feed medium, were carried out with extreme care, by swabbing any connection ports liberally with alcohol, as it is these linkage points that could prove to be the source of any contamination that may occur.

The fermenter itself was sterilized *in situ*, by the two heating elements inserted into the base of the fermenter working together to bring the temperature up to 116°C. During the sterilization cycle, it was important to fit the protective metal jacket around the fermenter, as a safety measure, should the glass cylinder explode at the higher temperatures or pressures. The vessel was also fitted with a safety thermostat, which would switch off the heaters should the temperature exceed 125°C.

The feed medium was sterilized in one of two ways. One method was to prepare 5 litres of medium in an aspirator bottle and to sterilize it in the autoclave. This had a great disadvantage, as the medium took a long time to heat up and to cool down - with the central section of the medium remaining at a high temperature for a long period of time - such that caramelization and, hence, degradation of the medium was occurring - giving reduced enzyme yields. The improved method was to sterilize empty aspirator bottles in the autoclave, and sterilize the medium itself in the 16 litre New Brunswick Fermenter. The medium was then aseptically transferred through sterile tubing to the aspirator bottles. This method was advantageous as the sterilization cycle was much shorter, due to the mixing of the medium in the 16 litre fermenter - and was much quicker than the first method, as 10 litres of medium could be sterilized in the 16 litre fermenter at a time. The sterilization temperature of the medium was $116 \pm 0.5^\circ\text{C}$, and was held there for 20 minutes. The aspirator bottles were sterilized at $121 \pm 1^\circ\text{C}$ for 20 - 30 minutes, as it was not possible to over-sterilize empty glass bottles.

5.4. Analytical Techniques.

The course of the fermentation was monitored using the various analytical techniques described in the following section, including determination of the cell growth and dextransucrase activity.

5.4.1. Cell Growth Determination.

It was necessary to follow the progress of the cells' growth during the course of the fermentation - particularly with respect to the fed-batch fermentation where cell growth is continually increasing until the stationary phase is reached. A simple method was used to determine the cell growth of the fermentation, which measured the turbidity, or optical density, of the culture. A sample was removed from the fermenter (at hourly or half-hourly intervals for a fed-batch process, and at longer intervals for a continuous fermentation), and diluted by a factor of 25 with saline (0.9% w/v NaCl solution). The optical density of the sample was determined at a wavelength of 590 nm on a UV photospectrometer (the model used at Aston was a Pye Unicam SP 1800 UV visible photospectrometer) using plastic cuvettes. The photospectrometer was initially set to zero using saline. It was seen that once the OD₅₉₀ readings passed the value of 0.6, the linearity of the photospectrometer was in question, so the sample was diluted further, with the resulting OD₅₉₀ value multiplied by the dilution factor. During the lag phase of the fermentation, the OD₅₉₀ value was typically less than 0.1, but during the exponential phase of the culture, the OD₅₉₀ increased to a maximum value of up to 1.1 or 1.2, depending on the fermentation conditions.

5.4.2. Cell Dry Weight Determination.

The cell dry weight determination was a lengthy procedure that was carried out to correlate the culture turbidity with the cell dry weight, and as such, was not carried out during every fermentation. Figure 5.7 shows the calibration curve drawn up for OD₅₉₀ against cell dry weight (g/l). The cell dry weight was determined as described below.

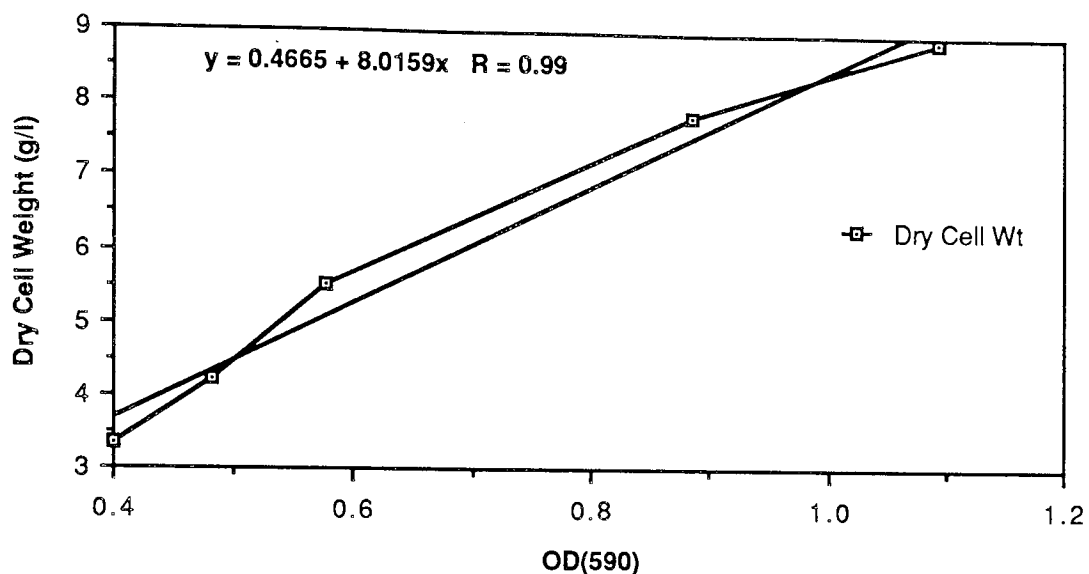


Figure 5.7: Dry cell weight calibration curve

Firstly, clean centrifuge tubes were dried in an oven at 90°C to remove all moisture and were allowed to cool in a dessicator, before being accurately and carefully weighed. Samples from the fermenter, with known OD₅₉₀ values, were put into the dry tubes, with the volumes being measured. The tubes were then placed in a centrifuge, and centrifuged at 300 rpm for 30 minutes, with the supernatants then being decanted and discarded. The remainder was then resuspended in saline and centrifuged as before. This was again repeated and the resulting cell precipitate was dried at 90°C for at least 48 hours. The dried tubes and cells were weighed and the cell dry weight was determined by subtracting the weight of the tubes from the combined weight of tube and cells. The weight of any solids in the medium was determined by carrying out the above procedure using sterile medium, and subtracting the final weight from the cell dry weight already determined. The cell dry weight was expressed in units of g/l by dividing the final weight by the volume of broth used for each tube. This procedure was carried out twice for each sample, with the two values being averaged to give a final result.

5.4.3. Enzyme Activity Determination.

The activity of the dextransucrase was determined using one of two methods - Hostettler's Method, or the HPLC (High Performance Liquid Chromatography) Method. These two techniques will be discussed in the following sections.

5.4.3.1. Hostettler's Method.⁽⁹⁹⁾

Enzyme activity was determined by reacting a sample of broth with a known amount of sucrose, and estimating the rate of fructose released by Hostettler's Method. This technique used the theory that 3-amino-5-nitrosalicylic acid is produced when 3,5-dinitrosalicylic acid reacts with reducing sugar. The amount of coloured product produced can be determined using a UV photospectrometer (as in Section 5.4), which is proportional to the fructose present. Dextransucrase, when incubated with sucrose, produces dextran and fructose and it was this fructose production that was analysed with this technique. The reagent used with this technique is known as "Sumner's Reagent" and consisted of 10 g 3,5 dinitrosalicylic acid and 300 g potassium-sodium-tartrate dissolved in 1 litre of 0.4 M NaOH. The activity assay was carried out as follows :-

A sample of enzyme containing 30 - 50 DSU/cm³ (1.44 - 2.41 U/cm³) or an appropriate dilution of it was diluted by a factor of 5 with 6.25% w/v sucrose in sodium acetate buffer (pH = 5.2 - optimum pH for the production of dextran) and shaken. An initial sample of 0.5 cm³ of this mixture was removed, and the remainder was incubated in a water bath at 25°C for the course of the assay. The withdrawn sample was added to 1 cm³ of Sumner Reagent, and boiled for about 5 minutes, to denature any enzyme present and to allow the fructose formed to convert the 3,5-dinitrosalicylic acid to 3-amino-5-nitrosalicylic acid. This sample was then cooled and diluted with 11 cm³ of distilled water, providing the unincubated sample (see Equation 5.1). Further 0.5 cm³ samples were withdrawn from the water bath at five minute intervals, up to a period of 20 minutes, with the above procedure being carried out for each sample. The absorbances of these samples at a wavelength of 530 nm were then determined and the enzyme activity calculated using the following Equation:-

$$DSU = \frac{(OD_{530t} - OD_{530u}) \times d \times 60 \times 2}{0.2 \times 0.52 \times t \times OD_{530f}} \quad 5.1$$

where OD_{530t} = Optical Density at 530 nm of incubated sample at time t
 OD_{530u} = Optical Density of unincubated sample
d = Dilution Factor of Enzyme
 OD_{530f} = Optical Density of a 2 g/l fructose solution on which Hostettler's Method has been carried out as before
t = incubation time (minutes)

The enzyme activity in DSU/cm³ was then converted to the standard Dextranucrase unit, U, using Equation 5.2.

One DSU (Dextranucrase Unit) refers to the amount of enzyme which will convert 1 mg of sucrose to dextran in 1 hour at 25°C and a pH of 5.2.

It has recently become necessary to use a standard, international unit for dextranucrase activity - the "U" - which is defined as follows :-

One U refers to the amount of enzyme that will release 1 μmole of reducing sugar (in this case fructose) from sucrose in one minute.

$$U = 20.77 \times DSU \quad 5.2$$

All future references to enzyme activity will be given in units of both DSU and U, with the DSU value being calculated from Equation 5.1, and the U value from Equation 5.2.

When taking the OD_{530} readings it was necessary to produce a blank sample in order to zero the photospectrometer. This was done by adding 0.5 cm³ water to 1.0 cm³ Sumner Reagent, and carrying out the process described for the enzyme. This was checked with a sucrose blank (0.5 cm³ of 6.25% sucrose solution in pH 5.2 acetate buffer) prepared in the same way. If these two samples had the same OD_{530} values, it indicated that the sucrose solution was not contaminated, and could be used in the assays.

The OD₅₃₀ value was checked by analysing solutions of various fructose concentrations, and plotting the values obtained to ensure linearity (See Figure 5.8).

5.4.3.2. HPLC Method.

The Hostettler Method for determining enzyme activity is quick (less than 30 minutes) and reasonably accurate. However, the HPLC Method for assaying enzyme activity can also be used, although this process is considerably more time-consuming, and does not result in much greater accuracy, and as such, tends not to be carried out. However, it is a useful check, to ensure that Hostettler's Method is being carried out correctly. This method is based on the determination, by HPLC analysis, of sucrose consumed by the enzymic reaction over a time period of, say, 2 hours, or of fructose released by the same reaction. The concentrations of sucrose, fructose and dextran were determined using HPLC analysis, every 10 to 20 minutes, during the reaction, where 20 cm³ of enzyme (diluted to give approximately 50 DSU/cm³ of activity) were added to 80 cm³ of a solution at pH 5.2 containing 2.0 g of sucrose. The activity was calculated using the following Equations :-

Activity based on sucrose consumption :

$$\text{DSU/cm}^3 = \frac{M_1}{1.0} \times 50 \times d \quad 5.3$$

Activity based on fructose released :

$$\text{DSU/cm}^3 = \frac{2 \times M_2}{1.052} \times 50 \times d \quad 5.4$$

where M₁ and M₂ are the initial slopes for the sucrose and fructose curves respectively (from a concentration versus incubation time plot), expressed in % w/v/h and d, the enzyme dilution factor.

The HPLC operating parameters were set as described below in Section 5.4.4.

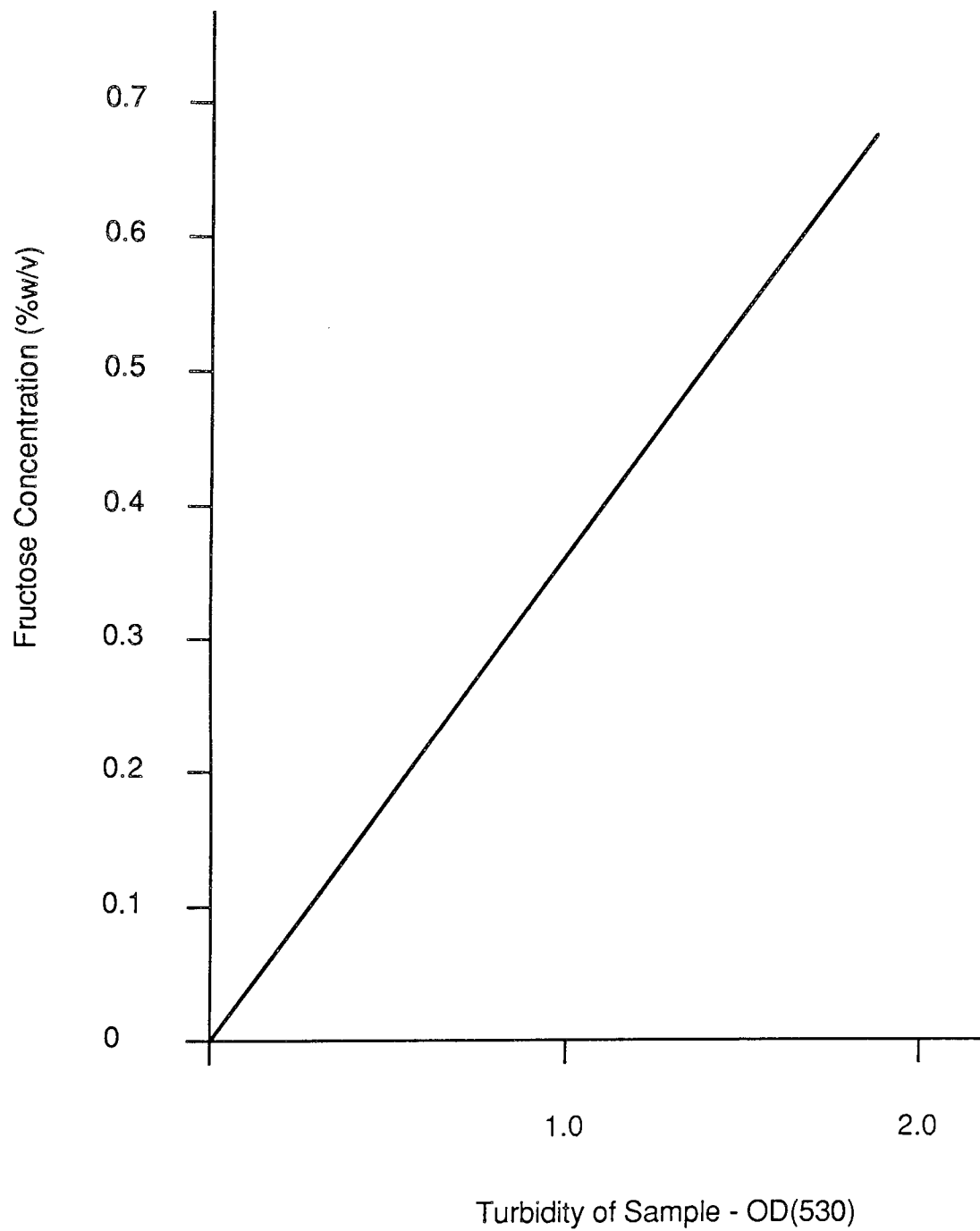


Figure 5.8

Fructose Calibration Curve

5.4.4. Fermentation Broth Analysis.

It was necessary, especially during continuous fermentations, to carry out analysis of the fermentation broth, to determine substrate concentration, as well as to monitor fructose, dextran and other carbohydrate levels in the culture fluid. A HPLC (High Performance Liquid Chromatography) system was used for this analysis, and is described below :-

The system used an Aminex Carbohydrate HP x 87 C column, supplied by Bio-Rad Laboratories, Watford, who also supplied the pump, column heater, and refractometer. The system also consisted of a Hewlett Packard Model 3390 A Integrator, where the various peak areas were calculated, and the actual concentrations obtained by comparing peak size, with that of a known concentration. Figure 5.9 shows a diagram of the HPLC, and Table 5.6 gives the operating conditions of the system.

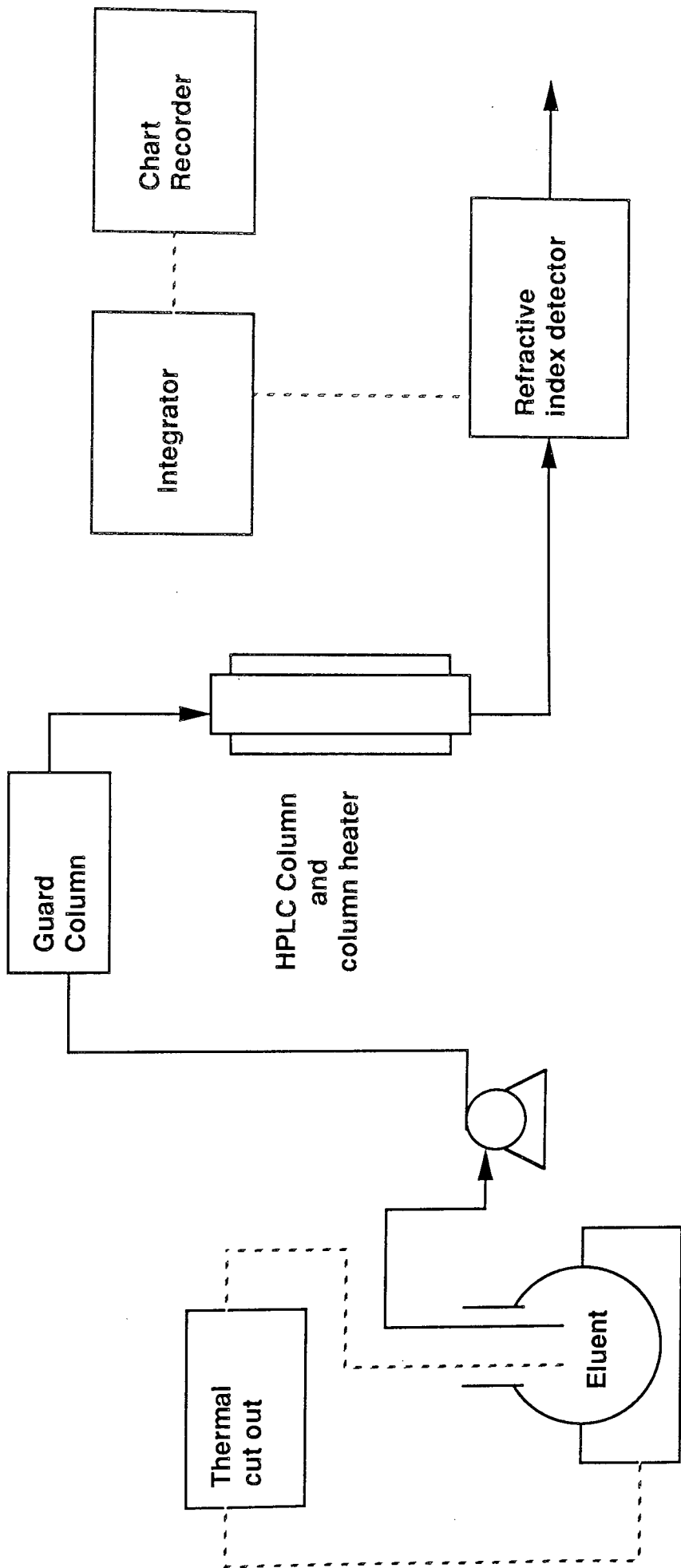
Table 5.6
HPLC Operating Conditions.

Eluent Flow Rate	0.5 (cm ³ /min)
Operating Pressure	up to 100 (bar)

Retention Times (mins.).

Dextran	8.03
Sucrose	10.02
Glucose	12.00
Fructose	15.32

All samples were passed through a 0.80 µm disposable filter, to remove any cells, before injection into the HPLC, with each sample then passing through the guard column, which acts as a prefilter - removing any remaining cells. This is necessary to prevent blockage of the system, and increase the life of the column.



Isomantle

Figure 5.9

HPLC Analytical System

5.4.5. Exit Gas Analysis.

The dissolved oxygen concentration in the fermenter was monitored continuously throughout the experiment with a DO probe. However, it was also possible to perform an analysis of the exit gases from the fermenter (namely O₂, CO₂ and N₂), using a Pye Unicam Series 204 gas-liquid chromatograph (glc). Samples were removed from the air space above the medium through a sterile tube that connected the vessel to a stainless steel bicycle pump, via a sterile filter. The samples were collected in stainless steel gas cylinders, and analysed by Mr. D. Walton, a member of the Technical Staff in the Department of Chemical Engineering and Applied Chemistry, at the University of Aston.

5.4.6. Gram Stain Sterility Check.

This is a differential staining procedure used to identify eubacteria, and to determine the presence of any contaminant micro-organisms in a sample. The process was carried out by fixing a smear of bacteria onto a microscope slide, and staining it with a solution of crystal violet, followed by a dilute iodine solution and then cleaning in ethanol or acetone. Gram-negative bacteria are completely decolourised by the solvent, although Gram-positive bacteria (such as *Leuconostoc mesenteroides*) retain the stain. A counter-stain can also be used, such as Safranin, which stains the negative cells, enabling them to be distinguished on microscopic examination. Using this technique, any Gram-negative contamination can be detected, and can explain a failed fermentation.

6. RESULTS - BATCH AND FED-BATCH FERMENTATIONS.

Batch and fed-batch fermentations were carried out on various scales, ranging from preliminary batch fermentations with a volume of 300 cm³, in shake flasks, to larger volume fed-batch experiments. These were carried out with working volumes of approximately 1 litre (MBR vessel) and 6 litres (New Brunswick vessel), along with scale-up fed-batch studies on vessels with working volumes of 600 litres (Birmingham University fermenter) and 800 litres (Fisons fermenter, Holmes Chapel). This chapter will discuss the experimental work carried out in each phase of the project mentioned above, along with the results obtained.

Before commencing this project, it was necessary for the author to become familiar with the various microbiological techniques, such as inoculum preparation, sterility checking, and various other culture handling skills in order to proceed successfully with the experimental work. These skills were duly acquired at the Microbiology Department of Fisons plc, Pharmaceutical Division, Holmes Chapel, in October 1987.

6.1. Shake Flask Experiments (Batch Fermentations).

These preliminary batch fermentations were carried out in flasks with a total volume of approximately 500 cm³, although a working volume of 300 cm³ was employed for the actual experimental work. These experiments were undertaken to determine whether it would be worthwhile running any full fed-batch fermentations with peptone as the medium nitrogen source as well as, or instead of, the yeast extract currently used. The peptones used during this section of experimental work were supplied by Oxoid Ltd., who claimed that "... chemo-organotrophic organisms grow best in culture media containing a partially digested protein ..." (100). Proteins have a chemical structure of one or more chains of alpha-amino-carboxylic acids (amino acids) consecutively linked covalently between the alpha-amino group of one molecule and the alpha-carboxylic group of the next, with the elimination of water. This linkage is known as the "peptide bond", with chains of three or more amino acids being termed "polypeptides", whilst larger structures with a molecular range of between 5,000 and several millions become the proteins. The hydrolysis of proteins which breaks them down to their constituent amino acids and peptides can be achieved by the use of strong acids, strong bases, or proteolytic enzymes. The result of such protein breakdown is a peptone

containing a chemically undefined mixture of peptides and amino acids. The manufacturing process of peptone produces syrups and powders. The syrups can be stored for long periods at room temperature, because the high dissolved solid content inhibits bacterial contamination and they can be used in fermentation processes without being dried to a powder. The peptones used in these preliminary experiments were Bacteriological Peptone, Peptone P, Special Peptone, Casein Hydrolysate and Oxoid yeast extract.

6.1.1. Peptones Used in Batch Experiments.

The following sections give a brief description of the various types of peptones mentioned above.

6.1.1.1. Bacteriological Peptone.

This is a very nutritious all purpose peptone prepared by the enzymatic digestion of selected animal proteins, and contains possibly the widest molecular weight spread of peptides in any of the meat peptones. This product is used in industry to produce antibiotics, interferon, pasteurilla vaccine and as a stabiliser for other vaccines.

6.1.1.2. Peptone P.

This is a peptic digest of fresh meat used in bacteriological culture media. It has a high sulphur content and is often used as a component of media used to demonstrate hydrogen sulphide production. This peptone is also used in media to produce interferon.

6.1.1.3. Special Peptone.

This is a specially prepared mixture of peptones, including meat, plant and yeast digests designed to encourage the growth of the most demanding organisms. It contains a wide spectrum of peptide sizes together with those minerals, vitamins, nucleotides and other carbon compounds present in the individual peptones. Special peptone is a component of media used for the culture of fastidious organisms.

6.1.1.4. Casein Hydrolysate.

This is prepared by the hydrolysis of casein with hydrochloric acid under pressure, followed by a neutralisation with sodium hydroxide. The product is then processed to remove impurities and form a light coloured peptone. Casein Hydrolysate provides the necessary nitrogenous material for culture

media. As acid hydrolysis is non-specific, attacking all peptide bonds and degrading proteins and polypeptides to low chain length peptides and amino acids, Casein Hydrolysate may also be used in media when amino acid mixtures are specified.

6.1.1.5. Yeast Extract.

This is a dried yeast autolysate which is a good source of nitrogen and vitamins, particularly the water soluble B-complex vitamins. Its addition, often in small quantities, to many media or fermentation broths, increases the yield of organisms.

These peptones were used in the preliminary shake flask experiments mentioned previously. In all cases 300 cm³ of the prepared medium were added to each flask before sterilization in the Adelphi Autoclave at 116 - 121°C for 20 minutes. The flasks were kept in a water bath held at 24°C, and shaken manually at intervals. The inoculum was developed in one stage, with one slope of culture being used to inoculate two 15 cm³ MRS broth bottles, which were subsequently incubated at 25°C for 12 hours.

The flasks were then inoculated with 6 cm³ of culture from one of the broth bottles. The flasks were sampled at various intervals using sterile syringes, supplied by Sterilin Ltd., Feltham, England.

6.1.2. Results Obtained With Various Peptones.

The various peptones discussed above were incorporated into media containing 1% w/v sucrose, 2% w/v K₂HPO₄, 0.5% v/v R* salts, and 4% w/v of the peptone, with the pH being adjusted to a value of 7.0. A flask containing 4% yeast extract was similarly prepared as a control sample. The flasks were each sampled for pH, OD₅₉₀ and enzyme activity (if necessary) throughout the duration of the fermentations.

Figure 6.1 shows the pH profiles recorded for each of the peptone media. It can be seen that the Special Peptone medium produced the greatest pH drop - decreasing from the initial value of 7.0 to a value of 6.2 after 13 hours. The Bacteriological Peptone produced a similar pH profile, with the lower pH value of 6.3 being slightly higher than that obtained with the Special Peptone. Casein Hydrolysate produced a smaller pH drop over 20 hours, whereas both

the Peptone P and the yeast extract media allowed no pH drop, implying no bacterial growth.

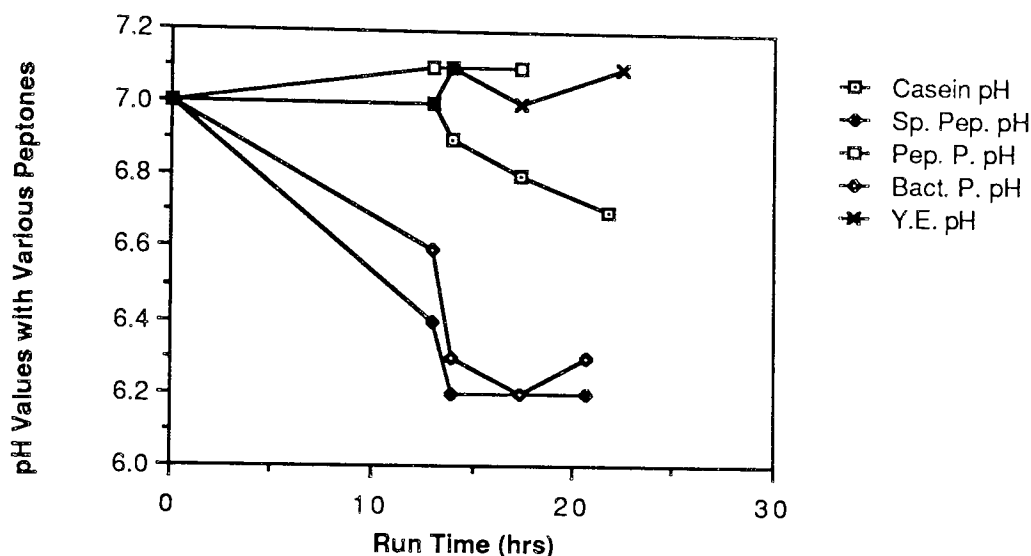


Figure 6.1: pH Profiles with Various Peptones

Figure 6.2 shows the OD₅₉₀ profiles recorded for the various peptone media. It clearly shows that, as indicated by Figure 6.1, Special Peptone and Bacteriological Peptone supported much higher growth levels than the other peptones, with a limited amount of growth occurring with Casein Hydrolysate and no growth with either Peptone P or the yeast extract. It is not clear why the yeast extract supported no growth, which would normally be expected as Gistex, DCL and Orhly extracts all allow some growth.

Table 6.1 gives the maximum enzyme activities achieved for the different media, apart from the yeast extract medium which supported no growth.

Table 6.1.
Enzyme Activity with Various Peptones.

Peptone	Activity (DSU/cm ³)
Casein Hydrolysate	5.6
Special Peptone	31.4
Peptone P	6.2
Bacteriological Peptone	47

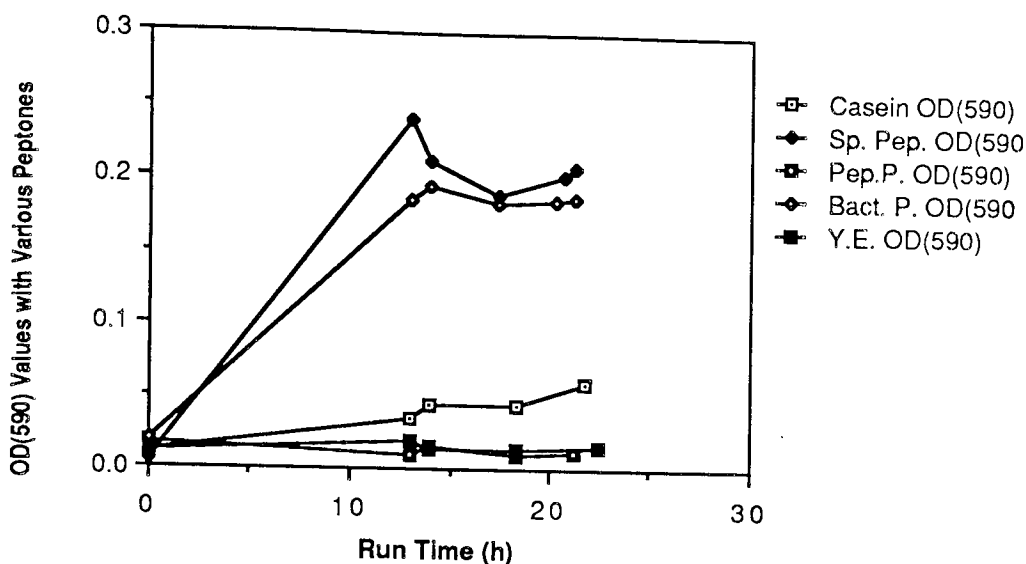


Figure 6.2: OD(590) Profiles with Various Peptones

These results indicated that, although Special Peptone allowed the best cell growth, the best enzyme yield occurred with the medium containing the Bacteriological Peptone, so this peptone was selected for use in further shake flask experiments.

6.1.3. Results Obtained With Bacteriological Peptone.

The shake flasks were prepared as detailed previously, but instead of containing 4% w/v of the peptone, the media were formulated with varying concentrations of Gistex yeast extract and Bacteriological Peptone. Each flask contained 4% w/v of the nitrogen sources, although the quantity for Bacteriological Peptone varied from 25% of the nitrogen source, i.e. 1% w/v Peptone and 3% w/v Gistex, to 100% of the nitrogen source, i.e. 4% w/v Peptone. As in the previous experiments, the pH, OD₅₉₀ and enzyme activity were monitored during the course of the fermentation. The pH profiles for all four samples were very similar, with the pH dropping from an initial value of 7.0, to values of between 6.4 and 6.3 after approximately 13 hours. Figure 6.3 gives the OD₅₉₀ profiles from the various media, showing that there is very little difference between the various medium compositions, although the medium containing 25% Peptone appears to support slightly more cell growth than the other media. Table 6.2 gives the maximum enzyme activity achieved from each shake flask.

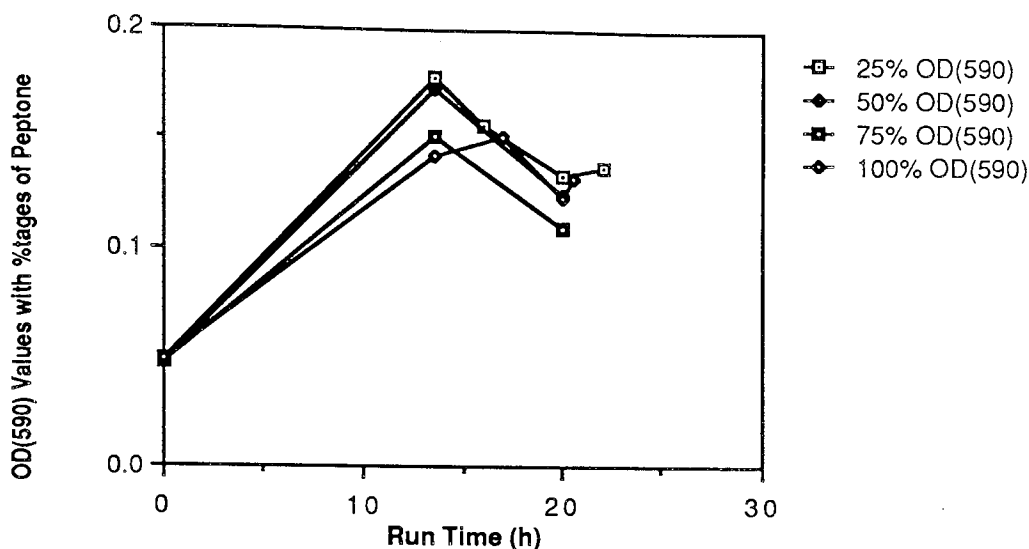


Figure 6.3: OD(590) Profiles with Bacteriological Peptone

Table 6.2.

Enzyme Activity with Bacteriological Peptone.

Percentage Peptone	Activity (DSU/cm ³)
25	46.2
50	40.8
5	-
100	21.0

The best results were obtained with medium containing 25% peptone and 75% yeast extract. From these results it would appear that reducing the concentration of peptone in the medium serves to increase enzyme activity. As no control experiment was carried out with medium containing 0% peptone, it is not known what activity would have been attained with medium containing 40 g/l yeast extract, but it is probable that the activity would have been higher than the value of 46.2 DSU/cm³ (2.22 U/cm³) obtained with medium containing 10 g/l Bacteriological Peptone and 30 g/l yeast extract. However, it would be necessary to carry out further peptone experiments to obtain more accurate results, although it is doubtful whether the substitution of peptone for yeast extract in the medium would be economically viable. The main advantage of using peptones is that the final fermentation broth can be easily analysed to determine the components in the medium, such as vitamins and minerals, that can affect the final result.

6.2. Fed-Batch Fermentations in the New Brunswick Vessel.

The majority of the fed-batch fermentations carried out during the course of this project, were in the New Brunswick 16 litre Microgen fermenter, as described in detail in Section 5.1.1. The fermentations carried out in the New Brunswick vessel were performed under both aerated and unaerated conditions, and therefore the results obtained from this fermenter are given in two sections, i.e. aerated and unaerated experiments.

6.2.1. Aerobic Fermentations.

Several previous researchers have claimed that optimum yields of dextransucrase are obtained under aerobic conditions, whereas work carried out by Ajongwen-Numfor (11) at Aston University has indicated that unaerated conditions allow for maximum enzyme levels. The author has continued the work commenced by Ajongwen-Numfor, and has carried out a number of fed-batch fermentations under aerated conditions to determine the enzyme activities it was possible to achieve with aeration. The vessel was fitted with an air flowmeter that was capable of passing between 1.0 and 24.0 litres of sterile air through the fermenter per minute. During all the aerobic fermentations, the pH was controlled at a level of 6.7, the optimum value for dextransucrase secretion. The inoculum for all the fermentations carried out in the New Brunswick fermenter was prepared as described in Section 5.1.2. In all the aerated fermentations carried out, the medium consisted of 10 g/l Sucrose, 40 g/l Gistex yeast extract and 20 g/l K_2HPO_4 as buffer, along with 0.5% v/v R* salts (see Table 5.1). The sucrose/NaOH feed was prepared as shown in Table 5.2. In all fermentations, the temperature was controlled at $23 \pm 0.5^\circ C$. Unless otherwise mentioned, the agitation rate during these fermentations was maintained at 100 rpm.

6.2.1.1. Results Obtained With Varying Aeration Rate.

Schneider *et al*, (101) in their U.K. Patent linked with Fisons plc, claimed that a dissolved oxygen level of 40 - 70% of the initial saturation value should be maintained during a fed-batch fermentation in order to achieve high enzyme activities. A fermentation was therefore carried out to determine the dextransucrase activity that can be attained under such conditions. An aeration rate of 24 litres/minute into the fermenter was used and the agitation rate was controlled between 300 and 600 rpm in order to maintain a dissolved oxygen level of 40% saturation value within the fermenter. Figure 6.4a shows the OD_{590} and enzyme activity profiles obtained during the course of the

experiment. It can be seen that the enzyme activity reaches a peak value before the cell turbidity does, with the maximum activity of 244 DSU/cm³ (11.75 U/cm³) corresponding to an OD₅₉₀ value of 0.752. The OD₅₉₀ factor reaches its maximum value of 1.071 approximately 2.5 hours from the time when the maximum activity is achieved, a value of about 0.1 of an OD₅₉₀ unit greater than that usually attained from an unaerated fed-batch fermentation. Figure 6.4b shows that, while the enzyme activity has peaked and begun to decrease after 12 hours of the experiment, the rate of sucrose consumption is still increasing by a constant amount, indicating that, while the cells do not require so much sucrose for enzyme production, it is still being consumed for cell growth and maintenance purposes. This phenomenon of enzyme activity reaching a maximum value before the end of the exponential phase of the cell growth cycle will be examined throughout the remainder of this section of aerobic fermentations.

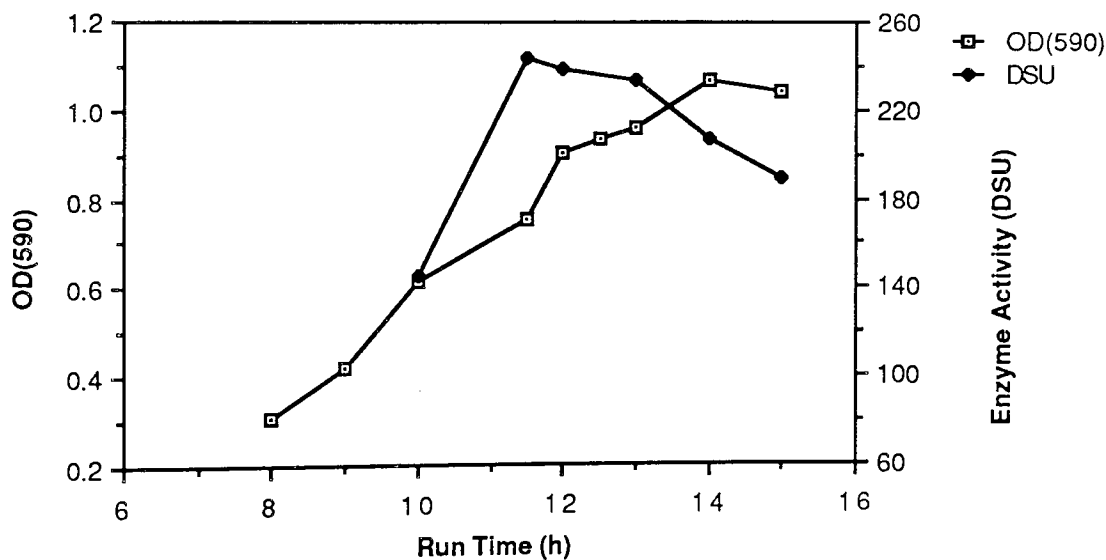


Figure 6.4a: OD(590) and Activity Profiles from R11

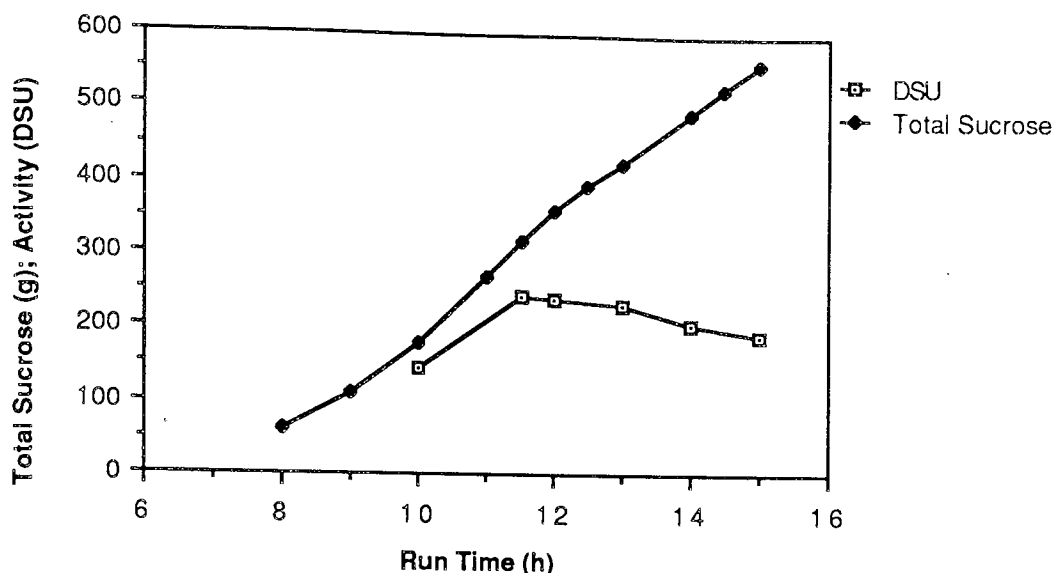


Figure 6.4b: Activity and Sucrose Usage from R11

Following this experiment, a number of fermentations were carried out with varying degrees of aeration, from a maximum rate of 24 litres/minute to the minimum level of 1 litre/minute, all with a constant agitation rate of 100 rpm throughout. The medium for all these fermentations was as previously mentioned in Section 6.2.1. Table 6.3 gives the results obtained from these experiments. The OD₅₉₀ and enzyme activity profiles for these fermentations are shown in Figures 6.5 - 6.8, and all exhibit the same basic structures, whereby the enzyme activity reaches a maximum value, and then decreases, before the OD₅₉₀ factor reaches its peak value. This confirms the results obtained from experiment R11, that exponential cell growth continues beyond the point of maximum enzyme yield, with sucrose demand being consistently high throughout this period. The maximum enzyme activity decreases with increasing aeration rate, indicating that increased dissolved oxygen concentration in the medium somehow inhibits enzyme production, although it does not appear to have a detrimental effect on cell growth. Indeed, aerated fermentations seem to result in increased OD₅₉₀ values, indicating that increased dissolved oxygen concentration in the medium certainly does not inhibit cell growth. These results will be discussed further in Section 7.1.1.1.

Table 6.3.

Results obtained from Aerobic Fed-Batch Fermentations with Varying Aeration Rates.

Run Number	Aeration Rate (litres/min)	Maximum OD ₅₉₀ Value	Maximum Activity (DSU/cm ³)	Total Sucrose Consumed (g)	Duration of Fermentation (h)
R34/11.5.88	1	1.0465	385.5	660	16
R23/24.2.88	12	1.042	249.4	564	17
R24/26.2.88	12	0.921	253.1	492	16.5
R37/7.6.88	24	1.25	236.4	522	16

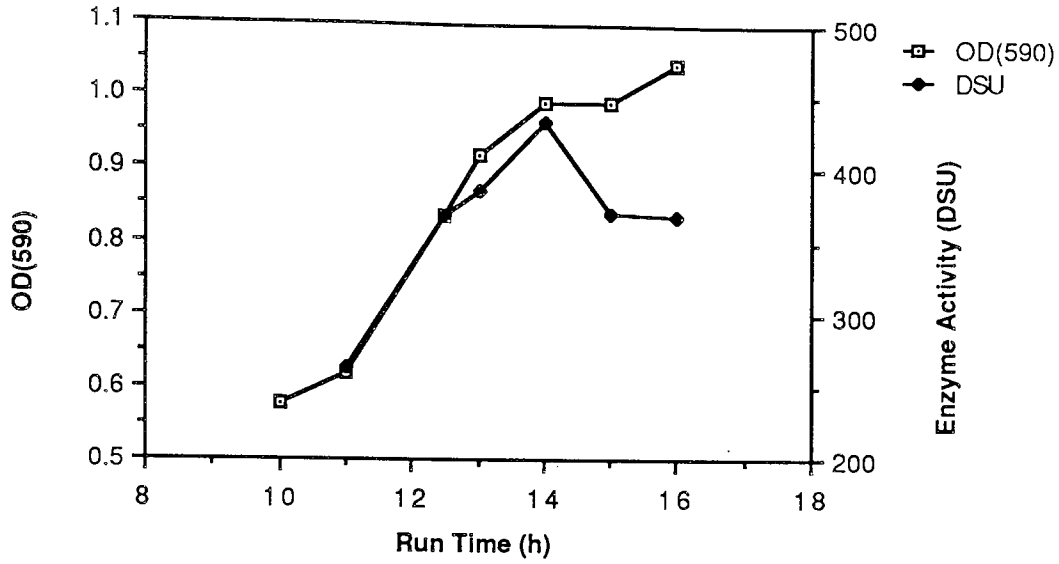


Figure 6.5: OD(590) and Activity Profiles from R34

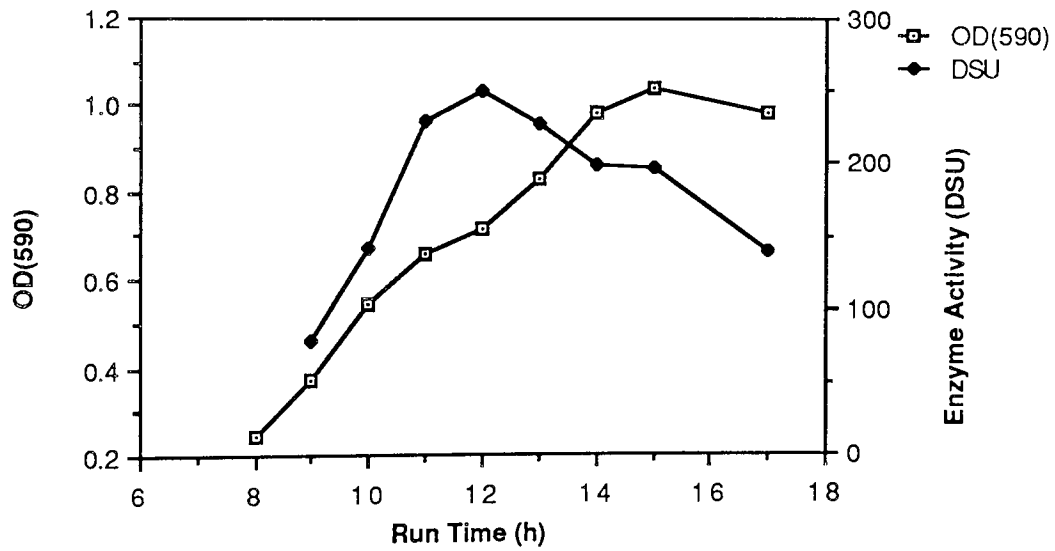


Figure 6.6: OD(590) and Activity Profiles from R23

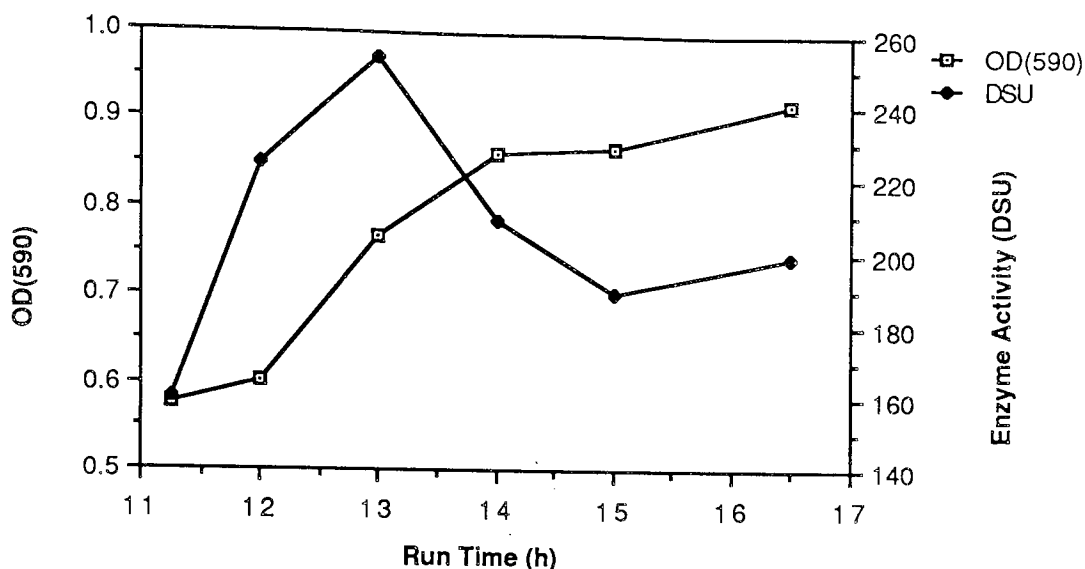


Figure 6.7: OD(590) and Activity Profiles from R24

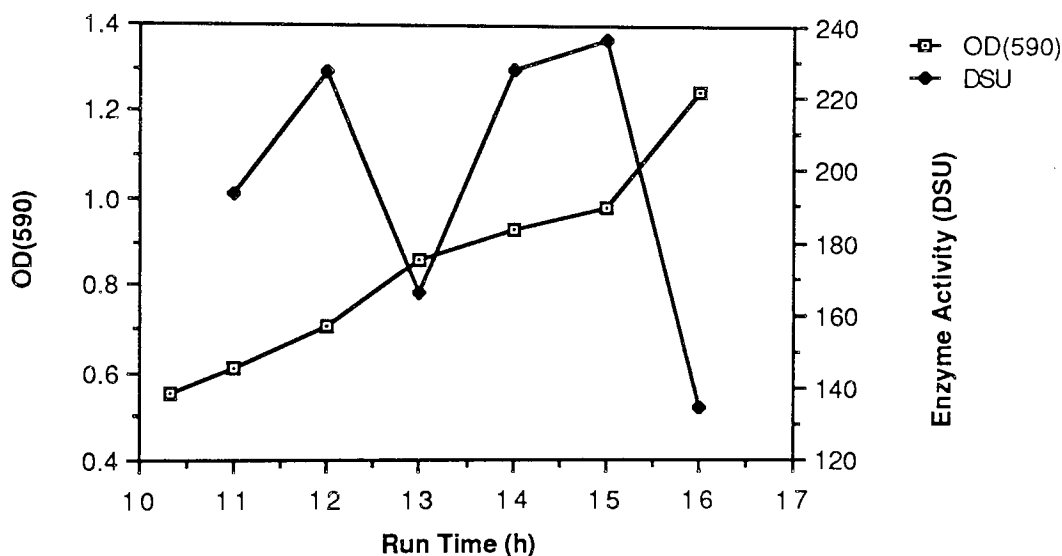


Figure 6.8: OD(590) and Activity Profiles from R37

6.2.1.2. Results Obtained with the Addition of Antifoam.

The experiments listed in Table 6.3 were repeated with the sole difference being the addition of a small amount (2 cm^3) of a silicone antifoaming agent. During a normal dextransucrase fermentation, where there is little or no aeration and a low agitation rate, there is little foam formation during the enzyme secretion stage of the fermentation, with any foam that is produced, corresponding to the termination of the experiment. When the fermentation is more highly aerated or agitated, there is increased foam formation throughout the whole process and so a number of fermentations were carried out under varying aeration conditions, with the addition of antifoam, in order to observe the effect that the presence of antifoam has on a dextransucrase fermentation.

Table 6.4 gives the results obtained from these experiments. Figures 6.9 - 6.12 show the OD₅₉₀ and enzyme activity profiles obtained from these experiments and again they show the activity peaking well before the OD₅₉₀ value. The OD₅₉₀ values obtained during aerobic fermentations with the addition of antifoam are as high as without the presence of the antifoaming agent, implying that the antifoam does not affect cell growth in any way. However, maximum enzyme yields are somewhat increased following the addition of antifoam to the medium, with the more highly aerated fermentation, of 12 litres/minute throughput of air, giving the more significant increase in enzyme activity. It would appear that the presence of antifoam in an aerated medium somehow reduces the rate of oxygen transfer to the cells, and therefore reduces the inhibitory effect that the oxygen seems to cause to dextransucrase production. These results will be discussed further in Section 7.2.2.

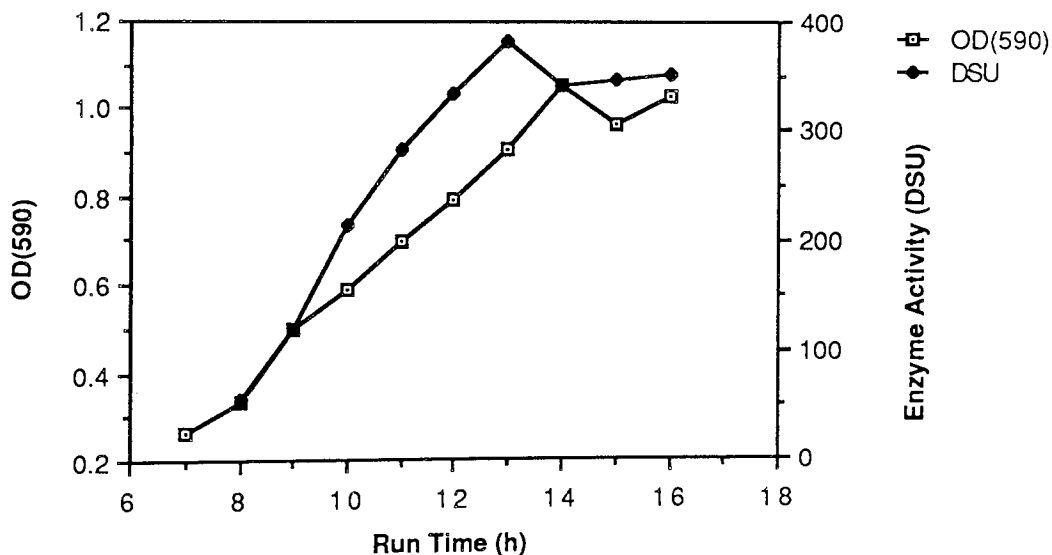


Figure 6.9: OD(590) and Activity Profiles from R27

Table 6.4.

Results obtained from Aerobic Fed-Batch Fermentations with the Addition of an Antifoam Agent.

Run Number	Aeration Rate (litres/min)	Maximum OD ₅₉₀ Value	Maximum Activity (DSU/cm ³)	Total Sucrose Consumed (g)	Duration of Fermentation (h)
R27/15.3.88	1	1.055	382.0	615	16
R28/17.3.88	1	0.994	392.3	540	16
R25/2.3.88	12	0.924	323.2	504	15
R26/9.3.88	12	1.014	306.2	600	16

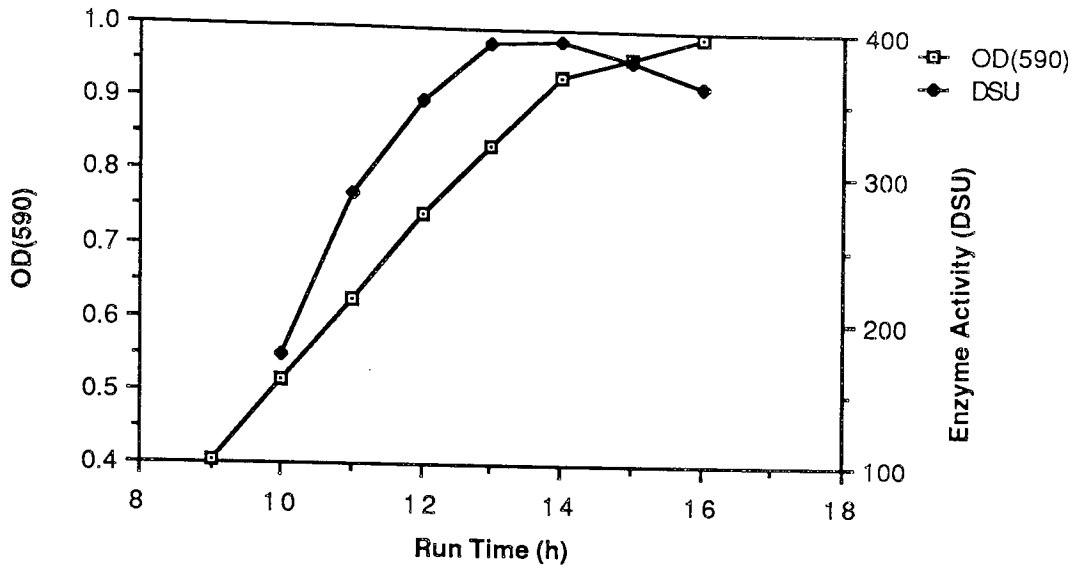


Figure 6.10: OD(590) and Activity Profiles from R28

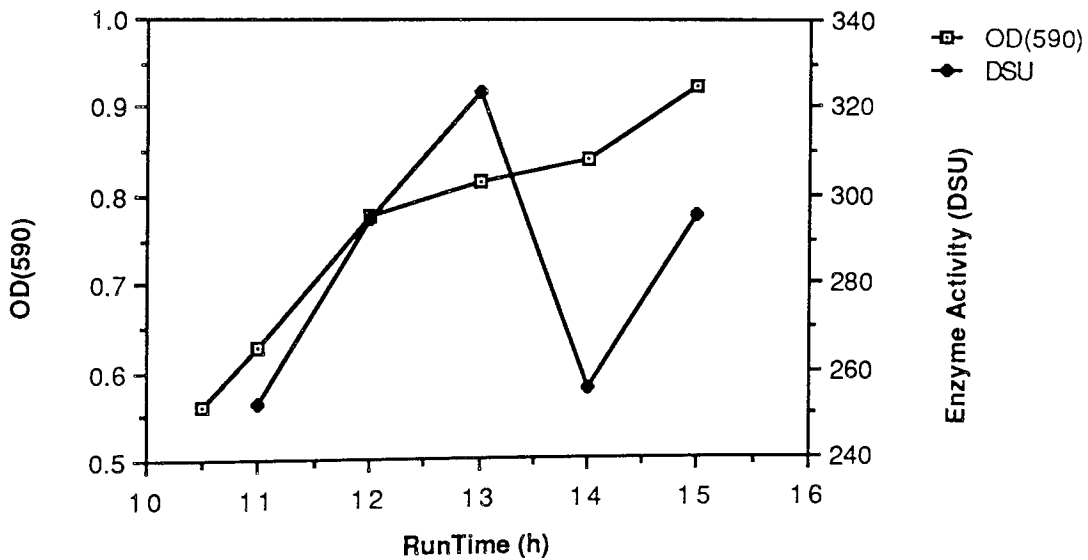


Figure 6.11: OD(590) and Activity Profiles from R25

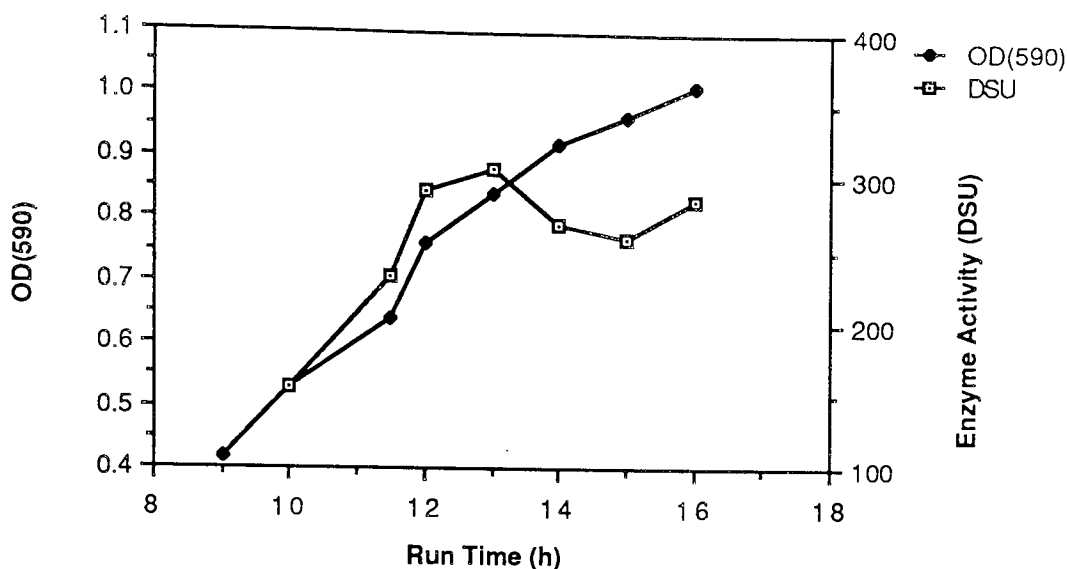


Figure 6.12: OD(590) and Activity Profiles from R26

6.2.2. Un-aerated Fermentations.

The majority of dextransucrase fermentations carried out by previous workers have been under conditions of various degrees of aeration. As discussed in Section 2.2, Schneider ⁽¹⁰⁾ believed that with an un-aerated fermentation, both biomass level and enzyme yield would decrease from the maximum obtainable at a dissolved oxygen concentration of 40 - 50% of saturation. Ajongwen-Numfor, in his 1988 PhD thesis ⁽¹¹⁾, was the first to obtain yields in excess of 400 DSU/cm³ (19.26 U/cm³) under conditions of low agitation and no aeration. A number of un-aerated fed-batch fermentations were therefore carried out to confirm these findings and to investigate a number of other crucial process parameters.

6.2.2.1. Results Obtained With Varying Agitation Rate.

A number of un-aerated fed-batch fermentations were carried out with varying degrees of agitation to determine the effect of shear on the organism and enzyme production. A standard experiment whereby the activity achieved exceeded 450 DSU/cm³ (21.67 U/cm³), was one such as experiment R6/12.11.87. The results from this fermentation are shown in Figure 6.13 and it can clearly be seen that enzyme production closely follows the growth of the organism, and they both reach maximum levels (OD₅₉₀ = 1.026; Activity = 483 DSU/cm³ (23.25 U/cm³)) at similar times. This confirms the statement from Chapter 2, that dextransucrase is a growth-related product when secreted by *Leuconostoc mesenteroides*. The OD₅₉₀ profile of this fermentation clearly shows the lag phase of the culture from the point of inoculation to the time,

approximately 7 hours into the fermentation, when the culture begins to grow exponentially.

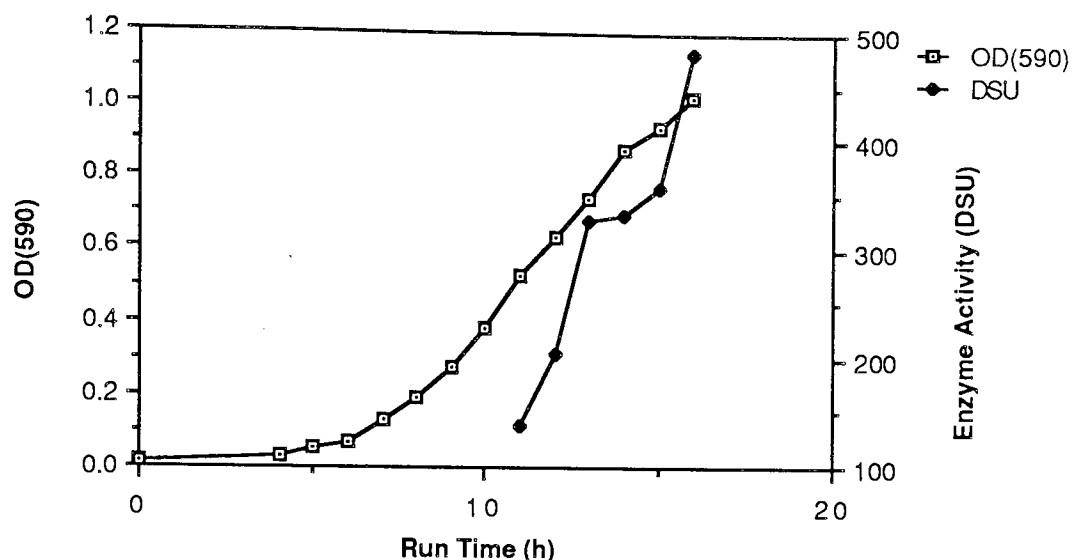


Figure 6.13: OD(590) and Activity Profiles from R6

The experiments discussed in the following section were carried out to determine the effect of increasing the agitation rate on enzyme production. Fermentations were carried out at agitation rates varying from 100 - 450 rpm, with the results from these experiments given in Table 6.5. A number of standard 100 rpm agitation fermentations are listed in the table, showing that it is possible to reach activities in excess of 450 DSU/cm³ (21.67 U/cm³), even when problems occur with the fermentation. For example, experiment R1/14.10.87 encountered many problems including the absence of a heating element to provide temperature control. The temperature was maintained at approximately 24.4°C by wrapping an insulating jacket around the vessel during the cooling period of the sterilization cycle. The inoculum was also not fully grown on inoculation and there was, therefore, a long lag phase of approximately 12 hours during the actual fermentation, leading to a long culture time of 28 hours, after which period a satisfactory activity of 482.9 DSU/cm³ (23.25 U/cm³) was achieved. This fermentation also consumed much less sucrose than usual, indicating once again that the cells were not growing as quickly as they usually do - a fact caused, most probably, by incorrect timing of the inoculation of the fermenter. This experiment shows the

Table 6.5.

Results obtained from Un-aerated Fed-Batch Fermentations with Varying Agitation Rates.

Run Number	Agitation Rate (rpm)	Maximum OD ₅₉₀ Value	Maximum Activity (DSU/cm ³)	Total Sucrose Consumed (g)	Duration of Fermentation (h)
R1/14.10.87	100	1.16	482.9	573	28
R10/27.11.87	100	0.9835	491.5	921	14
R29/23.3.88	100	1.044	511.4	753	16
R14/20.1.88	250	1.121	527	864	16.25
R15/27.1.88	350	1.145	456	792	15
R19/8.2.88	450	0.984	416.7	588	15

importance of transferring the culture from one stage to another at the correct time, i.e. during the exponential growth phase of the cell cycle. However, experiment R10/27.11.87 shows quite a different profile, with a duration of only 14 hours. This fermentation is much more ideal for commercial purposes, obviously, as the process time is much shorter, leading to a greater number of fermentations being carried out during a particular time period. Figures 6.14a and 6.14b give the OD₅₉₀ and enzyme activity profiles of the two fermentations, clearly showing the time differences between the two experiments.

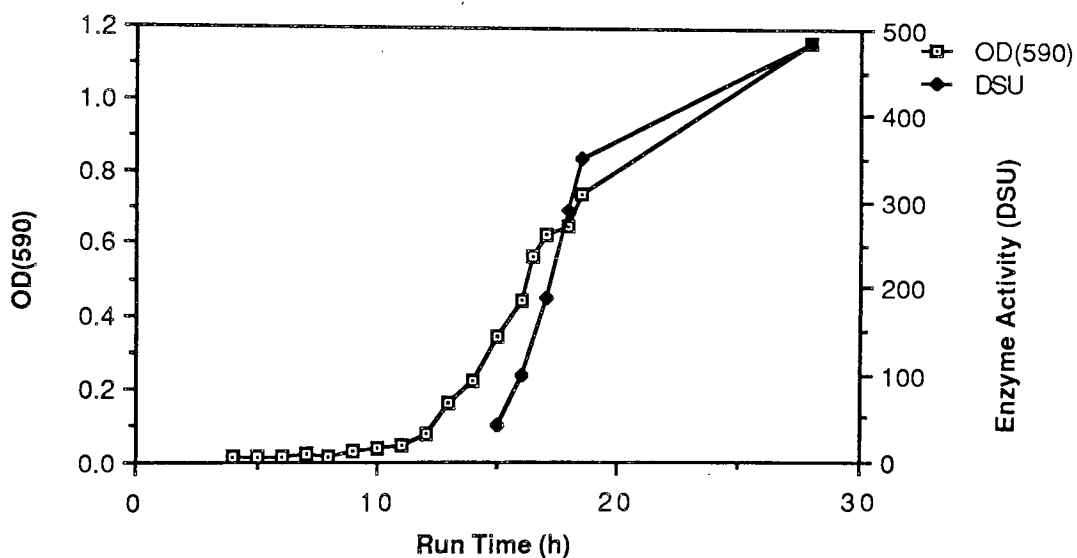


Figure 6.14a: OD(590) and Activity Profiles from R1

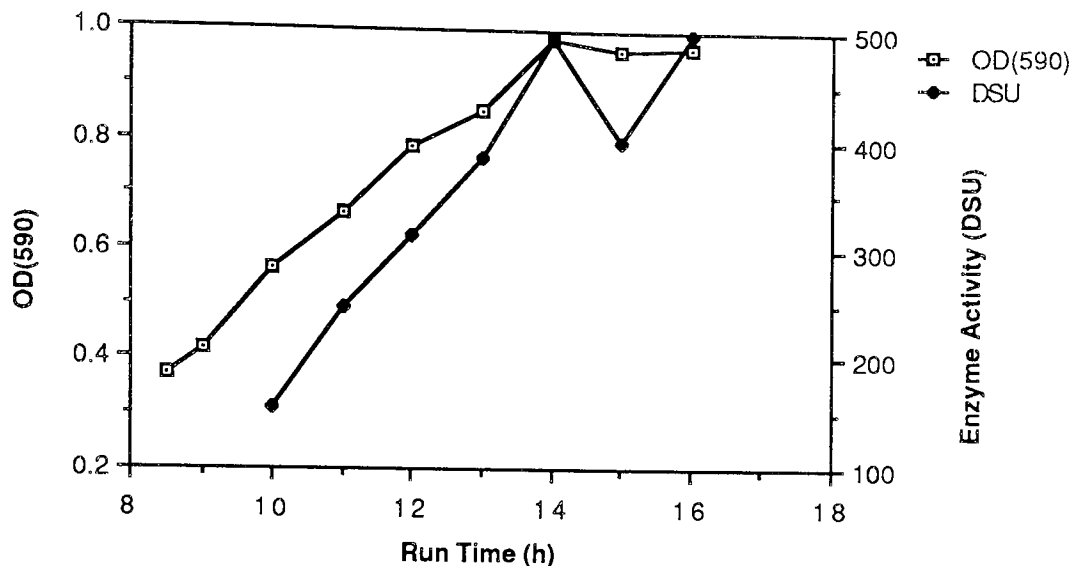


Figure 6.14b: OD(590) and Activity Profiles from R10

The other fermentations included in Table 6.5 show that even with agitation rates of 450 rpm, activities in excess of 400 DSU/cm³ (19.26 U/cm³) can be comfortably achieved, levels greater than those attained aerobically. It would appear though that enzyme yields reach peak levels at agitation rates up to 250 rpm, and above this level, the maximum enzyme activity drops slightly. It is, of course, preferable to have a lower agitation rate, as the power input into the fermenter and, hence, the operating cost of the fermentation is reduced under such conditions, provided there is adequate mixing within the vessel.

The importance of the effect of agitation rate on cell growth and enzyme production will occur again in the course of this project, especially during scale-up studies and continuous enzyme production runs.

6.2.2.2. Results Obtained With the Addition of Antifoam.

As a continuation of the studies mentioned in Section 6.2.1.2, whereby antifoam was added to the medium of aerated fermentations, a number of experiments were carried out with the addition of antifoam to unaerated fermentations which were operated at various degrees of agitation (see Section 6.2.2.1). It has already been noted that the addition of antifoam to a highly aerated fermentation reduces the rate of oxygen transfer, which in turn allows for greater enzyme production. This was compared to experiments carried out with no aeration nor addition of antifoam. Fermentations were carried out with agitation rates of between 100 and 600 rpm, with the results obtained being shown in Table 6.6. These values should be directly compared to those in Table 6.5. It was previously noted that an agitation of up to 450 rpm would still allow for an enzyme activity of greater than 400 DSU/cm³ (19.26 U/cm³) to be produced by the organism. The addition of antifoam to a low agitated (100 rpm) fermentation broth produces no appreciable decrease in enzyme activity and visual examination of the culture fluid through the window at the front of the fermenter can provide a reasonable explanation of this. The low agitation rate is not sufficient to allow for thorough mixing of the antifoam throughout the medium. The antifoam can, in fact, be clearly distinguished in the form of globules floating on the surface of the broth, with no mixing taking place. At a slightly higher agitation rate of 300 rpm, there appears to be a deviation in results obtained, with activities between 302.3 DSU/cm³ (14.55 U/cm³) and 417.6 DSU/cm³ (20.11 U/cm³) being recorded. This is due to the fact that different amounts of

Table 6.6.

Results obtained from Un-aerated Fed-Batch Fermentations with the Addition of an Antifoam Agent.

Run Number	Agitation Rate (rpm)	Maximum OD ₅₉₀ Value	Maximum Activity (DSU/cm ³)	Total Sucrose Consumed (g)	Duration of Fermentation (h)
R22/19.2.88	100	1.115	479.3	792	16.5
R4/4.11.87	300	1.229	340	909	19
R5/10.11.87	300	0.9385	202.3	849	20
R21/17.2.88	300	1.055	417.6	774	16
R20/10.2.88	450	1.074	285.9	633	16.5
R2/23.10.87	600	0.954	97.3	630	18
R3/28.10.87	600	0.914	133.4	690	20.75

antifoam were added to the medium of all the 300 rpm fermentations, resulting in different amounts of antifoam being mixed into the medium. An agitation rate of 300 rpm is not sufficient to disperse all of the antifoam throughout the culture fluid, but if a larger volume of antifoam is added, a correspondingly greater amount will be mixed into the medium, resulting in a lower final enzyme activity. Antifoaming agents, such as the silicone antifoam used during these experiments, are surface active agents and can affect fermentations in many ways. Viesturs *et al* (102) carried out research into the effect of antifoams on fermentation processes, and concluded that some of the ways in which antifoams can adversely affect a process are by somehow altering the metabolism of the cells, inhibiting the formation of a product, or preventing its secretion. The greater the degree of dispersion of the antifoam throughout the medium, the greater the influence the antifoam can exert on the process, and so, increasing the agitation rate should result in a greater decrease in enzyme activity. Increasing the agitation rate to 450 rpm results in a low maximum activity of 285.9 DSU/cm³ (13.77 U/cm³), and increasing it still further, to 600 rpm, causes an even greater drop in activity - to values between 97.3 (14.68) and 133.4 DSU/cm³ (6.24 U/cm³). These results all confirm the explanation given above into the action of antifoam on a highly agitated fermentation broth. Figures 6.15a and 6.15b compare the OD₅₉₀ and enzyme activity profiles for two fermentations carried out at 450 rpm agitation, with and without antifoam. The activity achieved is clearly lower from the fermentation where antifoam has been added, although cell growth appears comparatively unchanged.

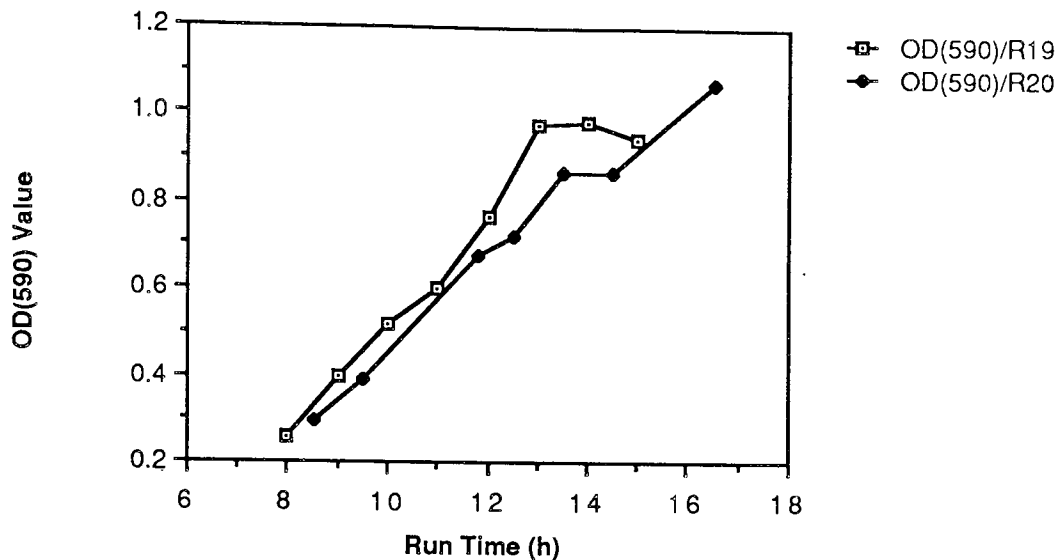


Figure 6.15a: OD(590) Profiles from R19 and R20

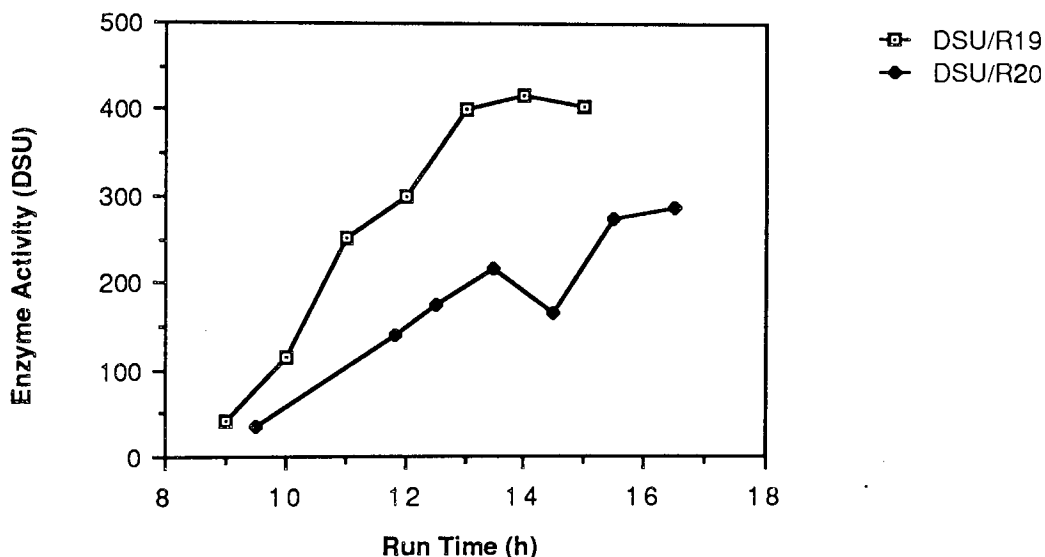


Figure 6.15b: Activity Profiles from R19 and R20

Figures 6.16a and 6.16b show the activity and cell growth profiles recorded for the two fermentations carried out at 600 rpm with the addition of antifoam. Again, the OD₅₉₀ value increases steadily, as would be expected, but the activity values peak early on in the process, and fluctuate quite rapidly without increasing to a value greater than approximately 120 DSU/cm³ (5.78 U/cm³). It appears, therefore, that the addition of a silicone antifoam agent to a *Leuconostoc mesenteroides* fermentation inhibits only dextransucrase production and not cell growth.

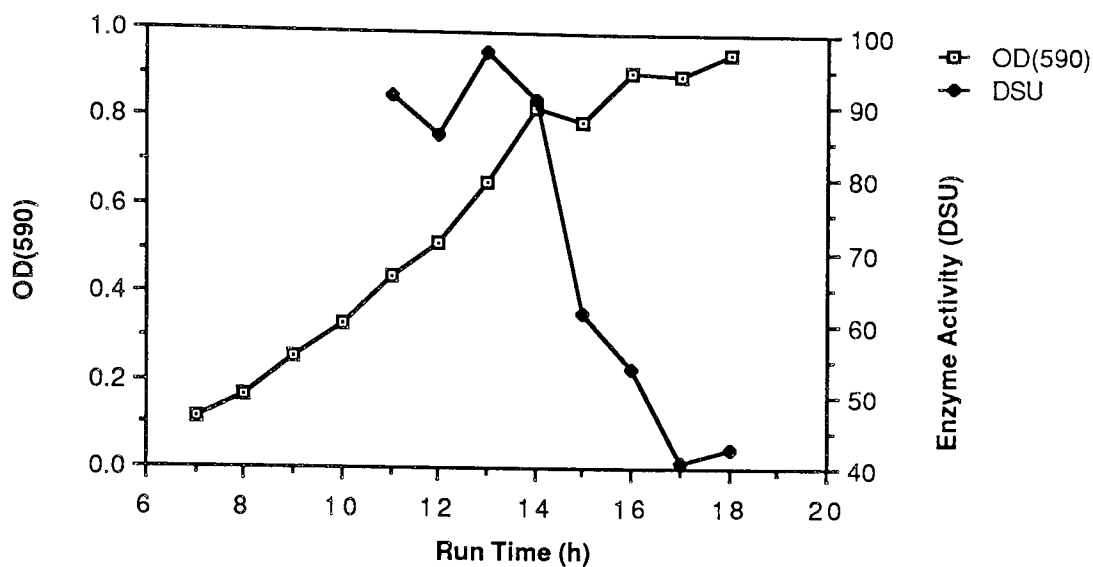


Figure 6.16a: OD(590) and Activity Profiles from R2

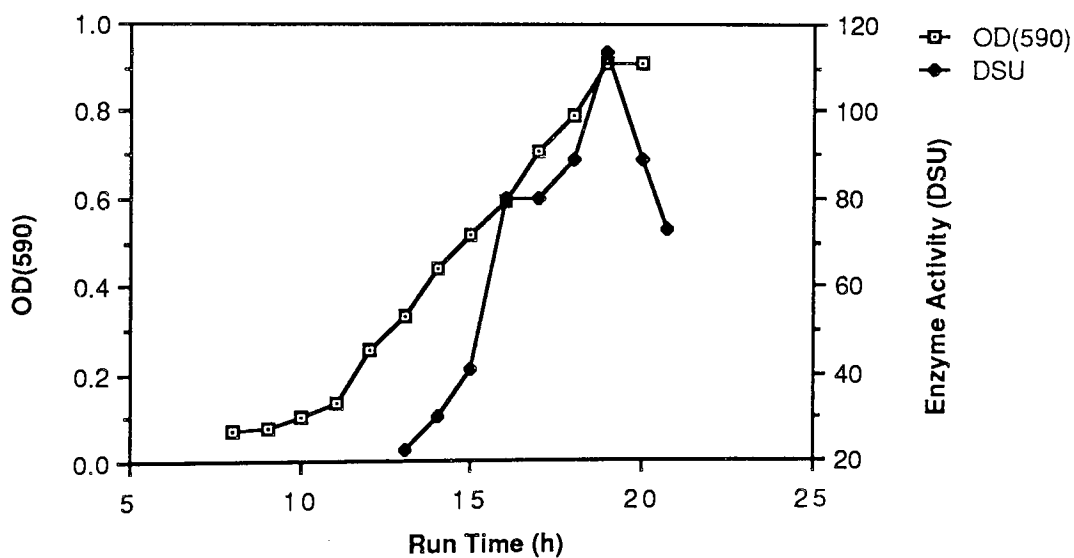


Figure 6.16b: OD(590) and Activity Profiles from R3

6.2.2.3. Results Obtained With Different Yeast Extracts.

Several fermentations were carried out with a number of different yeast extracts - either with different batches of the standard Gistex paste supplied by Gist-Brocades, or with different types of yeast extract such as Orhly, Distiller's Co. Ltd. (DCL) or Gistex powder. This series of experiments was carried out to determine whether another yeast extract would allow for high yields of dextransucrase, so that the producers of the enzyme would not have to be wholly dependent on one type of yeast extract and, hence, one yeast extract manufacturer for dextransucrase production. DCL yeast extract is used by Fisons plc during their whole cell production of dextran as the medium nitrogen source and it allows for high yields of dextran, and so it was necessary to determine its performance for dextransucrase production. It was also important to discover how consistent the different batches of Gistex were, with regard to enzyme production - whether different Gistex batches could be relied upon to allow for consistently high final enzyme activities. The results of these experiments are shown in Tables 6.7 and 6.8. Table 6.7 gives the results obtained with the use of various types of yeast extract, showing clearly that the best enzyme activity was achieved with Gistex Standard paste yeast extract. Gistex powder gave an activity of almost 400 DSU/cm³ (19.26 U/cm³) - a high value, but one that was approximately 100 DSU/cm³ (4.81 U/cm³) or 20% lower than that achieved with the paste. The DCL and Orhly yeast extracts both gave poor activity values, with the DCL producing approximately half the activity provided by the Gistex yeast extract. The Orhly brand not only gave lower activities, but also yielded lower cell growth as the maximum OD₅₉₀ value of 0.798 indicates. It would appear that Gistex paste is clearly the most suitable yeast extract to be used in the production of dextransucrase.

The results given in Table 6.8 show that the use of different batches of Gistex paste can result in different final results. Various batches of the yeast extract were supplied by Gist-Brocades in Holland, and corresponding unaerated fed-batch fermentations were carried out to determine and compare the maximum activities attained. It was possible to achieve activities of greater than 400 DSU/cm³ (19.26 U/cm³) on several occasions, with yeast extract batches CPN CGN 8-34, DGN4-04 and CPN CGN 8-13, although Gistex batch 7-25-16 gave only 360.9 DSU/cm³ (17.38 U/cm³) and batch 7-40-07 gave an even lower activity of 269.9 DSU/cm³ (12.99 U/cm³). There have also been results given in previous tables of experiments carried out with Gistex yeast extract, where the batch number was not recorded, which have

Table 6.7.

Results obtained from Un-aerated Fed-Batch Fermentations with Different Yeast Extract Types.

Run Number	Yeast Extract Type	Maximum OD ₅₉₀ Value	Maximum Activity (DSU/cm ³)	Total Sucrose Consumed (g)	Duration of Fermentation (h)
R7/19.11.87	DCL	1.098	225.6	933	16
R9/25.11.87	DCL	0.961	225.0	846	17
R8/24.11.87	Orhly	0.798	190	600	17
R10/27.11.87	Gistex Paste	0.9675	497.4	921	16
R102/5.3.90	Gistex Powder	0.97	392.4	744	17.25

Table 6.8.

Results obtained from Un-aerated Fed-Batch Fermentations with Different Batches of Gistex.

Run Number	Gistex Batch Number	Maximum OD ₅₉₀ Value	Maximum Activity (DSU/cm ³)	Total Sucrose Consumed (g)	Duration of Fermentation (h)
R35/26.5.88	7-40-07	1.093	269.9	567	16
R90/8.1.90	7-25-16	0.922	360.9	762	17
R95/29.1.90	CPN CGN 8-34	0.926	427.4	1212	19.5
R96/5.2.90	DGN 4-04	1.074	396.5	936	16.5
R99/13.2.90	DGN 4-04	1.146	412.3	876	18.25
R101/26.2.90	CPN CGN 8-13	1.17	432.4	936	15.25

given activities of approximately 500 DSU/cm³ (24.07 U/cm³), such as experiment R10/27-11-87 (Table 6.7). It is clear then, that whilst Gistex paste appears to be the only yeast extract of the ones studied that is capable of giving enzyme yields of 450 - 500 DSU/cm³ (21.67 - 24.07 U/cm³) it can also be widely inconsistent in the activities it produces with different batches. It would seem at this point that some detailed analysis of the composition of the Gistex yeast extract, particularly with regard to carbohydrate, amino acid, vitamin and mineral content, should be carried out. This was done, and the findings will be reported in Sections 7.3.4 and 7.3.5 of this thesis. From Table 6.8 it can be seen that, although Gistex batch composition affects enzyme activity, it does not appear to affect cell growth in the same way. It can be concluded then that composition of the yeast extract is crucial to the enzyme production, but not the cell growth.

6.2.2.4. Results Obtained With the Addition of Folic Acid.

As mentioned previously in Section 2.2.1, Brown and McAvoy ⁽¹⁴⁾ claimed that the addition of 0.0206 g/l of folic acid to the fermentation medium prevented the severe limitation of cell growth and enzyme production that they observed to be occurring towards the end of a fermentation. They identified folic acid as the limiting component of the medium, by a process of elimination, and claimed that its addition would restore the metabolic rate of the culture and enhance enzyme production. A series of experiments was therefore carried out to test this claim. Two different batches of Gistex yeast extract were used - one that had previously given an activity of approximately 500 DSU/cm³ (24.07 U/cm³), as in R10/27-11-87, and batch number 7-40-07 as used in R35/26:5:88, that had produced only 269.9 DSU/cm³ (12.99 U/cm³) of dextransucrase. Folic acid was added to the medium in two separate concentrations of 0.003 g/l and 0.0206 g/l as specified by Brown and McAvoy ⁽¹⁴⁾, to determine the effect the addition of different concentrations of folic acid would have on enzyme production. The results from these experiments are shown in Table 6.9. It can be seen that the addition of folic acid to a medium that was already producing enzyme yields well in excess of 450 DSU/cm³ (21.67 U/cm³), served only to cause a reduction in activity to occur. In these cases, it would appear that the excess folic acid could even act as an inhibitor to dextransucrase production - contradicting the results of Brown and McAvoy. The difference in maximum activity between R39/15.6.88 and R42/30.6.88 is puzzling, and can be explained by the fact that on occasions, fermentations do not proceed in the accepted way, a situation that

Table 6.9.
Results obtained from Un-aerated Fed-Batch Fermentations with the Addition of Folic Acid.

Run Number	Folic Acid Added (g/l)	Maximum OD ₅₉₀ Value	Maximum Activity (DSU/cm ³)	Total Sucrose Consumed (g)	Duration of Fermentation (h)
R39/15.6.88 (Good /Gistex)	0.003	1.227	392.3	900	16
R40/22.6.88 (Gistex 7-40-07)	0.003	1.069	254.2	681	16
R41/28.6.88 (Gistex 7-40-07)	0.003	1.015	262.0	747	15
R42/30.6.88 (Good Gistex)	0.003	1.01	267.3	576	15.2
R43/8.7.88 (Good Gistex)	0.0206	1.045	411.6	795	16
R44/13.7.88 (Gistex 7-40-07)	0.0206	1.085	297.6	774	16
R45/15.9.88 * (Good Gistex)	0.0206	-	301.9	795	17

* Antifoam Added to Medium

can be attributed to a poor culture, or simply to human error in preparing the medium or analytical error. However, there is good cell growth from R42/30.6.88, but poor activity and sucrose demand. It is known that Gistex paste can be of inconsistent quality even from the same container, and an unfortunate dosage of Gistex could be responsible for the abnormal result. With regard to Gistex batch 7-40-07, the addition of folic acid seems to have little effect on enzyme production, although the inclusion of 0.0206 g/l of the acid to the medium appears to slightly enhance enzyme activity. This is in agreement with the findings of Brown and McAvoy. It could be possible that folic acid was actually limiting in Gistex batch 7-40-07, such that its addition did serve to boost enzyme production, although not to any great extent. On the whole, however, there has been no real agreement with the results of Brown and McAvoy, except that to state that in the case where the Gistex yeast extract was not allowing for high enzyme yields, there is some possibility that the addition of 0.0206 g/l of folic acid to the medium could enhance enzyme activity. In all the folic acid fermentations, a good deal of foam formation was observed and so in experiment R45/15.7.88, a small amount of antifoam was added to the medium. This served to successfully disperse the foam, but resulted in a dramatic decrease in activity of over 100 DSU/cm³ (4.81 U/cm³), indicating that severe limitation of some kind was taking place.

6.3. Fed-Batch Fermentations in MBR Vessel.

Following the purchase of the MiniBioreactor from Sulzer (U.K.) Ltd. in 1988 for the purpose of carrying out continuous fermentations of dextransucrase, a number of standard fed-batch fermentations were carried out in the vessel to determine whether it was possible to achieve greater than 400 DSU/cm³ (19.26 U/cm³) of activity from such a fermentation. These experiments were carried out using the standard medium, with the only differences being with the batch of Gistex yeast extract used. These fermentations were carried out at two distinct agitation rates of 450-490 rpm and 700 rpm, although the relevance of this and its effect on the continuous fermentations will be discussed in detail in Section 9.1.4.

6.3.1. Results Obtained with Different Batches of Gistex Yeast Extract.

The results obtained from this section of the project are shown in Tables 6.10 and 6.11, where it can clearly be seen that there is no incidence of a high activity (in excess of 450 DSU/cm³ - 21.67 U/cm³) being achieved - indeed,

the maximum enzyme yield obtained with the MBR fermenter was an activity of only 329 DSU/cm³ (15.84 U/cm³) from experiment R55/21.2.88. Experiments R66/20.3.89 and R65/8.3.89 were carried out to compare the maximum activity achieved from both MBR and New Brunswick fermenters under identical experimental conditions. These experiments were slightly different from the usual standard fed-batch fermentations that would usually give 450 DSU/cm³ (21.67 U/cm³) of dextransucrase in that the lower pH dihydrogen phosphate (KH₂PO₄) was used in the medium in place of the high pH dipotassium phosphate (K₂HPO₄). Fermentation R65/8.3.90, which was carried out in the New Brunswick vessel, would normally be expected to give an activity of at least 450 DSU/cm³ (21.67 U/cm³), but with the use of KH₂PO₄, the maximum activity achieved was only 343.4 DSU/cm³ (16.53 U/cm³). This can be compared to the corresponding activity of 196.3 DSU/cm³ (9.54 U/cm³) which was attained in the MBR vessel. It would appear that, in general, results obtained from the MBR fermenter are approximately 33% lower than those from the New Brunswick vessel. This is probably due to scale-up/scale-down procedures, and will be discussed more fully in Section 7.3.6. It is also interesting to note experiment R53/9.12.88, which was carried out with Bacteriological Peptone (see Section 6.1.2) as the nitrogen source. The peptone supported poor cell growth, as the maximum OD₅₉₀ value of 0.437 indicates, along with a very low activity of only 18-32 DSU/cm³ (0.87 - 1.54 U/cm³). It would appear from this fermentation that it would not be possible to substitute Gistex yeast extract for Bacteriological Peptone, and still carry out successful dextransucrase fermentations. Experiment R52/30.11.88 also gave a very poor final enzyme activity of only 50.7 DSU/cm³ (2.44 U/cm³), despite the culture being well grown - with an OD₅₉₀ value of 0.958. Although the Gistex yeast extract batch used in this fermentation had previously given an activity in excess of 450 DSU/cm³ (21.67 U/cm³), the reason for the failure of this experiment appears to lie with the yeast extract, as it permitted growth to take place, but with very little enzyme production. Alternatively, this fermentation could be one of those mysterious fermentations, the failure of which cannot be satisfactorily explained. The increase of the agitation rate in the MBR vessel appears to result in a slight increase in cell growth as indicated by the OD₅₉₀ values, although it seems to have little success in boosting enzyme activity. Figures 6.17a and 6.17b compare the OD₅₉₀ and activity profiles recorded from experiments R65/8/3.89 and R66/20.3.89, which were discussed earlier, and

Table 6.10.

Results obtained from Un-aerated Fed-Batch Fermentations Carried out in MBR Vessel.

Run Number	Gistex Batch Number	Agitation Rate (rpm)	Maximum OD ₅₉₀ Value	Maximum Activity (DSU/cm ³)	Total Sucrose Consumed (g)	Duration of Fermentation (h)
R52/30.11.88	Good Batch	490	0.958	50.7	158.4	17
R53/9.12.88	Bact- Peptone	480	0.437	18.32	12	16.25
R55/21/12/88	New Batch	480	0.890	329	120	19
R66/20.3.89 *	New Batch	450	-	196.3	145.8	19.75
R65/8.3.89 *	New Batch	100 (N.Brunswick Vessel)	1.032	343.4	762	16.25

* With KH₂PO₄ in the medium instead of K₂HPO₄.

Table 6.11.

Results obtained from Un-aerated Fed-Batch Fermentations Carried out in MBR Vessel.

Run Number	Gistex Batch Number	Agitation Rate (rpm)	Maximum OD ₅₉₀ Value	Maximum Activity (DSU/cm ³)	Total Sucrose Consumed (g)	Duration of Fermentation (h)
R67/4.4.89 *	New Batch	450	0.805	190.4	97.2	21.75
R77/24.7.889	New Batch	450	0.881	266.3	136.2	17
R78/31.7.89	8.16.25	450	0.688	276.5	73.2	17.5
R87/27.11.89	2nd New Batch	700	1.112	277	105	18
R93/22.1.90	CPN CGN 8-13	700	1.026	313.5	143.4	17

* With KH₂PO₄ in the medium instead of K₂HPO₄.

clearly show the lower rate of enzyme elaboration that occurred in the MBR vessel.

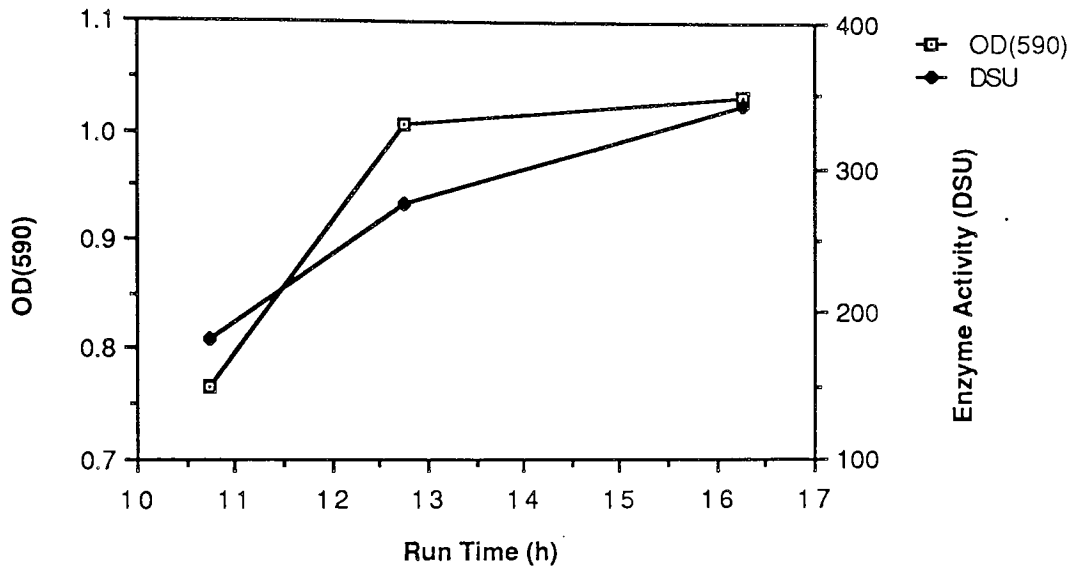


Figure 6.17a: OD(590) and Activity Profiles from R65

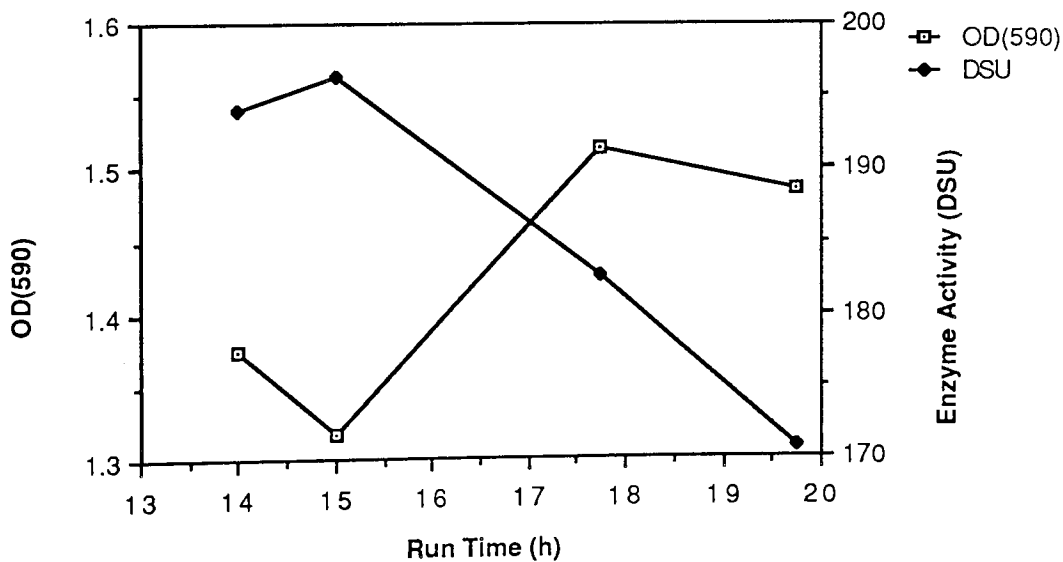


Figure 6.17b: OD(590) and Activity Profiles from R66

6.4. Large-Scale Industrial Fermentations.

It was very fortunate that during the course of this project, opportunities arose in which the unaerated fed-batch fermentation was carried out on the

Industrial Scale. The two fermenters used for these studies were the 1,000 litre seed vessel located on the Fisons Pharmaceuticals site at Holmes Chapel and the other, a newly commissioned 800 litre BioLafitte vessel located at the Centre for Biochemical Engineering at the University of Birmingham. These large scale fermenters are both described in detail in Section 5.2.

6.4.1. Results Obtained With the Fisons 1,000 Litre Vessel at Holmes Chapel.

The scale-up studies on the 1,000 litre seed fermenter at Holmes Chapel were carried out at two different times, with two fermentations being carried out in September 1987, and four further experiments taking place during April and May 1988. The first two fermentations were carried out to determine if it were possible to produce dextransucrase at a level of greater than 250 DSU/cm³ in the 1,000 litre seed fermenter, and then to immediately react the enzyme with a non-sterile sucrose solution in the main 50,000 litre fermenter to synthesize dextran. It was then desirable to compare this type of process with the current routine dextran process practised at Fisons, based on the whole cell fermentation technique. It was also necessary to analyze the final dextran to see if it had the same structure and quality as the dextran produced by the normal whole cell fermentation method. During the normal production of dextran by Fisons plc, 8.7 tons of sucrose are converted to dextran in the 50,000 litre fermenter in 30-40 hours, and it was found that an enzyme level of 4-6 DSU/cm³ (0.19 - 0.29 U/cm³) is present in the main fermenter to facilitate this synthesis. This means that an enzyme activity of 250 DSU/cm³ (12.04 U/cm³) is required to be produced in the seed vessel in order to provide 5 DSU/cm³ (0.24 U/cm³) in the main fermenter and, hence, produce the required amount of dextran. The inocula for all the plant trials carried out at Holmes Chapel were prepared as described in Section 5.2.2.1, with the fermentation media having been prepared and sterilized as detailed in Sections 5.2.3.1 and 5.2.4.1. The main results from these fermentations are shown in Table 6.12.

Table 6.12.
Results Obtained from the Dextransucrase Production Trials

Parameter	HZ38T	HZ39T
Maximum OD ₅₉₀ Value	0.67	0.86
Maximum Activity (DSU/cm ³)	262	442
Duration of Fermentation (h)	27	25.5
Temperature (°C)		
- Observed	23 ± 1	23 ± 1 (20.5 hrs) then 29 ± 1
- Actual	17 ± 1	17 ± 1 then 23 ± 1
Lag phase (h)	6	6
pH	6.6 - 6.8	6.6 - 6.8

These results show that an activity of 442 DSU/cm³ (21.28 U/cm³) was achieved from fermentation HZ39T, although HZ38T was not as good. The reason for this is that during HZ38T and for 20.5 hours of HZ39T the temperature of the fermentation was being monitored at 24°C, which was the required temperature. However, after 20.5 hours of HZ39T had progressed, the temperature was checked manually and found to be only 17°C - too low for maximum enzyme production. The temperature was therefore increased to a value of 23°C, giving a reading of 29°C on the fermenter instruments. This temperature increase served to boost the enzyme activity, which had begun to drop away as can be seen in Figures 6.18a and 6.18b, which show the OD₅₉₀ and activity profiles of the two fermentations. The 442 DSU/cm³ (21.28 U/cm³) enzyme was then transferred to the main 50,000 litre fermenter for the synthesis of dextran from sucrose. It was found that, without the need for any enzyme purification, the necessary yield of dextran was produced from a reduced sucrose input (6.25 tons compared to 8 tons) with the final product being of the quality that allowed it to become clinical dextran. The staff of the Natural Products Development Department (NPDD) at Holmes Chapel

estimated quite significant cost savings from the enzyme process of approximately £32,000 p.a. (50 batches of clinical dextran) with the saving mainly being due to the use of less sucrose in the main fermenter. Other cost savings were thought possible due to the improved downstream processing that the enzymatic route allowed, but there was insufficient data to estimate these savings. The main conclusion drawn from these two trials was that, although it was possible to produce high yields of dextransucrase on the Industrial Scale, further trials would have to be carried out to prove that this could be done consistently.

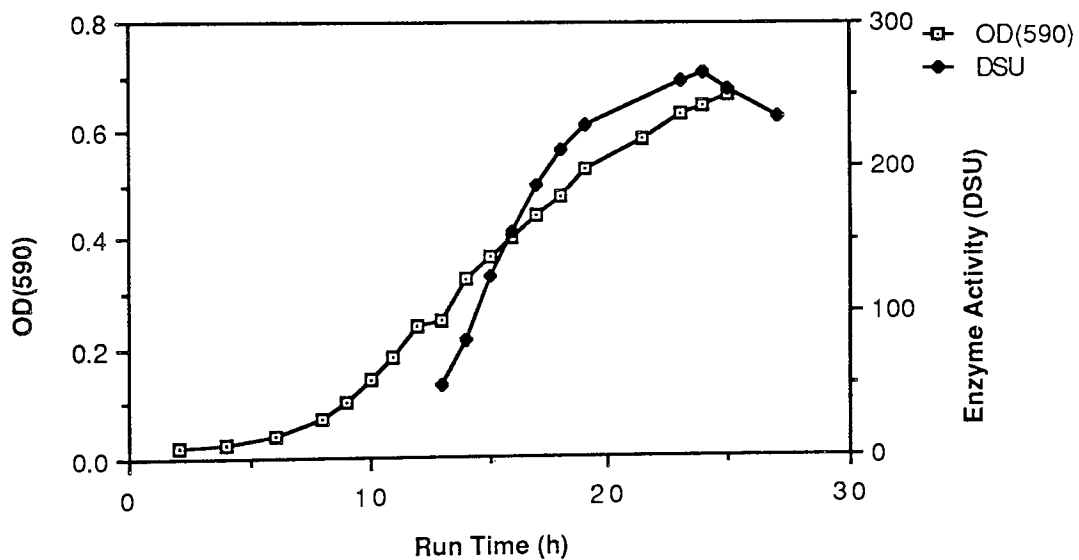


Figure 6.18a: OD(590) and Activity Profiles from HZ38T

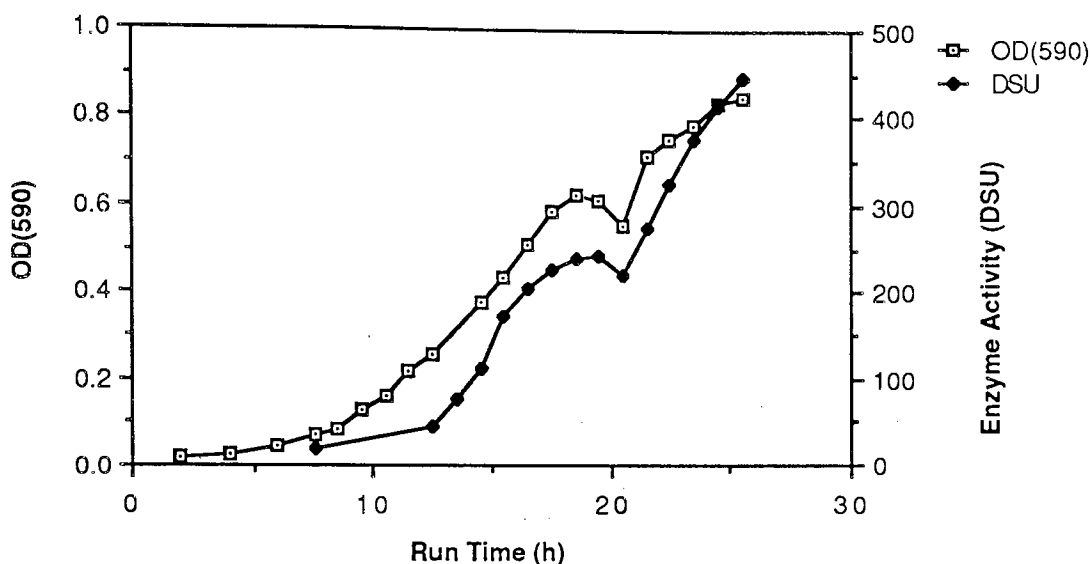


Figure 6.18b: OD(590) and Activity Profiles from HZ39T

To this end, a second set of four large-scale fermentations were carried out during April and May 1988. The enzyme from these experiments was due to be transported down to Aston University for purification and use in the Semi-Continuous Chromatographic Reactor-Separator studies at Aston. The aim of these fermentations was to reproduce the results obtained from experiment HZ39T, and to provide the Plant Operators at Holmes Chapel with further experience in operating the seed vessel under controlled conditions. The temperature probe that had caused problems during the previous experiments had been recalibrated and was monitoring the temperature correctly. The results from these fermentations are given in Table 6.13 and they show that the experiments were not as successful as the previous set of plant trials. Experiment 313/26 failed due, in the main, to poor pH and temperature control, with the major problem being the pH increase to 7.01 on the addition of the sucrose/NaOH solution. A medium pH of greater than 7.0 causes a pH gradient inversion to occur, with the cell interior becoming acidic with respect to the exterior, greatly inhibiting dextransucrase formation (see Section 2.3.2), although in this case the cells grew well, albeit slowly. Figure 6.19 shows the OD₅₉₀ and activity profiles recorded from this fermentation. The second fermentation, 319/29, proved to be much more successful than the first, with good cell growth being achieved, along with a maximum activity of 296 DSU/cm³ (14.25 U/cm³). However, whilst this was an improvement on 313/26, it was disappointing when compared to HZ39T which yielded 442 DSU/cm³ (21.28 U/cm³) of enzyme. The pH was better controlled during the fermentation, although the temperature fluctuated between 21-27°C. This

Table 6.13.

Results obtained from the Second Set of Dextranucrase Production Trials at Holmes Chapel.

Run Number	Maximum OD ₅₉₀ Value	Maximum Activity (DSU/cm ³)	Temperature (°C)	pH Value	Duration of Fermentation (h)
R30/313/26	0.750	33.2	22 - 25	6.62 - 7.01	33.3
R31/313/29	0.942	296.0	21 - 27	6.59 - 6.73	21.5
R32/313/32	0.31	42.4	23 - 24.5	6.59 - 6.73	35
R33/313/35	0.960	179	23 - 24.5	6.59 - 6.72	21.4

lack of good temperature control should not, however, have caused such a large activity drop, although 27°C is the temperature at which dextransucrase is degraded. It seems unlikely, though, that the length of time that the fermentation was operating at 27°C was the reason for such a loss of activity, although it could possibly have been the case. The OD₅₉₀ and activity profiles from this fermentation are shown in Figure 6.20. The results from Experiment 313/32 are extremely surprising considering that the pH and temperature were both controlled quite adequately. Figure 6.21 gives the OD₅₉₀ and activity profiles from this fermentation, and it can be seen that after approximately 16 hours of the fermentation there was so little growth (OD₅₉₀ value of 0.108) and the activity was so low (8.8 DSU/cm³ - 0.42 U/cm³) that it was thought that the R* salts had been omitted from the medium, and so 1 litre of triple concentrated R* salts was autoclaved and added to the fermenter through the septum using sterile 50cm³ syringes. By the following morning the OD₅₉₀ value had increased to 0.261. Although Figure 6.21 indicates that it had done so in a linear as opposed to an exponential manner, it would appear that the addition of the R* salts had little effect on the progress of the fermentation. The enzyme activity peaked at a value of 42.4 DSU/cm³ (2.04 U/cm³), considerably lower than expected. The final fermentation in this set, 313/35, gave a good cell growth profile (see Figure 6.22) but a disappointingly low final activity of only 179 DSU/cm³ (8.62 U/cm³). This was most unexpected as the fermentation had progressed well, with good inoculum development (as with all four experiments) and adequate temperature and pH control. It was then suspected that one of the medium components was at fault. It could not have been the sucrose, as it had been used successfully for dextran production, or the K₂HPO₄, as it was manufactured to standard specifications. It was therefore thought that the batch of Gistex yeast extract could have degraded in some way. It was found that the Gistex had been stored incorrectly, and that instead of holding it in a cool environment such as a fridge or cold room, it had been kept near the 50,000 litre fermenters which were operated at a temperature of 25°C and reached sterilization temperatures of 121°C. A sample of this Gistex (batch number 7-40-07) was returned to Aston University, where a standard unaerated fed-batch fermentation was carried out using it (experiment R35/26.5.88). A maximum activity of only 269.9 DSU/cm³ (13.00 U/cm³) was achieved with this Gistex, although there was good cell growth (see Table 6.8). It was concluded that the use of degraded Gistex yeast extract

was responsible for the poor results from the second set of Plant Trials, and that in future, all the medium components would be checked using the 16 litre New Brunswick Fermenter, which was capable of producing 450 DSU/cm³ (21.67 U/cm³) of enzyme, before any further large-scale fermentations were to take place.

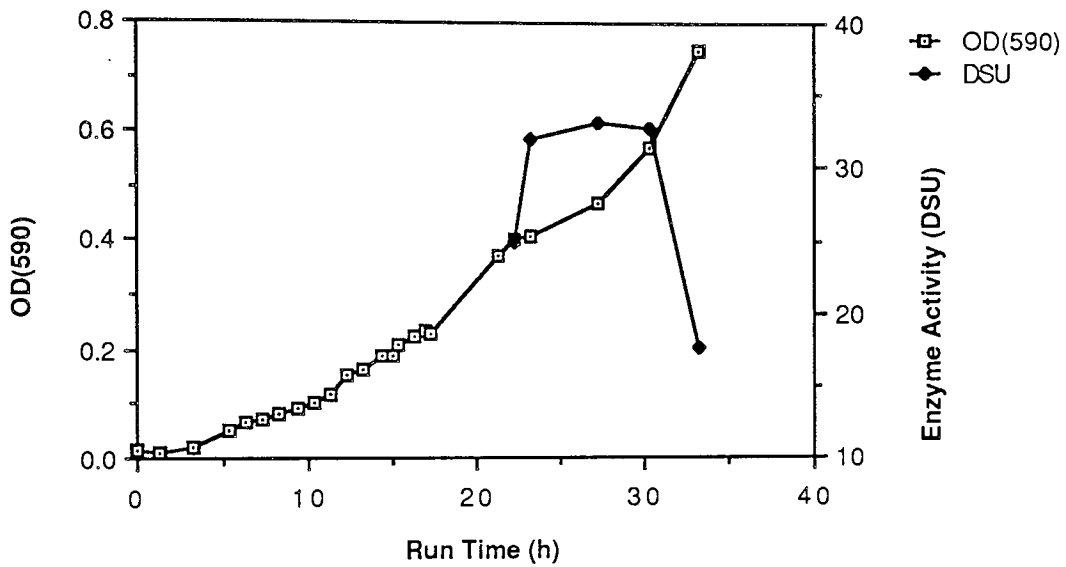


Figure 6.19: OD(590) and Activity Profiles from R30

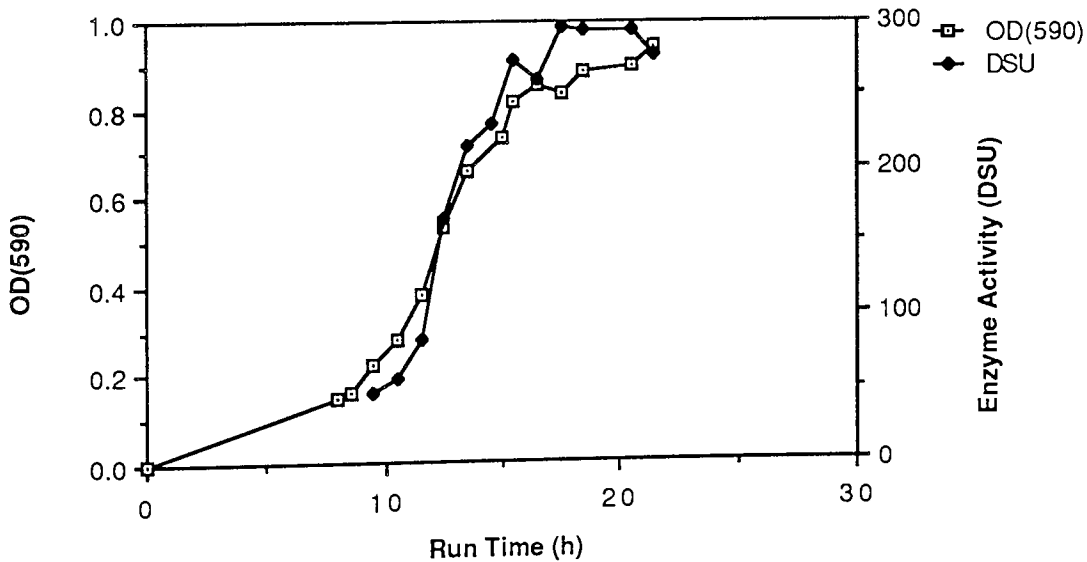


Figure 6.20: OD(590) and Activity Profiles from R31

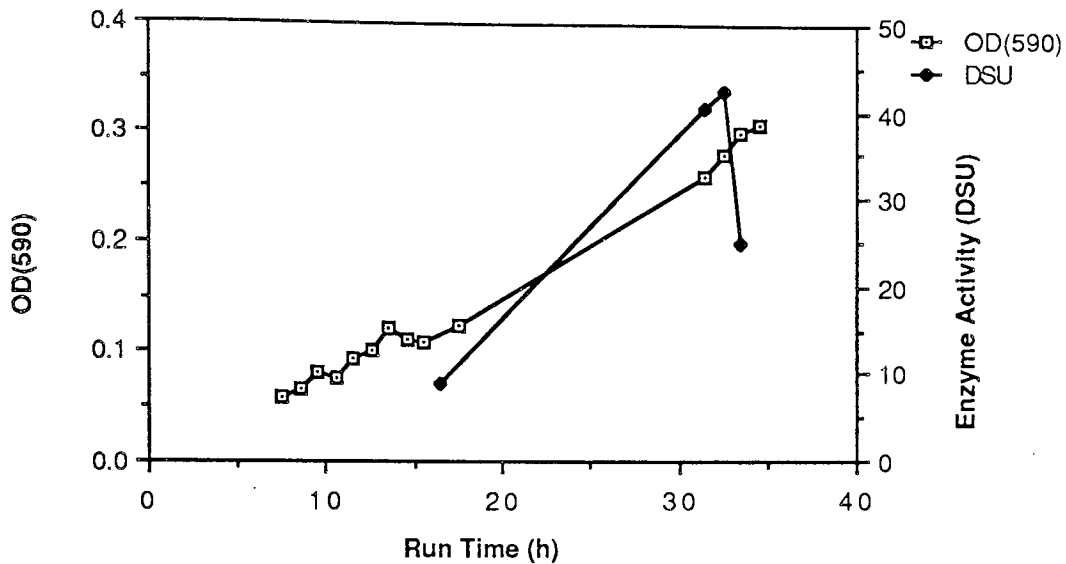


Figure 6.21: OD(590) and Activity Profiles from R32

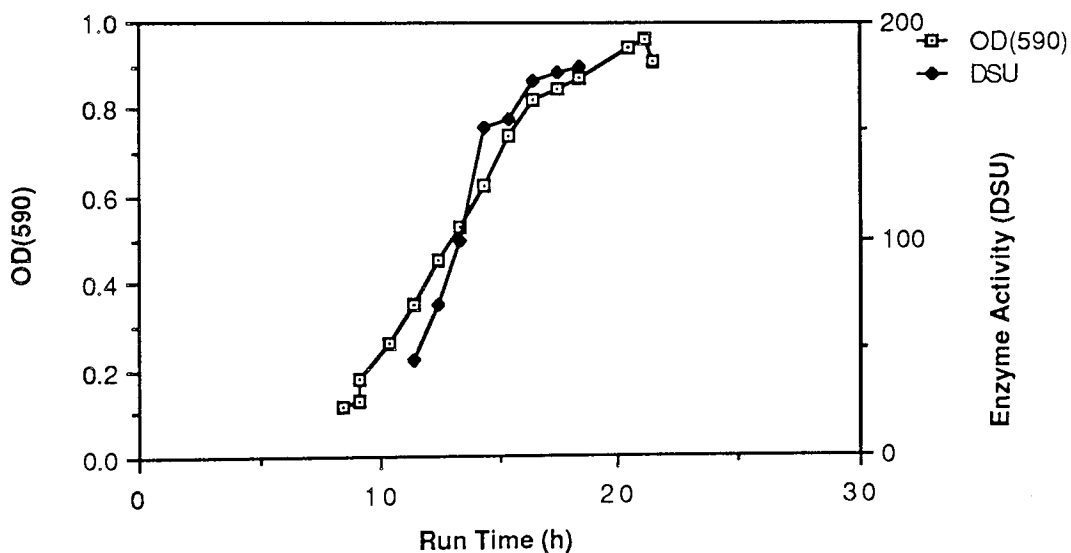


Figure 6.22: OD(590) and Activity Profiles from R33

6.4.2. Results Obtained with the 800 Litre Vessel at Birmingham University.

One large scale fermentation was carried out using the 800 litre BioLafitte vessel at the Centre for Biochemical Engineering, University of Birmingham, during July 1990. This was a very accurately controlled fermenter which was capable of controlling the operating temperature exactly at 23.5°C throughout the entire experiment, although the pH was only controlled between the limits of 6.63 and 6.69 throughout most of the fermentation. The fermenter was linked to a computer which recorded various parameters (data logging) throughout the whole experiment, and a number of outputs from this computer

are shown in Appendix A, where the profiles of various co-ordinates such as Dissolved Oxygen Tension, pH, Vessel Temperature, Sterilization Temperature and Set Point, Vessel Pressure and pH Control Signal Outputs are recorded. The exit gas from the fermenter was continuously analyzed with the use of a mass spectrometer, and Appendix A also contains a number of exit gas profiles from the experiment. The data shown in Appendix A is given in three distinct groups, with Figure A.1 showing an 18 hour profile, including the inoculation of the fermenter, and Figure A.2 showing a second 18 hour profile, to the termination of the fermentation. Figure A.3 gives a 4 hour profile of the sterilization of the vessel, and Figure A.4 shows a 20 hour profile of the vessel as it cools down from the sterilization procedure, and stabilizes itself prior to inoculation. Figures A.5 - A.9 each show 6 hours of the fermentation and together provide a complete profile of the whole process, being a more extended version of Figures A.1 and A.2. Figures A.10 - A.13 show the exit gas profiles determined during the fermentation in terms of CO₂, N₂, O₂ and Ar. This fermentation has been the most accurately controlled and monitored of all of the fermentations carried out during the entire project, and provides some very interesting on-line data which, together with the off-line data recorded, gives a very complete picture of an unaerated fed-batch fermentation. Unfortunately, the actual fermentation was not as successful as it was hoped it would be. The inoculum was prepared as described in Section 5.2.2.2 and was well grown on transfer to the main fermenter, with the OD₅₉₀ values of the three final inoculum bottles being 0.137, 0.126 and 0.127. The culture was gravity fed into the fermenter, with this process taking approximately 1.5 hours to complete. The culture remained in the lag phase for 9 hours until it began to grow slowly, although it grew in a linear rather than an exponential manner. Figure 6.23 shows the OD₅₉₀ and dextransucrase activity profiles recorded from the fermentation, showing a maximum OD₅₉₀ value of 0.722, and activity of 133.2 DSU/cm³ (6.41 U/cm³). This was a very disappointing final result, the reasons for which cannot be fully explained. It was noticed, however, that after 17 hours of the fermentation a great deal of foam began to be formed and soon filled up the whole of the head space of the vessel. Up to this point the fermenter had been agitated at a rate of only 100 rpm, and the pH was being controlled between values of 6.3 and 6.9. It was thought, therefore, that the low agitation rate was providing inadequate mixing so that the sucrose, that was being added on demand with the alkali, was not being dispersed throughout the medium at a sufficiently high rate and hence not being passed to the cells,

and as a consequence, the cells were being starved of sucrose and had ceased to grow. The appearance of foam has traditionally signalled the end of a fermentation, when the cells reach the end of their growth cycle, which can occur prematurely if the cells are starved of sucrose. In this case, foam formation at an OD₅₉₀ value of 0.385, and an activity of 61.6 DSU/cm³ (2.97 U/cm³) seemed to point to cell starvation, and so after 19 hours of the fermentation, the agitation rate was increased to 250 rpm to provide better mixing and to attempt to boost cell growth and activity. Almost immediately, the pH began to be controlled much more accurately, between the values of 6.64 and 6.66, and CO₂ production increased (see A.10 - A.13) indicating increased cell growth. The foam was quickly dispersed and the cells began to grow at a quicker rate, secreting more enzyme. However, as Alsop stated, once cell starvation has occurred, it cannot be reversed and in this case, although there were probably a number of viable cells left in the fermenter, it was not possible to successfully continue the experiment. The final OD₅₉₀ value of 0.722 and activity of 133.2 DSU/cm³ (6.41 U/cm³) were extremely disappointing, but now that some experience has been gained with this fermenter, further runs would be desirable. This experiment, along with the other scale-up studies, will be discussed in greater detail in Section 7.3.7.

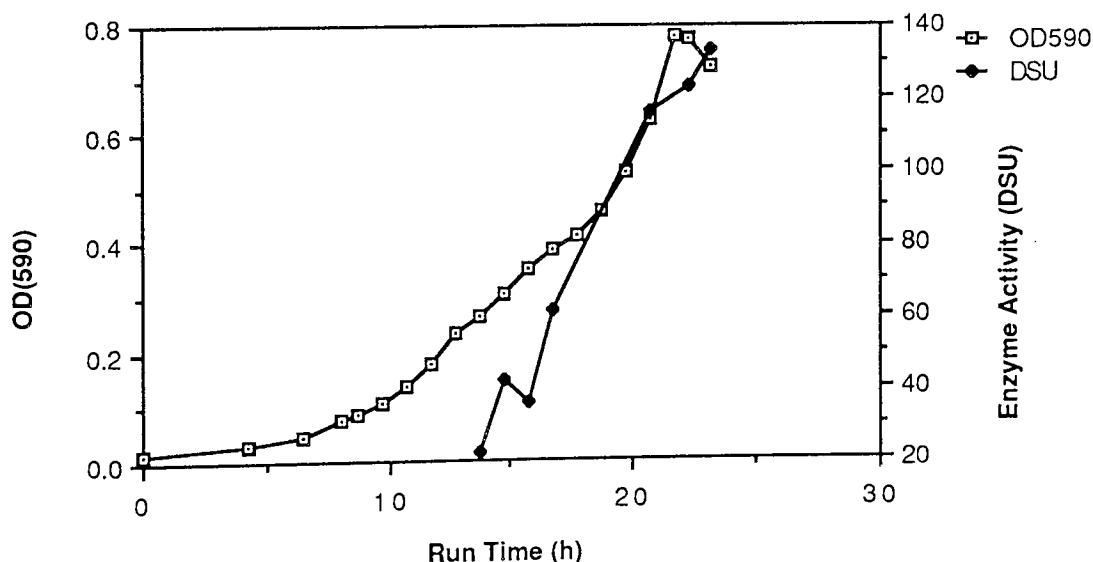


Figure 6.23: Results obtained from 800 litre Vessel

7. DISCUSSION OF BATCH AND FED-BATCH FERMENTATION RESULTS.

The experiments carried out during the course of this project were split into two distinct groups; fed-batch and continuous fermentations. The results obtained from the two sections of work and, hence, the discussions of those results will be in separate chapters with the current chapter concerning batch and fed-batch fermentations. This work was carried out in order to produce high yields of dextransucrase under both fed-batch and continuous fermentation conditions - to improve upon the results achieved by previous researchers using this technique. This chapter also contains a considerable amount of studies into the scale-up of the fed-batch production of dextransucrase to an 800 litre and a 1,000 litre vessel.

7.1. Fed-Batch Fermentations.

The results obtained from these experiments are recorded in detail in Sections 6.2, 6.3 and 6.4, dealing with the fermentations carried out in the New Brunswick vessel, the MBR vessel and the Industrial Scale vessels respectively. Various parameters were investigated during this section of the project, including a comparison between aerated and unaerated fermentations, an investigation into the effects of antifoam and folic acid on product formation, alteration of the agitation rate of the process and variations in the medium composition with regard to the nitrogen source.

7.2. Aerobic Fermentations.

To date, the majority of researchers in the field of dextransucrase have produced the enzyme under conditions of high aeration, the most notable being Schneider *et al* ⁽¹⁰¹⁾ who, in their 1984 Patent maintained a dissolved oxygen level of 40-70% of the initial saturation value in order to obtain optimum enzyme yields. A number of aerobic fermentations were therefore carried out during this work, either to reproduce the conditions specified by Schneider, or to pass known constant volumes of sterile air into the fermenter. A group of experiments that were carried out with the addition of an antifoaming agent to the medium will also be discussed here.

7.2.1. Effect of Aeration Rate on Product Formation.

An experiment was carried out to confirm the findings of Schneider *et al* ⁽¹⁰¹⁾ in his work for Fisons plc. The results from this fermentation can be seen in Figure 6.4, and show that the activity value reaches a peak well before the

OD₅₉₀. This fermentation yielded an activity of 244 DSU/cm³ (11.75 U/cm³), along with a maximum OD₅₉₀ value of 1.071 - although this did not coincide with the maximum activity. There are a number of reasons for the poor results achieved from this experiment, for example, the operating parameters of the fermentation could have led to considerable denaturation of the protein, according to Thomas (82). He claimed that shear damage to proteins, including enzymes, was less severe than had at first been thought. It would appear that, to subject proteins to high levels of shear alone does not lead to a great amount of denaturation, compared with high shear levels combined with air-liquid interfaces in the medium. In other words, for shear damage to proteins to occur to any great extent, there must be high levels of both agitation and aeration. Thomas claimed that it was possible to eliminate shear damage in an unaerated stirred tank, and this can be seen from the results obtained from experiment R11/3.12.87, given in Figures 6.4 and 6.5. Figure 6.4 shows the activity and cell growth profiles recorded from R11, and clearly shows that the enzyme activity reaches a peak and begins to decrease well before the cell growth. This confirms the claims made by Thomas concerning enzyme denaturation as this fermentation was operated at a high aeration rate of 24 litres/minute, with the agitation rate being controlled between 300 and 600 rpm in order to maintain a dissolved oxygen concentration within the vessel of 40%. These conditions were sufficient to cause degradation of the protein in the broth with the cell growth rate (μ) of 0.35 h⁻¹ that was recorded, being a high value for dextransucrase fermentations. It would seem that *Leuconostoc mesenteroides* cells are not greatly affected by high shear conditions.

Another possibility for the reduced enzyme yield from this fermentation could involve the metabolism of the cells. Figure 6.5 shows the total sucrose consumed during the experiment, compared to the enzyme yield. As seen from the graph, the sucrose was still being consumed at quite a high rate even when the enzyme activity began to decrease. It is reasonable, therefore, to assume that the sucrose was being consumed predominantly for cell growth and maintenance. Energy is required by the cells for growth, maintenance and product formation and is provided in the form of ATP molecules, with the number of ATP molecules being produced in the presence of oxygen far surpassing the number produced in the absence of oxygen. Therefore, under aerated conditions, there are sufficient ATP molecules available in the medium for all the cell processes to take place, so that the cells do not have to

break down any extra sugars, fatty acids or amino acids from the medium, in order to obtain further metabolic energy. The total sucrose consumed during experiment R11/3.12.87 was 561 g, which was the average value amongst aerated fermentations (see Table 6.3), with the sucrose consumption varying between 494 and 660 g. The sucrose consumption under unaerated conditions was considerably higher (see Table 6.5), varying between 573 and 921 g. However, this section is concerned with aerobic fermentations and so the discussion will be limited mainly to those experiments. It is therefore possible that, as there is such an abundance of ATP molecules in the medium, sufficient for cell usage, the sucrose is utilized as a growth and product substrate and the cells do not have to break down any further sucrose to provide energy. This could be a reason why there is less sucrose consumed during a typical aerobic fermentation compared to an unaerated fermentation. The lower enzyme yield in these cases could be due to the fact that the cells do not have to secrete excess dextransucrase in order to break down the extra sucrose, as could be the case with unaerated fermentations.

The cell growth rate is relatively unaffected by the aeration conditions of the fermentation, which is not an unexpected phenomenon as *Leuconostoc mesenteroides* is a facultative anaerobe.

A further factor that could limit enzyme production under aerobic conditions involves the transport of the enzyme from the cells to the medium. When there is no aeration, more carbon dioxide is produced by the cells, with the presence of carbon dioxide being thought to improve the transport of dextransucrase (11). Therefore, an aerobic fermentation will produce less CO₂, resulting in less efficient transport of enzyme to the medium from the cells and, hence, a lower enzyme yield.

These three reasons could therefore provide explanations for the reduced yields of enzyme under aerobic conditions, although the transport mechanism theory does not provide an adequate explanation as to why there is much less sucrose consumption during aerated fermentations, unless the extra sucrose utilized under unaerated conditions is used mainly for dextransucrase formation. However, it is more likely that the metabolism of the cells alters between aerobic and unaerated fermentation conditions, with the amount of ATP present governing the final sucrose consumption and enzyme yield. The theory put forward by Thomas, concerning the denaturation of

proteins under conditions of high aeration and agitation, could also possibly be true in the case of experiment R11/3.12.87, where the maximum enzyme activity achieved was 244.0 DSU/cm³ (11.75 U/cm³).

Table 6.3 gives the results obtained from a further set of aerobic fermentations, although these were all carried out at an agitation rate of 100 rpm. The maximum activities achieved from these fermentations vary between 385.5 DSU/cm³ (18.56 U/cm³) at a very low aeration rate, to 236.4 DSU/cm³ (11.38 U/cm³) at the same aeration rate that was previously used in experiment R11/3.12.87. It can therefore be stated that increasing the aeration rate of the fermentation results in a decrease in enzyme yield, caused either by a reduction in enzyme formation, or a denaturation of the enzyme following formation. It is probable that less enzyme is actually formed during aerobic fermentations, justifying the consumption of a lower amount of sucrose, although the high aeration rates could cause some enzyme denaturation to occur.

7.2.2. Effect of Antifoam on Product Formation.

During a standard unaerated fed-batch fermentation of dextransucrase, there is little or no foam formation, until the end of the experiment, when there is often a certain amount of foam that builds up in the vessel. Indeed, substantial foam formation often indicates the termination of a fermentation. However, experiments that are highly aerated, agitated, or both often exhibit a greater than usual amount of foam formation during the exponential period of the cells' growth, when the majority of the enzyme is secreted. It was not known whether this foam inhibited dextransucrase production in any way, and so a set of aerobic fermentations were carried out in which approximately 2 cm³ of a silicone antifoaming agent were added to the medium before sterilization. The results from these experiments can be seen in Table 6.4, although they need to be compared with the results in Table 6.3 where there was no addition of antifoam to the medium. Experiments R27/15.3.88 and R28/17.3.88 can be compared to R34/11.5.88, with the latter fermentation having been carried out without the addition of antifoam. The enzyme yields from these three fermentations were all very similar to each other, all being approximately 390 DSU/cm³ (17.77 U/cm³). It would appear that the addition of the antifoam had little effect on the enzyme yield on aerobic fermentations through which was passed 1 litre of sterile air per minute and agitated at 100 rpm. The cell growth was also unaffected by the addition of antifoam.

Experiments R25/2.3.88 and R26/9.3.88 were carried out at an aeration rate of 12 litres/minute and an agitation rate of 100 rpm, identical to R23/24.2.88 and R24/26.2.88, but for the addition of antifoam. The fermentations that included the antifoam produced approximately 60 DSU/cm³ (2.89 U/cm³) of enzyme more than the comparable fermentations that did not contain antifoam. It can therefore be stated that the use of antifoam in an aerobic fermentation serves to enhance the enzymic activity of the final broth, providing there is sufficient mixing to allow for thorough dispersion of the antifoam throughout the broth. When the antifoam was added to the fermentations that were aerated at 1 litre/minute, there was insufficient mixing and the antifoam could be seen floating on the surface of the medium in the form of oily globules. This was the reason the antifoam had no effect on the fermentation. However, when there was a greater amount of aeration, the antifoam dispersed throughout the medium, leading to a reduction in the oxygen transfer capability of the broth, as the oily antifoam somehow coated the surface of the cells, preventing as much oxygen from becoming available to the cells as was previously the case. This would have the effect of apparently reducing the aeration of the fermentation, which would, as discussed in Section 7.2.1, have the effect of increasing the overall enzyme yield of the fermentation. These findings are confirmed by the results given in Table 6.4, when compared to those in Table 6.3..

7.3. Unaerated Fermentations.

The majority of the fermentations that were carried out over the course of this work were not aerated, although they cannot be strictly termed "anaerobic", as air was allowed to enter the fermenter through an inlet filter to allow for a positive pressure in the head-space of the vessel in order to prevent the formation of a vacuum within the fermenter during post-sterilization cooling. This served to eliminate the possibility of any contaminant organisms being sucked into the vessel. However, because of this, these fermentations are described as "unaerated" as opposed to strictly "anaerobic".

Ajongwen-Numfor (11) was the first to successfully obtain yields of dextransucrase in excess of 450 DSU/cm³ (21.67 U/cm³) under unaerated conditions, and so a number of fermentations were carried out to determine whether these results could be consistently reproduced, with the process parameters then being varied to investigate the results subsequently obtained..

7.3.1. Effect of Agitation Rate on Product Formation.

When Ajongwen-Numfor (11) obtained 450 DSU/cm³ (21.67 U/cm³) of enzyme, there was very little agitation in the vessel, with the stirrer being rotated at a speed of 100 rpm. A number of experiments were therefore carried out at varied agitation rates to determine whether increased mixing would increase enzyme production in any way, or whether it would serve to limit cell growth at all. Table 6.5 shows the results obtained from this group of fermentations, with the agitation rate varying between 100 and 450 rpm. The sucrose consumed during these unaerated fermentations was considerably greater than that utilized during the aerated fermentations discussed in Section 7.2.1 with the exception of R1/14.10.87 and R19/8.2.88. Experiment R1/14.10.87 suffered from poor temperature control, with the fermenter being lagged with an insulation material following sterilization to maintain the required fermentation temperature. This experiment was also operated for 28 hours until the maximum activity was achieved, indicating that the cells grew in a linear manner, rather than exponentially, during this fermentation. Therefore, R1/14.10.87 cannot be considered a standard fermentation in terms of its operation, although the final results were satisfactory. Experiment R19/8.2.88 was carried out at an agitation rate of 450 rpm, at which level the substrate became available to the cells at a greatly increased rate, such that a greater amount of the residual sucrose was utilized by the cells, leading to a reduced sucrose demand. However, the higher agitation rate may also in some way have disrupted the cells so that they would not require as great an amount of sucrose as previously. The other experiments listed in Table 6.5 consumed between 750 and 920 g of sucrose during the course of the fermentation, compared with values of between 520 and 660 g consumed aerobically (see Table 6.3). This factor has been discussed in Section 7.2.1, where a change in the cells' metabolism was thought to be responsible for the increased sucrose consumption and, hence, higher enzyme yield, as dextransucrase was being produced by the cells to break down excess sucrose in order to provide the cells with extra energy for their metabolic processes that were not being provided for by ATP molecules, the production of which decreased in the absence of oxygen.

However, an increased agitation rate of up to 350 rpm does not greatly affect the overall fermentation in any way, with the OD₅₉₀ value, maximum enzyme activity and growth rate all being reasonably consistent between experiments

R10/27.11.87, R29/23.3.88, R14/20.1.88 and R15/27.1.88. When a fermentation produces an activity of at least 450 DSU/cm³ (21.67 U/cm³) and an OD₅₉₀ value of approximately 1.0, it is said to have been a successful experiment, and the fermentations mentioned above all achieved these values, despite the agitation rate being increased from 100 to 350 rpm. Experiment R19/8.2.88 was carried out with an agitation rate of 450 rpm, and although the final results were slightly lower than those obtained in the previous experiments, the enzyme yield was still greater than values obtained aerobically. Therefore, it can be said that increased agitation does not cause any "shear damage" to the cells, at least up to a speed of 350 rpm in the New Brunswick vessel. Above this value, the cell growth and enzyme yield drop slightly, but not dramatically. An agitation rate of 250 rpm produces an OD₅₉₀ value of 1.121 and an activity of 527 DSU/cm³ (25.37 U/cm³) - the highest results achieved in any fed-batch fermentation in these studies, although an experiment carried out at 100 rpm, such as R29/23.3.88 produced 511.4 DSU/cm³ (24.62 U/cm³) of enzyme with an OD₅₉₀ value of 1.044. It was therefore decided that, although an increased agitation rate of above 100 rpm was not detrimental to the project, it was not advantageous either, and as higher agitation rates required a higher power input and hence increased operating costs of the fermentation, the agitation rate for dextransucrase production should be maintained at 100 rpm with the New Brunswick vessel.

7.3.2. Effect of Antifoam on Product Formation.

The experiments carried out in this section were very similar to those discussed in Section 7.2.2, where approximately 2.0 cm³ of a silicone antifoaming agent were added to the medium before sterilization and fermentations were carried out at different levels of aeration. In this section, 2.0 cm³ of the same antifoam were added to the medium and a number of fermentations were carried out with varying agitation rates. The results from these experiments are given in Table 6.6, where it can be seen that, at an agitation rate of 100 rpm, the results are almost identical to those obtained without antifoam addition. The reason for this is the same as the reason why antifoam did not affect fermentations such as R27/15.3.88 (see Table 6.4), because there was insufficient mixing (due to low agitation and/or aeration rates) to allow complete dispersion of the antifoam throughout the medium. Indeed, as in R27/15.3.88 and R28/17.3.88, the antifoam could be seen floating on the surface of the medium, and had, therefore, not been thoroughly mixed. At increased agitation rates, the antifoam became more dispersed

throughout the culture fluid and although this did not affect cell growth greatly, the final activity was very much reduced. For example, at an agitation rate of 600 rpm, an activity of only 133.4 DSU/cm³ (6.42 U/cm³) was achieved. This was due to the fact that when the antifoam was thoroughly distributed throughout the medium, it coated the cells and reduced the amount of sucrose becoming available to the cells (indicated by the decreased sucrose consumption levels at higher agitation rates) and also preventing the cells from secreting high levels of enzyme.

Therefore in unaerated fermentations, where the agitation conditions are sufficient to allow thorough dispersion of the antifoam throughout the medium, the antifoam acts to prevent enzyme formation or secretion. It is possible that, as claimed by Viesturs and co-workers (102), the surface active antifoam could cause morphological changes in the cell structure leading to alterations in the physiological function of the cells and, hence, the elaboration of less dextransucrase. As the effect that antifoam addition has on unaerated fermentations is the opposite to the effect on aerated fermentations, it seems likely that some kind of change occurs within the cells due to the presence or absence of oxygen, with respect to the cells' ability to secrete dextransucrase. The activity achieved under unaerated conditions is still much higher than that obtained under highly aerated conditions with the addition of antifoam, despite the fact that the presence of antifoam serves to reduce the aeration of the fermentation by decreasing the oxygen transfer rate of the medium. Technically, a fermentation that is sufficiently aerated to provide adequate mixing of the antifoam throughout the broth, could have its oxygen transfer rate reduced such that the cells could be said to be growing in an unaerated medium. However, as the metabolism of the cells was probably altered by the addition of air, due to the presence of more ATP molecules in the medium, the cells would never be capable of producing as much enzyme as under unaerated conditions. This supports the theory that cell metabolism is altered between aerated and unaerated fermentations.

7.3.2. Effect of Folic Acid on Product Formation.

A set of fermentations was carried out to investigate the claims made by Brown and McAvoy (14), that folic acid was the limiting component in dextransucrase fermentations and that the addition of 0.0206 g/l of folic acid to the medium would prevent the severe limitation of cell growth and enzyme formation that appeared to occur at the end of a typical fermentation and would increase the duration of the exponential growth phase of the fermentation - during which phase the enzyme was secreted. These fermentations were carried out with the addition of two different quantities of folic acid, with 0.0206 g/l and 0.003 g/l being added. The lower amount of folic acid was added to determine whether the concentration specified by Brown and McAvoy would cause any enzyme inhibition. Whiteside-Carlson and Carlson (6) carried out extensive studies into the vitamin and amino acid requirements of various strains of *Leuconostoc* and found that *Leuconostoc mesenteroides* ATCC 683, among others, required the presence of folic acid in the medium for growth, but also in sucrose-based media, that there were some inhibitory effects with various strains of *Leuconostoc mesenteroides* when riboflavin, biotin, folic acid, p-aminobenzoic acid, pyridoxal and/or pyridoxamine were present in excess. These fermentations would also determine whether a larger quantity of folic acid added to the medium would inhibit dextransucrase production in any way.

The results from this section of the project are shown in Table 6.9, with two separate batches of Gistex yeast extract having been used as the medium nitrogen source. The batch termed "Good Gistex" had produced enzyme with an activity in excess of 450 DSU/cm³ (21.67 U/cm³), although, unfortunately, the batch number had not been recorded. Batch 7-40-07 did not appear to be capable of producing dextransucrase of a high activity - indeed in R35/26.5.88 which was carried out under standard conditions, a maximum activity of only 269.9 DSU/cm³ (12.99 U/cm³) was obtained. The folic acid was added to the medium prepared with Gistex batch 7-40-47 in an attempt to boost the activity obtained, which would occur if folic acid was limiting in Gistex 7-40-07.

The enzyme activity achieved when 0.003 g/l of folic acid was added to the medium containing the good Gistex varied between 267.3 and 392.3 DSU/cm³ (12.87 and 18.89 U/cm³). Despite the differences between the two results, it could be seen that the addition of even a small quantity of folic acid caused a reduction in the subsequent enzyme activity. When 0.0206 g/l of

folic acid was added to the medium prepared with the good Gistex, the final enzyme yield was slightly increased, at 411.6 DSU/cm³ (19.82 U/cm³), although still considerably below the expected activity of 450 - 500 DSU/cm³ (21.67 - 24.07 U/cm³). It would appear that folic acid addition did indeed cause some limitation in enzyme production although it is not clear why the final enzyme yield was greater with the addition of a larger amount of folic acid. It is possible that a particular level of folic acid present in the medium would cause some enzyme inhibition, but as the folic acid concentration increased beyond this point, dextransucrase production was again stimulated, although these results have to be compared to those obtained when the medium was prepared with Gistex 7-40-07. During the folic acid fermentations there was a high incidence of foaming, so an experiment was carried out which included an antifoam addition to the medium to prevent this. Experiment R45/15.7.88 (see Table 6.9) was carried out, although, according to the findings from Section 7.2.2, there was insufficient agitation in the vessel to disperse the antifoam throughout the medium. However, the high foam formation acted as its own mixing agent and was therefore broken down by the antifoam. The activity achieved from this fermentation was considerably lower than the other folic acid fermentations with the good Gistex, with the exception of R42/30.6.88, confirming the earlier findings that antifoam addition to an unaerated fermentation caused a reduction in enzyme activity.

A number of fermentations were carried out with Gistex batch 7-40-071 and with the addition of the lower quantity of folic acid, the activity achieved was approximately 260 DSU/cm³ (12.52 U/cm³). This was very similar to the value obtained from R35/26.5.88 in which standard conditions were used to obtain high enzyme yields with Gistex batch 7-40-07. The addition of 0.0206 g/l of folic acid to the medium increased the activity only slightly, to 297.6 DSU/cm³ (14.33 U/cm³), still considerably lower than the experimental maximum. It is possible that folic acid was a limiting component in Gistex batch 7-40-07, and the addition of extra folic acid boosted enzyme production. However, folic acid may not have been the only limiting component in Gistex 7-40-07, although the only method of making sure of this fact would be to carry out detailed analyses of both Gistex batches used in these folic acid experiments and to compare the results. However, *Leuconostoc mesenteroides* has very complex growth requirements, which would make this study very difficult to carry out successfully. The yeast extract composition will be discussed further in Sections 7.3.4 and 7.3.5. The

claims made by Brown and McAvoy have not been confirmed by the results from this project, although it can be said that in certain cases, when a Gistex batch is unable to produce high yields of dextransucrase, this could be due, in part, to folic acid limitation and in these cases, the addition of folic acid to the medium would serve to slightly increase dextransucrase production.

7.3.4. Effect of Different Yeast Extract Types on Product Formation.

A number of fermentations were carried out with various yeast extract types and nitrogen sources. The simplest of these were the shake-flask "batch" fermentations that were carried out using various types of peptones supplied by Oxoid U.K., including Bacteriological Peptone, Special Peptone, Peptone P and Casein Hydrolysate. The results of these preliminary experiments can be found in Tables 6.1 and 6.2. These batch fermentations were carried out to determine whether the peptones would prove to be more capable of allowing high yields of dextransucrase to be produced from the cells. As cells grow they produce acid, and so the pH of these peptone-containing media were monitored to determine which allowed the greater pH drop. Figure 6.1 clearly shows that the best results were obtained with Bacteriological and Special Peptones, although the Bacteriological peptone gave an activity of 47 DSU/cm³ (2.26 U/cm³) compared to 31.4 DSU/cm (1.51 U/cm³) obtained with Special Peptone (see Table 6.1). The Bacteriological peptone was then used for further trials in combination with various concentrations of Gistex yeast extract, with the results from those experiments shown in Table 6.2. Increasing the percentage of Bacteriological Peptone - and hence decreasing the percentage of Gistex - in the medium served to decrease the final enzyme yield and, although no control experiment containing 100% Gistex was carried out, it would appear that Bacteriological Peptone would not be as successful as Gistex yeast extract for the purpose of dextransucrase fermentations.

Following these experiments, a series of fed-batch fermentations was carried out using different types of yeast extract as the nitrogen source, including Gistex paste, Gistex powder, Orhly and DCL yeast extracts. The results from these experiments are shown in Table 6.7, where it can be clearly seen that only Gistex paste was capable of giving dextransucrase yields in excess of 450 DSU/cm³ (21.67 U/cm³) Gistex powder yielded approximately 400 DSU/cm³ (19.26 U/cm³), whereas DCL allowed only 225 DSU/cm³ (10.83 U/cm³) of enzyme to be produced, and Orhly only 190 DSU/cm³ (9.15 U/cm³).

The continued use of Gistex paste as a nitrogen source was obviously not in dispute, although the reasons for these wide variations in enzyme yield from the various yeast extract types should be discussed. In this way, the various medium requirements for *Leuconostoc mesenteroides* growth and dextransucrase production may possibly be determined.

A comparison can first be made between Gistex paste and Gistex powder, both manufactured by Gist-Brocades. These yeast extracts are prepared from the yeast *saccharomyces cerevisiae*, with the yeast being produced in a batch process and aeration and sugar from molasses being used to increase the yeast biomass. The yeast is then harvested, washed and then undergoes autolysis at 50°C, after which point the yeast cell components are removed by centrifugation and clarified by filtration. The clear liquid extract is then either concentrated under vacuum to form Gistex paste, or spray-dried to form Gistex powder which is oil-coated to prevent dusting and reduce hygroscopicity. These two products, as well as being used in many food production processes, are often used in industrial fermentations, being excellent sources of vitamins and amino acids. These yeast extracts contain approximately 43% proteins and 10% carbohydrates (as oligosaccharides), with the powder containing approximately 0.4% fat. Table 7.1 gives an analysis of various components of the two products (104).

Table 7.1.
Analyses of Gistex Paste and Gistex Powder Yeast Extracts.

Component	Paste	Powder
% total solids	80	96
% NaCl	16	38
% flavour solids	64	58
% total N ₂	7	6.8
pH (2% solution)	5.5	5.3
Copper	< 10 ppm	< 10 ppm
Arsenic	< 2 ppm	< 2 ppm
Lead	< 5 ppm	< 5 ppm

These analyses were the only ones made available to the project by Gist-Brocades and they show a considerable difference in solids and NaCl content between the two products. The nitrogen compositions of the two yeast extracts are very similar and the slight pH differences would be compensated by the addition of the alkaline buffer K_2HPO_4 to the fermentation medium. Therefore, it would appear that the solids content and NaCl content provide the major composition differences between Gistex paste and powder. The solids content was accounted for by the use of only 30 g/l of powder in the medium compared to 40 g/l paste. This, in turn, reduced the NaCl content in the powder medium to nearer the paste level, but it also reduced the N_2 content in the powder medium still further below the paste value. An amino acid content of Gistex has been obtained, but with no distinction between the paste and the powder, so this data will be better used along with the amino acid contents of the DCL and Orhly yeast extracts, that were provided by the manufacturers of those yeast extracts. The DCL and Orhly information also contained data concerning the vitamin contents of the products, although it must be stressed that all the values presented in Tables 7.2 and 7.3. are only approximate averages and can vary by up to $\pm 20\%$ between batches. A number of analyses were therefore carried out by Alta Bioscience - located at the University of Birmingham Science Park - who analysed several batches of the various yeast extract types with respect to carbohydrate and amino acid content. These results show that significant variations occur between batches of the same yeast extract type, as well as between different yeast extracts.

Table 7.2.
Approximate Average Analyses of Various Yeast Extract Types.

Component	Gistex (104) (% w/w)	Orhly (105) (% a.a)	DCL (106) (% a.a)
Alanine	3.4	2.0	6.1 - 6.7
Aminobutyric acid	0.3	0.1	0.59 - 0.61
Arginine	2.6	0.8	3.6 - 3.7
Aspartic acid	4.7	1.4	12.7 - 14.3
Cysteine	-	0.4	17.4 - 20.3
Glycine	2.3	1.4	4.1 - 4.7
Histidine	1.3	0.4	1.7 - 1.8
Isoleucine	1.9	1.6	4.6 - 5.4
Leucine	2.1	2.1	5.7 - 6.7
Lysine	3.9	2.0	8.4 - 10.7
Methionine	0.4	0.9	1.1 - 1.3
Ornithine	-	0.6	0.67 - 1.6
Phenylalanine	1.7	1.3	4.0 - 4.8
Proline	1.7	1.0	3.6 - 3.7
Serine	2.3	1.1	4.4 - 4.6
Threonine	2.5	1.0	7.0 - 7.1
Tryptopan	0.5	0.3	Trace
Tyrosine	1.8	0.6	2.3 - 2.5
Valine	2.9	1.7	4.8 - 6.0

Table 7.3.
Approximate Average Vitamin Content in Orhly and DCL Extracts.

Component	Orhly (105) (ppm)	DCL (106) (ppm)
Thiamin	10 - 15	15
Riboflavin	50 - 70	75
Pyridoxine	15 - 25	16
Nicotinic acid	350	400
Pantothenic acid	80	75

As already mentioned, the various yeast extracts were analysed for carbohydrate and amino acid content. The data obtained from the various batches of Gistex will be discussed in the following section which deals with fermentations carried out with different Gistex batches. However, three batches of DCL yeast extract, and two of Orhly were analysed and will be discussed below.

The batches analysed were DCL 1580, 1653 and 1822, and Orhly 47-9 and 321 (107). It was found that the amino acid levels in DCL batches 1580 and 1653 were very similar, except that DCL 1653 contained slightly higher levels than DCL 1580 overall. However, both these levels were higher than those in DCL 1822. All the DCL samples contained arginine, but the levels of ornithine were low in all three cases, particularly with DCL 1580. However, the two Orhly samples were both free of arginine, with low levels of ornithine. Indeed, the levels of amino acids generally were much lower in the Orhly yeast extracts than in the DCL yeast extracts, with levels being very depleted in Orhly 321. However, the Orhly samples contained extremely high levels of glutamic acid - in excess of 700 nM/mg, although the exact value is not known as the reading went off-scale. Apart from the normal amino acids there were also indications of a major unknown with an elution time of approximately 75.17 minutes in both Orhly samples, along with DCL 1822 - with only small amounts present in DCL 1653 and 1580. It should be restated here that DCL allows slightly more enzyme production than Orhly, although cell growth with DCL is considerably higher than with Orhly. DCL yeast extract is used by Fisons plc as the nitrogen source for their whole cell production of dextran. These yeast extracts were also analysed for bound amino acids which were released by acid hydrolysis, thus boosting the overall amino acid content of the various yeast extracts quite considerably. For example, DCL 1580 contained 90 nM/mg of free aspartic acid, but after acid hydrolysis this figure had increased to 270 nM/mg. Generally, with the DCL samples, the levels of amino acids were greatest in DCL 1653, followed by 1580 and 1822, although all levels fell within a similar range. The levels of amino acids in the Orhly samples were much lower than with the DCL samples, except for extremely high levels of glutamic acid that were detected in both samples. It could be possible that an excess of glutamic acid suppresses the growth of the *Leuconostoc mesenteroides* cells in some way, as its level in the DCL

yeast extracts (and also Gistex samples) is much lower, approximately 30%, of the levels within the Orhly samples.

The carbohydrate levels within the yeast extracts were also tested and it was found that only DCL 1822 and 1580 contained any detectable amounts of mannose. However, yeast produces a surface polysaccharide, called a mannan, that gave high levels of mannose on acid hydrolysis, particularly in the three DCL samples, although the mannose detected in the Orhly samples after hydrolysis was less than half that in the DCL yeast extracts. Mannose is usually important in the carbohydrates of glycoprotein enzymes. Free glucose was present in all the samples tested, particularly DCL 1822, although after acid hydrolysis more was released from the high levels of sucrose, particularly in DCL 1822 and 1580. A smaller amount of glucose was also released on hydrolysis from maltose. Free sucrose was present in all the samples, with higher levels being once more detected in DCL 1822 and 1580. There was no free ribose present in the samples, although some did appear after acid hydrolysis. The ribose residues that were detected came from nucleosides and nucleotides within the yeast extract and are an indication of the concentration of certain coenzymes, such as NAD. Just as the Orhly samples had low levels of yeast mannan, so they had low liberated ribose levels - less than 50% that of DCL (and Gistex) yeast extracts. However, the Orhly samples seemed to have some glucose tied up in molecules other than sucrose or maltose since acid hydrolysis released very high levels of glucose in these samples, much higher than those detected in the DCL extracts. The DCL samples yielded very small amounts of xylose after acid hydrolysis which was not the case with the Orhly samples, although the Orhly samples did contain several unknown substances before hydrolysis, which were thought to be deoxynucleosides. These unknowns disappeared following hydrolysis, with the Orhly extracts being rather unique in terms of their concentrations of complex carbohydrates.

Following these analyses, all the yeast extract samples were examined by a U.V. scan between 200 and 400 nm. All the samples experienced their maximum absorption at 258 nm and the intensity values, along with the weight (g) of 10 cm³ of each sample, were recorded. The values of intensity/weight were calculated for each sample, and as all the samples absorbed at 258 nm, these final figures could be used on a comparative basis as an indication of the amounts of coenzymes like NAD, nucleosides and

nucleotides present in the samples. The DCL samples gave results of 0.5007, 0.5096 and 0.5760 for batches 1653, 1822 and 1580 respectively, whilst the Orhly samples gave values of 0.3649 and 0.3922 for batches 47-9 and 321. The materials present that absorbed at 258 nm could be clearly seen in the separation of each yeast extract on a Biogel P2 column. The period beyond 10 hours was particularly interesting as no sugars were detected beyond this point. This would indicate that any nucleosides would be eluted before 10 hours and they would be detected since they contained sugars (ribose or 2-deoxyribose). Any pyrimidine or purine components were eluted after 10 hours because of their aromatic-like interaction with the Biogel column. It is also possible that, due to their phosphate groups, nucleotides would not be bound in any degree to the polyacrylamide gel of the column and would be eluted first. Therefore, the DCL 1653 UV scan showed a nucleotide region with up to 5 hours elution time, a nucleoside region between 5 and 11 hours, and a pyrimidine/purine region beyond 11 hours. Any vitamins which are not nucleotides would also show UV absorption. Sugar scans revealed the presence of mono- and disaccharides, although it was not certain whether polysaccharides were present because any nucleotides with sugar moieties would be eluted in the same position as any polysaccharides. Between 9 and 10 hours a component was eluted from all the yeast extract samples (including Gistex) that did not absorb at 258 nm, did not react with cysteine to give H_2SO_4 , but reacted with IO_4^- to give HCHO . This could be a polyhydric alcohol being eluted between the monosaccharide at 7 - 8 hours and the disaccharide at 11 hours. The disaccharide did not react with IO_4^- to form HCHO and was therefore non-reducing, eg. sucrose. The monosaccharide(s) took part in both the cysteine and IO_4^- reactions and was therefore reducing, eg. fructose, glucose. There was also a component eluted around 14 hours, which reacted only with IO_4^- , that might be the phosphate component of a polyhydric alcohol phosphate, such as ribital phosphate, this component being present in all the yeast extract samples tested.

As mentioned earlier in this section, during a description of the production process of Gistex yeast extract, the state of the yeast extract depends very much on the progress of the yeast fermentation required to produce it. Any sucrose present would be broken down by the yeast invertase to give glucose and fructose, with some trisaccharides possibly being formed from invertase action on the sucrose. Generally the yeast will ferment the glucose component of the sucrose first, and the fructose second.

An analysis of the various yeast extracts showed that Orhly 321 and 14-9 had no monosaccharides but contained residual sucrose with a small amount of trisaccharides. The DCL yeast extracts were quite different, with DCL 1822 containing high levels of sucrose and monosaccharides and no trisaccharides, DCL 1580 containing high sucrose and lower monosaccharides levels, again with no trisaccharides and DCL 1653 having low sucrose and monosaccharide levels and some trisaccharides. The Gistex batches examined in this way will be discussed in the next section.

Sucrose is required to induce dextransucrase production from *Leuconostoc mesenteroides* cells, although glucose might be preferable for cell growth, followed by fructose. In many bacteria, the induction of a product does not occur until any glucose present has been utilized. For example, the Orhly yeast extracts contained no glucose or fructose, and would therefore probably give reduced cell growth, with dextransucrase being induced immediately. This, in fact, was the case with experiment R8/24.11.87 in which there was poor cell growth and consequently reduced enzyme activity. The DCL yeast extracts contain glucose, fructose and sucrose, allowing for good cell growth and, theoretically, good enzyme production. The fact that the DCL yeast extracts did not give good yields of enzyme was probably due to its amino acid and vitamin, rather than carbohydrate, content. The DCL yeast extracts had higher levels of amino acids than the Orhly extracts, but similar levels to Gistex yeast extracts. DCL samples contained higher levels of leucine and tryptophane than Gistex, which theoretically could cause enzyme inhibition, and lower levels of arginine, lysine and glutamic acid, which could be limiting. However, it was more likely to be a combination of factors that caused reduced enzyme yield in DCL yeast extracts.

As already mentioned, DCL yeast extracts are used to produce dextran during industrial fermentation and the three batches of DCL tested had all been used for this purpose. DCL 1822 was generally a good batch for cell growth and dextran production. DCL 1653 was a poor batch, although it was possible that dextran was produced and that the problems lay in the isolation of the product. DCL 1580 was an older batch, but was generally successful at the time.

The discussion above has described in detail the various analyses that were carried out in an attempt to determine the factors that are critical within a yeast extract, for a successful dextransucrase fermentation. However, as *Leuconostoc mesenteroides* has very complex growth requirements, it is difficult to specify exactly which components are limiting and which might cause any inhibition. It is useful to note, though, the differences in content between various batches of the same yeast extract type, showing that it is very difficult to obtain completely standard medium components for a dextran or dextransucrase fermentation.

7.3.5. Effect of Different Gistex Batches on Product Formation.

A number of fermentations were carried out with different batches of Gistex yeast extract, with the results obtained shown in Table 6.8. The batches used were 7-40-7, 7-25-16, CPN CGN 8-34, DGN4-04 and CPN CGN 8-13. The results achieved show that certain Gistex batches were clearly incapable of producing high yields of enzyme. For example, Gistex 7-25-16 gave 360.9 DSU/cm³ (17.38 U/cm³) and Gistex 7-40-07 gave only 269.9 DSU/cm³ (12.99 U/cm³). The texture and physical appearance of Gistex paste often vary from batch to batch, and even between two containers of the same batch, so it is not altogether surprising that the results obtained with Gistex paste should vary so dramatically with different Gistex batches. A series of analyses was carried out on three batches of Gistex, similar to those carried out on a number of Orhly and DCL yeast extracts that were discussed in Section 7.3.4, in a further attempt to define the precise parameters that determine whether a dextransucrase fermentation would be successful.

The Gistex batches analysed in this way were 7-40-07, as used in this project, 8-16-23 and 8-16-25, which was probably the batch mentioned in Table 6.7 as being a "Good Gistex". The findings will be discussed in terms of amino acid content, both free and after acid hydrolysis, followed by carbohydrate content and UV analysis. Of the Gistex batches analysed, 8-16-25 gave the highest yield of enzyme (approximately 450 - 500 DSU/cm³ or 21.67 - 24.07 U/cm³) alongside the best cell growth, with the enzyme yield from 8-16-23 being the poorest of the three samples, although there was good cell growth. Batch 7-40-07 was found to give poor enzyme yield along with reasonable cell growth.

The amino acid levels in the three samples were quite similar to each other, except for glutamic acid and proline where 8-16-25 contained the greatest amount (310 and 48 nM/g respectively) with 8-16-23 containing slightly less (290 and 46 nM/g), but the levels of these components in 7-40-07 were significantly lower, at 260 and 28 nM/g. In the cases of alanine and leucine, sample 8-16-23 contained the highest amounts (300 and 130 nM/g), with 8-16-25 close behind with 290 and 120 nM/g, although 7-40-07 was again significantly lower with respect to these amino acids, containing 240 and 100 nM/g of each respectively. However, the greatest difference between the free amino acid contents of these Gistex batches lay in the fact that both 8-16-23 and 8-16-25 contained no arginine, whereas 7-40-07 contained 85 nM/g of arginine. However, arginine is converted to ornithine on alkaline treatment, and all three samples contained ornithine at very similar levels. This indicates that Gistex batches 8-16-23 and 8-16-25 may well have contained some arginine and they might have undergone alkaline treatment of some sort that may have eliminated their arginine levels. It would seem unlikely that sample 7-40-07 experienced any extended alkaline treatment as it still contained high levels of arginine, although the presence in this yeast extract of ornithine would imply that some limited alkaline treatment took place. However, this would still indicate that the levels of arginine were originally much higher in 7-40-07 than in the other yeast extracts. As 7-40-07 was reported to be unable to support as good a cell growth as the other Gistex batches involved in these analyses, although this was not supported by the experiments carried out during this project, it is possible that high levels of arginine somehow inhibited cell growth, either in combination with ornithine or as a singular component. In Section 7.3.4., the elution of an unknown component at 75.17 minutes was reported in DCL 1822 and Orhly 47-9 and 321. This same unknown was also present in Gistex 7-40-07, with only very small quantities being detected in 8-16-25 and 8-16-23. As the nature of this component is not known, no firm conclusions can be drawn from this, except to comment that it might in some way have contributed to a reduced final dextranucrase activity.

The three samples were then each subjected to acid hydrolysis and analysed once again for amino acid content. As was the case with the free amino acids, the levels in all the three batches were very similar, except for arginine, which was significantly higher in Gistex 7-40-07 than in 8-16-23 and, in turn, 8-16-25. The amino acids - glutamic acid, proline, alanine and leucine - which

Gistex 7-40-07 contained in smaller quantities than did the other two samples, after free amino acid analysis were very similar in all three batches after acid hydrolysis. Therefore, Gistex batch 7-40-07 must have contained higher levels of bound amino acids, as opposed to batches 8-16-23 and 8-16-25, which contained higher concentrations of free amino acids.

The Gistex samples were also analysed for carbohydrate content, as were the DCL and Orhly yeast extracts. Very high levels of mannose were detected in all three samples, which were similar to the concentrations found in the DCL yeast extracts, although the Orhly extracts contained only half the mannose compared to the other samples. As mentioned in Section 7.3.4, mannose is an important factor in the carbohydrates of glycoprotein enzymes and this may prove to be a vital component in this process as both DCL and Gistex yeast extracts are used successfully to produce either dextran or dextransucrase. DCL yeast extract is obviously capable of producing high yields of enzyme, but only in the presence of high concentrations of sucrose that can be immediately converted into dextran. Indeed, the presence of dextran may also encourage dextransucrase secretion during a whole cell fermentation process using DCL yeast extract.

The Gistex yeast extracts all contained low levels of glucose and sucrose in the free state, which was similar to the case with the Orhly samples, although DCL 1822 contained high levels of both sugars, with DCL 1580 containing high sucrose levels. The Gistex samples did not contain any free ribose, although some appeared after acid hydrolysis, being liberated from 2 - deoxyribose and other nucleosides and nucleotides. The Gistex samples had high levels of liberated ribose, indicating high levels of coenzymes such as NAD in the yeast extract. Gistex 7-40-07 also contained high levels of certain unknown carbohydrates, which was not the case with Gistex 8-16-23 and 8-16-25, although very similar to the concentrations of unknowns detected in the DCL samples.

The three Gistex samples were then examined by a UV scan between 200 and 400 nm, in the same way as with the DCL and Orhly samples. The samples all reached their maximum absorption at 258 nm, and the values of Intensity were calculated in the same manner as for the DCL and Orhly yeast extracts. The results from these calculations were 0.5673, 0.5169 and 0.5028 for batches 8-16-25, 7-40-07 and 8-16-23 respectively, and as already

mentioned, these values can indicate the levels of coenzymes such as NAD and other nucleosides and nucleotides in the samples. The results obtained from the Gistex samples were similar to those obtained from the DCL extracts, with all being greater than those obtained from the Orhly extracts. These results agree with the findings determined from the levels of ribose in the samples following acid hydrolysis, where the Orhly extracts contained approximately 33% - 50% less than the other samples.

A discussion was included in the previous section concerning the elution of various components using a Biogel P2 column, so it will not be repeated here. However, the Gistex samples contained similar levels of the polyhydric alcohol, that was eluted after 10 hours, as did the DCL samples, both being considerably higher than the Orhly levels of this component.

The levels of mono-, di- and trisaccharides in the three Gistex samples were also determined, with the reason being that although sucrose is the substrate required for dextransucrase induction, most bacteria will utilize all the available glucose for growth and maintenance purposes before any induction is commenced. Gistex batches 8-16-25 and 8-16-23 both contained moderate levels of sucrose, but with low levels of trisaccharide and monosaccharide, the latter being eluted from the Biogel P2 column as two distinct peaks. Gistex 7-40-07 had the same features as the other Gistex samples but with lower sucrose levels, implying decreased ability to induce enzyme with a reasonable cell growth. However, despite the fact that batch 8-16-23 contains the same concentrations of saccharides as the high enzyme-yielding 8-16-25, it also does not have the ability to induce dextransucrase, even with good cell growth.

Gistex sample 7-40-07 was found to contain an extra component that was eluted at 10.5 hrs, which absorbed in the UV at 258 nm. Apart from this factor, the UV scans of the three samples were very similar. It is possible that some of the purine/pyrimidine/nucleosides had not been converted into the nucleotide coenzyme in sample 7-40-07, as was the case with 8-16-23 and 8-16-25, which could explain the nature of the unknown component in 7-40-07. The only other differences that occurred between the three Gistex samples were all very minor. Batch 8-16-25 eluted two unknown components between 15 and 16 hours. Batch 7-40-07 eluted a more prominent unknown at

approximately 4 hours that reacted with IO_4^- to form HCHO, indicating that it was a non-reducing sugar.

The three Gistex batches were all found to be quite dissimilar from each other in many ways, so it would be very difficult to accurately predict which medium components were limiting and which were inhibitory, as it could very easily be a combination of components that boosted or decreased cell growth and enzyme production.

7.3.6. Effect of MBR Fermenter on Product Formation (Scale-Down).

A 2.5 litre MBR fermenter was purchased in 1988 for the purpose of investigating the continuous production of dextransucrase. However, the vessel was also used to carry out a number of fed-batch fermentations, predominantly with Gistex batches that had previously produced good yields of dextransucrase. In particular it was necessary to determine whether it was possible to obtain in excess of 450 DSU/cm^3 (21.67 U/cm^3) of enzyme using the MBR vessel, with the same Gistex batch that had allowed the achievement of $450 - 500 \text{ DSU/cm}^3$ ($21.67 - 24.07 \text{ U/cm}^3$) enzyme using the New Brunswick vessel. These fermentations were carried out at various intervals during the continuous fermentation programme in order to determine whether the yields being obtained continuously could be improved with the use of a different vessel that would allow yields of 450 DSU/cm^3 (21.67 U/cm^3) of enzyme from the fed-batch mode.

The results from the various fermentations carried out in the MBR vessel can be seen in Tables 6.10 and 6.11. It is clear from this data that in none of the fermentations in the MBR vessel did the maximum enzyme activity reach a value of at least 450 DSU/cm^3 (21.67 U/cm^3). Experiment R52/30.11.88, for example, was extremely disappointing with respect to the final activity achieved, despite the fact that a yeast extract batch capable of supporting good cell growth and enzyme activity was used. This batch had been used in fed-batch fermentations with the New Brunswick vessel, such as experiment R29/23.3.88, where a final activity of 511.4 DSU/cm^3 (24.62 U/cm^3) was obtained. The extremely poor activity of 50.7 DSU/cm^3 (2.44 U/cm^3) achieved during R52/30.11.88 indicates that either there was a problem that occurred during the operation of the fermentation, or that the yeast extract had degraded quite considerably. As Gistex paste has a shelf life of only 12 months, it seems most likely that the latter of the two possible theories was

responsible for the poor results. Following this, a new Gistex batch was purchased and a series of experiments were carried out using this yeast extract. Tables 6.10 and 6.11 show the results obtained from these fermentations, although reference should be made to R65/8.3.89 which was carried out in the New Brunswick fermenter, with the same Gistex - and phosphate - as was used in R66/20.3.89. The lower pH KH_2PO_4 , which was used successfully during the continuous fermentations, was not used during standard fed-batch experiments, with the more alkaline K_2HPO_4 being preferred under such conditions. Experiment R65/8.3.89 corresponded exactly with R66/20.3.89 in that the same phosphate and Gistex batches were used. The fermentation carried out in the New Brunswick vessel yielded approximately 150 DSU/cm^3 (7.22 U/cm^3) more dextransucrase than the fermentation carried out in the MBR vessel. However, experiment R55/21.12.88, which was identical to R66/20.3.89 in all respects apart from the fact that K_2HPO_4 was used as a medium component, yielded approximately 329 DSU/cm^3 (15.84 U/cm^3) of dextransucrase. Assuming that the MBR vessel is only capable of producing 150 DSU/cm^3 (7.22 U/cm^3) enzyme less than the New Brunswick vessel, the conditions employed for R55/21.12.88 should theoretically yield an activity of 479 DSU/cm^3 (23.06 U/cm^3) in the larger fermenter. However, such an experiment was not carried out at this time, although the theory can be confirmed - that the MBR vessel is incapable of producing enzyme yields in excess of 450 DSU/cm^3 (21.67 U/cm^3) - when comparing the results obtained from R93/22.1.90 (see Table 6.11) and R101/26.2.90 (see Table 6.8), where corresponding fermentations were carried out in both vessels using the same phosphate (K_2HPO_4) and the same Gistex batch (CPN CGN 8-13). Experiment R101/26.2.90 produced dextransucrase with a maximum activity of 432.4 DSU/cm^3 (20.82 U/cm^3), whereas R93/22.1.90 only yielded 313.5 DSU/cm^3 (15.09 U/cm^3) of enzyme. The final yield from the MBR vessel was over 100 DSU/cm^3 (4.81 U/cm^3) lower than that obtained in the New Brunswick vessel, supporting the theory that the MBR vessel, being operated under the current conditions, was not able to produce the required high levels of dextransucrase being obtained by the New Brunswick fermenter. The reasons for this can be explained using the basic principles of scale-up - or, as in this case, scale-down. As already mentioned in Section 3.7, there are many factors that could be used in determining the nature of a scale-up procedure, including constant K_{La} , constant tip speed, constant volumetric power input and equal mixing times. When the MBR fermenter was purchased, a review of these parameters was

not carried out, as all the vessels that were suitable for use as a chemostat were very similar in all respects. The MBR fermenter was chosen mainly for its highly sophisticated controls and due to the fact that it was able to sterilize itself *in situ* - a feature that made it very attractive over the other models considered at the time. However, it now became necessary to consider the various scale-up factors in order to determine why it did not appear possible to obtain high yields of dextransucrase from this vessel.

An important parameter that needs to be considered when designing a bioreactor for a specific purpose is the shear effect on the fermentation. Certain cultures, such as plant and animal cells, can be extremely susceptible to shear damage and so require modified reactor designs. Bacterial cultures are less shear sensitive, with average laminar shear stresses that occur within the fermenter usually being insufficient to cause damage to most micro-organisms. However, the stresses formed by the turbulent break-up of the laminar jet from the agitator could cause shear damage to most organisms. Certain workers, such as Midler and Finn⁽¹⁰⁹⁾ (1966) and Tanaka, Mizugushi and Veda⁽¹¹⁰⁾ (1975) investigated the shear effects on micro-organisms and related shear damage, such as cell disruption and nucleotide leakage, to a function of impeller tip speed, ND_i , where N is the impeller rotational speed and D_i is the impeller diameter. It would therefore be interesting to determine the tip speed values for the New Brunswick and MBR fermenters at the various agitation rates used during the course of the fed-batch section of this project.

The volumetric transfer coefficient, K_La , of a fermenter is a measure of the aeration capacity of the vessel, with the greater the value of K_La , the higher the aeration capacity of the system. The degree of agitation assists oxygen transfer in a fermenter in many ways, including increasing the area available for oxygen transfer by dispersing the air in the culture fluid in the form of small bubbles, and delaying the escape of air bubbles from the system. The degree of agitation can be measured by the amount of power consumed in stirring the vessel contents. Several attempts have been made to correlate power consumption (and hence, agitation) with K_La values so that mathematical descriptions of the relationship can be used for predicting power requirements in design and scale-up exercises. It has generally been found that the K_La value is almost directly proportional to the power consumption per unit volume. Therefore, if the value of P/V is determined for each fermenter, where

P is the power exerted by the agitator and V is the volume of the fermenter, the resultant values can be said to be characteristic of the $K_L a$ value of the vessel. In other words, the higher the P/V value, the greater the aeration capacity of the fermenter. It should be noted here that these dextransucrase fermentations are carried out under unaerated conditions and so low values of P/V would be preferable.

There have also been various investigations into the relationship between power consumption and operating variables in baffled, agitated vessels using the technique of dimensional analysis. It was found that power absorption during the agitation of a Newtonian liquid could be represented by a dimensionless group known as the power number, defined as

$$Po = \frac{P}{\rho N^3 D^5} \quad 7.1$$

where

Po is the power number,

P is the external power from the agitator, kW,

ρ is the liquid density (taken as 1000 kg/m³), and

N and D have previously been defined.

The power number, therefore, is the ratio of external force exerted (P) to the inertial force imparted ($\rho N^3 D^5$) to the liquid. The power number can be regarded as a form of drag coefficient and, as is the case with most flows, the relationship can be divided into laminar and turbulent flow regimes. At sufficiently low Reynolds numbers (Re) - where

$$Re = \frac{ND^2\rho}{\mu} \quad 7.2$$

and μ is the liquid viscosity - the flow is laminar. The power number falls linearly with increasing Reynolds number. Therefore, low power numbers indicate high Reynolds numbers, leading to turbulent flow, with high power numbers corresponding to low Reynolds numbers and laminar flow.

The limiting factor of an aerobic fermentation is the transfer of oxygen to the cell surface and not the transfer of the gas into solution. It is also known that small impellers are superior to large impellers in transferring oxygen from the

gas phase to the microbial cells. Wang and Fewkes (111) (1977) found the critical dissolved oxygen concentration of a fermentation depended on the degree of agitation and the size of the impeller, agreeing that the limiting factor was the transfer of oxygen to the cell surface. These results were explained in terms of the impeller's ability to produce turbulent shear stress and pumping capacity. Turbulent shear stress is proportional to N^2D^2 and impeller pumping power is proportional to ND^3 . Thus, the ratio of impeller turbulent shear stress to impeller pumping is proportional to:-

$$\frac{N^2D^2}{ND^3} \text{ or } \frac{N}{D} (\text{cm. sec})^{-1} \quad 7.3$$

Wang and Fewkes demonstrated that the observed dissolved oxygen concentration decreased exponentially as the shear stress to pumping ratio increased. Therefore, an increase in the ratio of impeller shear stress to impeller pumping would decrease the transport resistance of oxygen to the cell surface resulting in a lower DOC value measured in the culture fluid. With unaerated dextransucrase fermentations therefore, the higher the value of N/D , the lower the DOC value and the greater the resultant dextransucrase activity achieved.

The results of the various parameters discussed above are given in Table 7.4 for the New Brunswick fermenter and the MBR fermenter at agitation rates of 450 and 700 rpm. The power input values for the New Brunswick and MBR fermenters were determined by measuring the current and voltage across the vessels. It should be stressed that these power measurements are only very approximate values and more specialised equipment such as strain gauges should be used to obtain more accurate results.

Table 7.4
Scale-Down Parameters for N.Br. and MBR Vessels.

Parameter	New Brunswick Vessel	MBR Vessel	
		450 rpm	700 rpm
Impeller Diameter D	0.0746 m	0.0572 m	0.0572 m
Agitation Rate N (rps)	1.667	7.5	11.667
Tip Speed (ND)	0.124	0.429	0.667
P (kW)	0.204	0.077	0.082
Po	19.1	0.30	0.08
N/D	22.3	131.1	204.0
P/V	0.034	0.074	0.086

The tip speeds of the two agitators at the different agitation rates are given in Table 7.4, from which it can be seen that the tip speed calculated for the New Brunswick fermenter, at an agitation rate of 100 rpm is considerably lower than those for the MBR fermenter. This means that there is less shear damage to the organism in the New Brunswick fermenter than in the MBR vessel. This agrees with the findings of improved results from the New Brunswick vessel. The tip speeds of the two agitation rates of the MBR vessel were very similar and would not result in any great variation in the degree of shear damage to the organism.

The P/V values of the agitators are also given in Table 7.4, with the value obtained for the New Brunswick vessel being slightly lower than the MBR P/V values. It is interesting to note that both the tip speed and the P/V values are greater for the MBR fermenter compared to the New Brunswick vessel, by factors of between 2 and 4. As already discussed, the P/V value is proportional to the aeration capacity of the fermenter, so that the MBR vessel is transferring a greater quantity of oxygen to the cells from the headspace gas by agitation alone, compared to the New Brunswick vessel and with the greater tip speeds of the MBR vessel, this all leads to increased oxygen transfer to the cells in the MBR fermenter, which should, theoretically, reduce the enzyme yield. These findings agree with the results from the dextransucrase fed-batch fermentations in the two vessels.

The power numbers calculated in each case are also shown in Table 7.4, with the value for the New Brunswick fermenter being higher than the values for the MBR vessel. It has already been stated that the higher the power number, the less turbulent the culture solution within the fermenter. It is possible that highly turbulent fermentation broths would result in some shear damage to the organisms, so that Po could be a similar scale-up parameter to tip speed. A high power number implies that the inertial force imparted to the liquid is reasonably low, resulting in reduced power-induced damage to the culture. This also corresponds to the increased enzyme activity and cell growth recorded in the New Brunswick vessel.

The N/D values for each fermenter at each required agitation rate are shown in Table 7.4. As previously mentioned, the higher the N/D value, the lower the DOC value of the fermentation and, hence, the greater the resultant enzyme activity. The calculated N/D values, however, are not as would be expected for these fermenters, with the New Brunswick N/D value being considerably lower than the MBR values. Therefore, the New Brunswick vessel actually allows a greater rate of oxygen transport to the cells than the MBR vessel, but, under conditions of little or no aeration, this parameter becomes relatively unimportant. However, this does imply that for an aerated fermentation the New Brunswick fermenter would produce improved results when compared to the MBR vessel.

These results show therefore that the New Brunswick fermenter can inherently produce improved results in the case of dextransucrase production when compared with the MBR fermenter. In fact, it would appear that it will not be possible to obtain the high yields of dextransucrase in the MBR fermenter that can be achieved in the New Brunswick fermenter.

The slightly improved results obtained in the MBR fermenter at the agitation rate of 700 rpm compared to 450 rpm can be attributed in the main to improved mixing (reduced mixing times) at the higher agitation rate as all the other parameters discussed with the scaling down of the process from the New Brunswick vessel were very similar at both agitation rates (see Table 7.4).

These parameters will also be used in the next section (7.3.7) concerning the scale-up of the process to the plant level, with the use of the Fisons plc 1,000 litre fermenter and the Birmingham University 800 litre vessel.

7.3.7. Industrial Scale Fermentations (Effects of Scale-up).

A number of attempts were made during the course of this research to scale-up the fed-batch process using fermenters with total volumes of 1,000 and 800 litres. The working volumes of these vessels were approximately 800 and 600 litres respectively, with the larger vessel being located at the Holmes Chapel site of Fisons plc, Pharmaceuticals Division, and the smaller vessel at the University of Birmingham, Department of Biochemical Engineering. The details of the fermentations are discussed in Section 6.4, with the results obtained from the first set of Dextranucrase Production Trials that were carried out at Holmes Chapel being recorded in Table 6.12. It can be seen that experiment HZ39T was extremely successful in allowing the production of approximately 442 DSU/cm³ (21.28 U/cm³) of dextranucrase, corresponding to an OD₅₉₀ value of 0.86. These results follow directly from HZ38T, which was disappointing with a maximum activity of 262 DSU/cm³ (12.61 U/cm³) and OD₅₉₀ value of 0.67 being achieved. During HZ38T, the cells grew in a linear rather than exponential manner with the stationary phase being reached at an unusually low OD₅₉₀ value. This profile was repeated during HZ39T, for no obvious reason as all of the parameters of the fermentation appeared to be at the correct control points. After 20.5 hours of experiment HZ39T, the fermentation broth was tested manually for pH and temperature and, although the pH was found to be adequately controlled between values of 6.6 and 6.8, the actual temperature of the fermentation was found to be approximately 6°C below the recorded value. The temperature was therefore adjusted in order to have an actual value of 23°C, with a recorded value of 29°C. This adjustment prompted an almost immediate boost in cell growth, along with the subsequent enhancement of enzyme production, until an extremely respectable activity of almost 450 DSU/cm³ (21.67 U/cm³) was achieved. Figures 6.18 a and b clearly show the profiles of these two fermentations, with the enhancement of cell growth and enzyme production being easily observed in Figure 6.18b. These fermentations show the importance of adequate temperature control at approximately 23°C for dextranucrase production. The optimum temperature for *Leuconostoc mesenteroides* growth is, in fact, 30°C, although at this point any dextranucrase produced will be very quickly degraded. The temperature of 23°C represents the point at which the cells have a reasonable growth rate and the dextranucrase formed is relatively stable. The enzyme produced from these fermentations was used to produce dextran in the 50,000 litre

fermenters, from which a considerable cost saving of approximately £637 per batch was achieved. The reduced costs (if any) of the resultant downstream processing of this dextran were not determined. Following this, four further large-scale fermentations were carried out, again on the 1,000 litre vessel at Holmes Chapel, with the aim being to consistently reproduce the results from HZ39T. The results from this series of fermentations are shown in Table 6.13. It can be seen that experiments 313/26 and 313/32 were both extremely unsuccessful, each yielding less than 50 DSU/cm³ (2.41 U/cm³) of enzyme. It was thought that the first fermentation (313/26) suffered from cell starvation early on in the process as the initial pH of the medium dropped from 7.40, after inoculation, to the low value of 6.53 before the pH control system allowed any sucrose to pass into the fermenter. With such a large pH drop it was feared that the cells had consumed all available sucrose and had been subsequently starved of the substrate for some time before there was any NaOH/sucrose addition. It is a known fact that the cells cannot resume any exponential growth following severe cell starvation, with limited linear growth being the best that can be achieved. Certainly 313/26 experienced this phenomenon with a maximum OD₅₉₀ value of 0.750 being recorded, following a prolonged period of linear cell growth - a factor which can be clearly observed in Figure 6.19. The pH control range during this fermentation, with values between 6.62 and 7.01 being observed during each addition of alkali, was also deemed to be unsatisfactory, especially as the pH value of 7.00 is the point at which a pH inversion occurs within the cells, leading to severe dextransucrase limitation. The pH control module was subsequently adjusted for the proceeding fermentations. Experiment 313/29 was carried out at a pH value between 6.59 and 6.73, with the initial medium pH having been adjusted to a value of approximately 7.0 before inoculation, so that there should be a shorter period of time before sucrose addition commenced than had been the case with 313/26. In this way, cell starvation was avoided, with the culture growing exponentially until an OD₅₉₀ value of 0.942 was reached. However, the maximum activity achieved was only 296 DSU/cm³ (14.25 U/cm³) - considerably less than that obtained from HZ39T. The temperature control throughout this fermentation had been poor, with values ranging from 21 to 27°C, a temperature high enough to allow denaturation of the enzyme. The process parameters during 313/32 were controlled much more accurately and yet the fermentation yielded an activity of only 42.4 DSU/cm³ (2.04 U/cm³) corresponding to an OD₅₉₀ value of 0.31. The reasons for this failed fermentation were by no means easy to determine,

although it was thought that there was some loss of viability of the culture. The inoculum used for the final trial - 313/35 - was supplied by Aston University instead of by the Microbiology Department of Fisons Pharmaceuticals plc., and was well grown at all transfer points. This fermentation gave good cell growth, with an OD₅₉₀ value of 0.960, but this corresponded to a poor enzyme yield of only 179.0 DSU/cm³ (8.62 U/cm³). It was finally decided that the Gistex batch was at fault and had degraded somehow - the differences in the compositions of various batches of Gistex already having been discussed in detail in Section 7.3.5. The Gistex batch used in these trials was Number 7-40-07, which was one of those extensively analysed for amino acid and carbohydrate compositions. A fed-batch fermentation was subsequently carried out in the New Brunswick vessel at Aston University, using Gistex batch 7-40-07, to determine whether the yeast extract was at fault during the plant trials. A corresponding fermentation was also carried out at Holmes Chapel and the results from these tests are shown in Table 7.3.7.5. It is clear that this Gistex batch was not capable of producing high yields of dextransucrase, although the claim by Fisons, that it was incapable of allowing good culture growth was not supported by the findings from Aston. It is clear, however, that the Gistex batch used is a critical component of any dextransucrase fermentation and as such, should be tested in the laboratory before use in any future large-scale trials.

Table 7.5.
Results of Fermentations carried Out with Gistex 7-40-07.

Parameter	Aston Trial (R35/26.5.88)	Fisons Trial
OD ₅₉₀	1.093	0.840
Activity (DSU/cm ³)	269.9	205.0
Sucrose Used (g)	567	-
Growth Rate (h ⁻¹)	0.28	-
Duration (h)	16	17.5

Following these industrial fermentations, an experiment was carried out using the 800 litre vessel at Birmingham University. This fermenter is described in Section 5.2.1.2 and was the most sophisticated and highly controlled piece of equipment used during the course of this project. The graphs shown in Appendix A represent very accurately the course of the entire fermentation, with Figures A.10 - A.13 showing the exit gas analyses that were continually analysed, in terms of Ar, N₂, CO₂ and O₂. However, despite the accurate controls and detailed representations of this fermentation, the final results achieved were extremely disappointing, being an activity of 133.2 DSU/cm³ (6.41 U/cm³) and OD₅₉₀ value of 0.722. The agitation rate chosen for this fermentation was similar to that used during the trials at Holmes Chapel. Unfortunately, it appeared that the agitation rate of 100 rpm did not provide adequate mixing throughout the Birmingham fermenter, indicated by the pH control range of 6.63 to 6.69. These values were thought to be satisfactory until the agitation rate was increased to 250 rpm, leading to a pH control range of 6.64 to 6.66. These figures indicated higher degrees of mixing than at an agitation rate of 100 rpm, although it was necessary to perform the various scale-up calculations to determine the precise mixing details.

The various parameters, such as tip speed, power number, P/V and N/D, that were calculated for the New Brunswick and MBR vessels were also determined for the Fisons plc 1,000 litre and Birmingham University 800 litre vessels. The results from these calculations are given in Table 7.6. The Fisons vessel was operated at an agitation rate of 140 rpm, whilst the Birmingham University vessel was operated at agitation rates of 100 and 250 rpm.

Table 7.6.
Scale-Up Parameters for Fisons and Birmingham Vessels.

Parameter	Fisons Vessel	Birmingham Vessel	
		100 rpm	250 rpm
Impeller Diameter, D	0.45 m	0.27 m	0.27 m
Agitation Rate N (rps)	2.33	1.667	4.167
Tip Speed (ND)	1.05	0.45	1.125
P (kW)	2.033	0.083	1.291
Po	0.009	0.012	0.012
N/D	5.18	6.17	15.43
P/V	2.54×10^{-3}	1.28×10^{-4}	1.99×10^{-3}

The tip speeds of the two agitators are given in Table 7.6, from which it can be seen that the Birmingham University vessel at the lower agitation rate of 100 rpm had the lowest tip speed. The tip speeds for the larger vessels are all considerably greater than those of the smaller vessels (see Table 7.4), but this is not unusual as larger vessels require greater tip speeds for good mixing. The 1,000 litre Fisons vessel would, therefore, need a greater agitator tip speed than the 800 litre BioLafitte Birmingham University vessel as it is 25% larger than the smaller vessel. However, the tip speeds of the vessels were all very similar and, as the 1,000 litre vessel was known to be capable of giving 450 DSU/cm³ (21.67 U/cm³) of enzyme, the tip speed at which it operated was not known to cause any shear damage to the organism. Therefore, the lower tip speeds of the slightly smaller 800 litre vessel were also be unlikely to cause any shear damage to the organism.

The P/V values of these vessels are also given in Table 7.6 and were all considerably lower than that of the New Brunswick fermenter which, of course, was capable of producing high enzyme yields. These values imply that the larger vessels could transfer even less oxygen to the cells from the headspace than could the New Brunswick vessel - a definite advantage with the unaerated dextransucrase production process. It was known that the Fisons vessel was capable of producing the optimum enzyme yields and therefore these figures imply that the Birmingham University fermenter could also, theoretically, produce high enzyme levels.

The power numbers calculated for the larger vessels are also shown in Table 7.6 and can be compared to the power numbers of the laboratory vessels shown in Table 7.4. The power numbers of the 1,000 and 800 litre vessels were considerably lower than those of the smaller vessels, in particular the Fisons vessel. It would appear, therefore, that the culture solution was in a turbulent condition in the 1,000 litre fermenter.

The Birmingham University vessel had very similar power numbers at the two agitation rates used during the fermentation, which were, in turn, significantly higher than the power number with the Holmes Chapel vessel. As already mentioned, the higher the power number, the less turbulent the fluid within the vessel, with the laboratory vessels having much greater power numbers. However, the Birmingham University vessel should provide less turbulent conditions than the Fisons vessel and hence cause less shear damage to the organism. These findings all agreed with the earlier statement, that the Birmingham University 800 litre vessel should be perfectly capable of producing the required high enzyme levels.

The N/D values are also given in Table 7.6 and are seen to be somewhat smaller than the N/D value of the New Brunswick vessel. These larger vessels would, therefore, allow a greater rate of oxygen transfer to the cells than would the laboratory vessels, resulting in increased enzyme production from aerated fermentations. It has already been stated that the N/D parameter is of less importance in unaerated compared to aerated fermenters, although it could be indicative of the mixing capabilities of the vessels.

The parameters determined for the larger vessel could be used to determine whether the 800 litre vessel was capable of producing enzyme yields of approximately 450 DSU/cm^3 (21.67 U/cm^3), with the knowledge that the Fisons vessel was definitely capable of producing these high enzyme levels. It would appear that the 800 litre vessel was indeed capable of successfully producing high enzyme yields, although an agitation rate of at least 250 rpm should be used to produce a sufficiently high tip speed and, hence, allow for sufficient mixing. It would be interesting to carry out a further large-scale fermentation in the Birmingham University vessel, at a higher agitation rate, to determine whether high enzyme yields could actually be obtained. In fact, for the 800 litre vessel to operate with a tip speed of 0.473π , the same as the

Fisons vessel, the agitation rate would have to be approximately 390 rpm. This high agitation rate should allow for improved mixing throughout the medium and, theoretically, high enzyme yields.

7.3.8. Effect of Temperature on Product Formation.

The effects of a low operating temperature on a dextransucrase fermentation have already been discussed during the preceding section, with the Plant Trials HZ38T and HZ39T suffering from low temperatures of between 17 and 18°C. A fermentation was then carried out in the New Brunswick vessel under standard conditions, with the single exception being that the operating temperature was controlled at 28°C. Experiment R38/9.6.88 yielded approximately 430 DSU/cm³ (20.70 U/cm³) of enzyme at an OD₅₉₀ value of 1.17. The most noticeable aspect of this fermentation was, however, the growth rate of the cells which was approximately 0.36 hr⁻¹. The increased temperature, as expected, caused a significant increase in cell growth rate, resulting in a high OD₅₉₀ value of 1.17. However, the activity achieved, although above 400 DSU/cm³ (19.26 U/cm³) was somewhat lower than could have been expected from such well grown cells. It would appear, therefore, that the increased temperature of 28°C, whilst being extremely beneficial to the cells, was responsible for a limited amount of degradation of the enzyme, resulting in a lower activity than would otherwise have been expected. This factor was not explored further, although it would have been interesting to have done so, particularly with respect to results obtained at, or above, 30°C, which is the optimum temperature for the growth of *Leuconostoc mesenteroides*.

8. RESULTS - CONTINUOUS FERMENTATIONS.

The continuous fermentations carried out in the course of this project were undertaken in two separate fermenters - the New Brunswick C30 Bioflo Model, and the MBR MiniBioreactor Model. These vessels are both described in detail in Section 5.3 of this thesis, along with inoculum preparation procedures, media formulation and sterilization techniques. The results obtained from these fermentations will, therefore, also be given in two sections, but will concentrate mainly on the results achieved with the superior, more accurately controlled MBR fermenter.

8.1. Results Obtained with the New Brunswick Bioflo C30 Model.

This fermenter was loaned to the author by Dr. P Lambert of the Department of Pharmaceutical Sciences, Aston University. It had a total volume of 1 litre, but due to the fixed position of the overflow weir, it had a continuous fermentation operating volume of 338 cm³. Three experiments were carried out using this fermenter, with the purpose being to undertake a few preliminary investigations into the optimum method of operating a continuous fermentation. The first fermentation carried out was experiment CR12/14.12.87, containing an initial medium of 10 g/l sucrose, 20 g/l K₂HPO₄ and 40 g/l Gistex yeast extract, as with fed-batch fermentations. The fermentation was operated as a fed-batch, with the pH being controlled at a value of 6.65, until an enzyme activity of 411 DSU/cm³ (19.79 U/cm³) was achieved. At this point, the feed medium containing 20 g/l sucrose and similar levels of K₂HPO₄ and yeast extract as in the initial medium was fed through to the vessel via a peristaltic pump, with the overflow weir being opened to allow steady-state conditions to occur. The fermenter was operated for a total time of 72 hours, from inoculation, with the activity values being recorded up to 46.5 hours, as Figure 8.1 shows. This time, at about 38 hours from inoculation, where the feed was started to the fermenter, can be clearly seen on both the OD₅₉₀ and activity profiles from this fermentation as the peak values reached immediately begin to decline. The condition of steady state was, in fact, never achieved, as at some point during the period between 46.5 and 72 hours the feed tube leading to the fermenter became blocked and the experiment was aborted. This fermentation did, however, indicate that the best method for operation was not to switch to the continuous mode at levels of high enzyme activity as such high levels of growth cannot be supported and

the activity drops to a value below 50 DSU/cm³ (2.4 U/cm³), but to commence continuous operation as the cells enter their exponential growth phase.

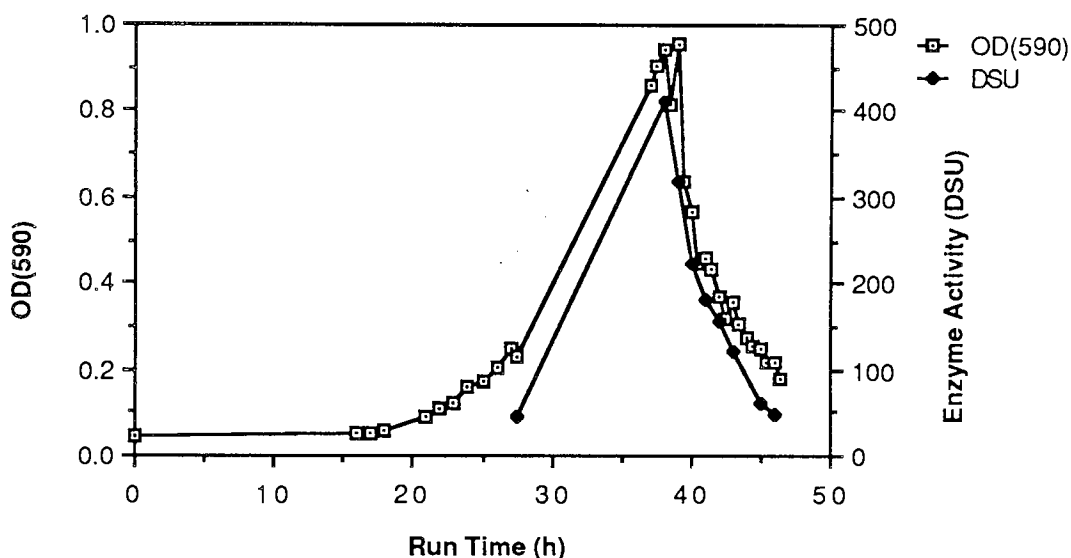


Figure 8.1: OD(590) and Activity Profiles from CR12

The following fermentations, CR13/11.1.88 and CR17/2.2.88, that were carried out followed the recommendations made after experiment CR12/14.12.87. Experiment CR13/11.1.88 was operated as a fed-batch with a 10 g/l sucrose medium for 12.5 hours until a 10 g/l sucrose feed was commenced, at a dilution rate of 0.2 h⁻¹. The activity at this time was 13.6 DSU/cm³ (0.65 U/cm³) with an OD₅₉₀ value of 0.142 and, although these values increased slightly to 22.6 DSU/cm³ (1.09 U/cm³) and 0.169, they decreased to levels of approximately 15.1 DSU/cm³ (0.73 U/cm³) and 0.145 respectively. The dilution rate was then increased to a value of 0.25 h⁻¹ which resulted in a drop in activity to a level of 6.3 DSU/cm³ (0.30 U/cm³) and OD₅₉₀ value of 0.103. This indicated that washout was occurring (a situation where the dilution rate is greater than the maximum growth rate of the cells) and so the feed was changed to medium containing 20 g/l sucrose. The fermentation was operated at the same dilution rate of 0.25 h⁻¹, resulting in an activity of approximately 9.6 DSU/cm³ (0.46 U/cm³) and OD₅₉₀ value of 0.135. An increase in the dilution rate to 0.3 h⁻¹ corresponded with an activity increase to 11.7 DSU/cm³ (0.56 U/cm³) and OD₅₉₀ increase to 0.141, with a further dilution rate increase to 0.33 h⁻¹ causing the activity to increase to 13.5 DSU/cm³ (0.65 U/cm³) and OD₅₉₀ to 0.172. A subsequent increase in

dilution rate to 0.36 h^{-1} resulted in still higher results of 14.9 DSU/cm^3 (0.72 U/cm^3) activity and OD_{590} growth value of 0.226 being recorded. Raising the dilution rate still further to a value of 0.38 h^{-1} resulted in cell washout, and the fermentation was terminated after 112 hours of operation. Figure 8.2 shows the overall OD_{590} and activity profiles of the fermentation whilst Figure 8.3 shows the effect that the dilution rate has on these two parameters with a medium feed containing 20 g/l sucrose. The steady-state data recorded from this experiment are shown in Table 8.1, along with the steady-state results obtained from CR17/2.2.88, which was the final fermentation carried out in the New Brunswick fermenter. Experiment CR17/2.2.88 was operated in a similar manner to CR13/11.1.88, in that the medium feed was commenced when the cells were entering the exponential stage of growth. The feed for this fermentation contained 20 g/l of sucrose throughout the experiment. The results obtained from this experiment did not appear to be as consistent as those from CR13/11.1.88 as higher OD_{590} and activity values were achieved from lower dilution rates, with the best results coming from a dilution rate of only 0.20 h^{-1} . This experiment did, however, confirm the washout dilution rate determined in the previous experiment, of 0.37 h^{-1} (0.38 h^{-1} in CR13).

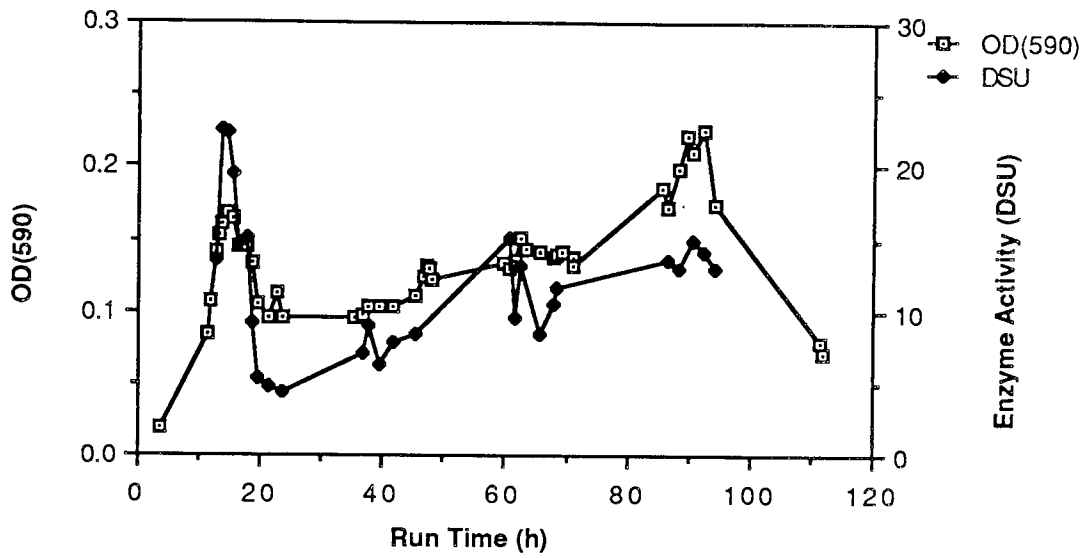


Figure 8.2: OD(590) and Activity Profiles from CR13

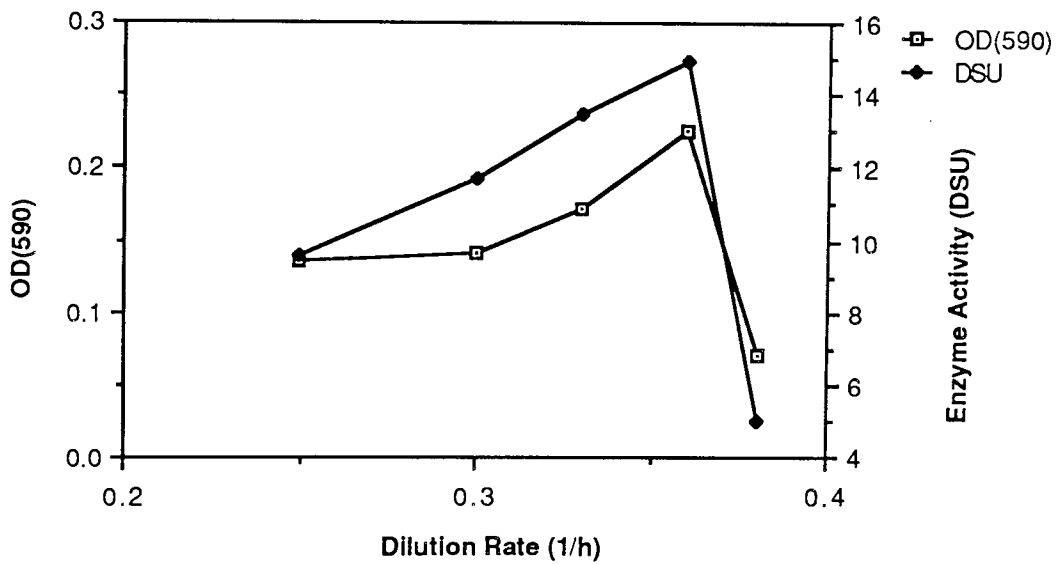


Figure 8.3: Dilution Rate Profiles from CR13

Table 8.1.
Results Obtained from Continuous Fermentations in the New Brunswick Bioflo Fermenter.

Run Number	OD ₅₉₀ Value	Activity (DSU/cm ³)	Dilution Rate (h ⁻¹)	Duration (h)
CR13/11.1.88	0.135	9.6	0.25	16
	0.141	11.7	0.3	6.5
	0.172	13.5	0.33	17
	0.226	14.9	0.36	7.5
	0.071	-	0.38 *	18
Total Fermentation Time = 112 hours.				
CR17/2.2.88	0.178	15.8	0.19	10
	0.250	24.3	0.20	15
	0.216	14.2	0.23	9.5
	0.233	17.2	0.28	21
	0.227	-	0.31	21.5
	-	-	0.37 *	23
Total Fermentation Time = 138 hours.				

(*) Cell washout occurred.

These were the results obtained from the continuous operation of the BioFlo fermenter. They were only carried out as preliminary experiments whilst the purchase of a new, more sophisticated and more highly controlled fermenter was being arranged.

8.2 Results Obtained with the MBR MiniBioreactor Model.

During the summer months of 1988, a fermenter was ordered from, and delivered by Sulzer (U.K.) Ltd. for use in continuous fermentations. The vessel had a total volume of 2.5 litres and contained an adjustable overflow weir which enabled it to be operated at several different working volumes. It was generally run at the lowest possible level which allowed complete immersion of the pH probe as the lower the working volume, the less the feed medium required to maintain a particular dilution rate ($D = \text{flow rate}/\text{working}$

volume). A detailed description of the fermenter is given in Section 5.3.2, with inoculum development stages, media formulation and sterilization described in subsequent sub-sections of Section 5.3.

8.2.1. Commissioning Experiments.

Following the purchase of the fermenter, a number of preliminary, commissioning fermentations were carried out in order to gain familiarity with the equipment. The major problems encountered are discussed below, although they were quickly overcome and did not occur again.

The first experiment carried out with the MBR vessel was CR48/1.1.88 which was, in many respects, a repeat of CR13 and CR17, in that, while the fermenter was operated as a batch culture, the initial medium contained 10 g/l and the feed medium contained 20 g/l sucrose. The agitation rate during the fermentation was controlled at 450 rpm which meant that the working volume was approximately 1,150 cm³. The first main problem that occurred was leakage from the overflow weir. The base of the overflow tube that is inserted into the bottom plate of the vessel contains two small holes that were designed to allow complete and thorough mixing of the medium during the sterilization cycle. A specific hyperdermic needle had been supplied by the manufacturers that would fit into the overflow tube and seal the holes, hence preventing leakage. Unfortunately, this fact was not realised initially, and so the incorrect hyperdermic needle was used for the output from the fermenter which, as it was too narrow, did not entirely seal the holes, leading to leakage of the culture fluid from the fermenter. Once this situation was realised, the correct needle was clearly marked, and the situation did not occur again. The experiment was operated for 44.75 hours without contamination, although, because of the leakage problems, steady-state could not be said to have occurred. Following this experiment, CR49/7.11.88 was carried out in an attempt to reach steady-state conditions and maintain them for a period of time. The initial medium for this fermentation contained 20 g/l sucrose, as did all the feed medium throughout. The agitation rate chosen for the experiment was 500 rpm, leading to a working volume of 1,035 cm³. In one case, a bottle of feed medium was connected to the fermenter by means of sterilized polyvinyl chloride tubing, with the tube being primed to ensure that it was capable of passing the required dilution rate into the vessel. However, overnight this tubing became distorted, as a result of the sterilization, and was

blocked, so that there was no feed to the fermenter overnight. It was realised from this that p.v.c. was not the correct material to use for tubing, and in all the following fermentations silicone rubber tubing was used, which did not warp at high temperatures. As there was a prolonged period without any feed, it was thought that the cells were starved of sucrose at these times, although HPLC analysis indicated that despite the fact that sucrose levels within the broth were low, there was still some residual sucrose in the medium. However, although there was good growth within the fermenter - as indicated by OD₅₉₀ values of approximately 0.275, the enzyme activity levels remained low - at values between 8 - 12 DSU/cm³ (0.39 - 0.58 U/cm³). It is probable that, in this case, the cells consumed most of the substrate for growth and maintenance purposes, and little for product formation. However, this fermentation proved to be quite successful as a commissioning run as, although steady state was not achieved, the experiment was operated continuously for 70.75 hours, without any contamination occurring and a great deal of experience was gained in operating the equipment. These two fermentations were the main commissioning runs that were carried out, and although some minor errors were made in the subsequent runs, no further major problems occurred.

8.2.2. Results Obtained with Controlled Sterilization of the Feed Medium.

During the first few continuous fermentations that were carried out, the medium was formulated and then sterilized in the Adelphi autoclave to a temperature of 121 - 125°C with a holding time of 20 minutes. This was a long process, taking approximately 2 hours from commencement of the sterilization cycle to the point where the full aspirator bottle had cooled down sufficiently to be removed from the autoclave, although even from this point, the medium remained quite hot for several hours as the interior cooled down to room temperature. This time period was due mainly to the fact that, as there was no mixing in the autoclave, the large volume (5 litres) of liquid was heated by the high temperatures slowly penetrating to the centre of the medium, with only minimum circulation occurring. This meant that the outer portions of the medium were at high temperatures for a much longer time during heating and that, as the inner portions of the medium took much longer to cool down than the outer portions, the centre of the medium was also at high temperatures for a very long time during cooling. There was no way that the heating or cooling periods could be reduced in the autoclave, so it was

decided to reduce the medium holding time from 20 to 10 minutes, to prevent any over-sterilization of the medium. It was thought that, should this be the case, then degradation of the yeast extract would occur, which may destroy some vital amino-acid or vitamin components in the medium, leading to reduced cell growth and/or product formation. Medium degradation can be detected visibly as having occurred in medium that has become a dark brown, or even black colour following sterilization. A holding time of 10 minutes in the autoclave resulted in a sterile medium of a golden brown colour. It was also important that decreasing the sterilization temperature allowed continued sterility in the medium which was the case with a holding time of 10 minutes, but reducing this period still further resulted in a feed medium that quickly became contaminated.

Experiments CR50/14.11.88 and CR51/21.11.88 were both carried out using a batch of Gistex in the medium that had been used for fed-batch fermentations over the previous year and was by then nearing the end of its shell life. (Gistex yeast extract should be used for approximately 12 months, provided it has been stored in a cold environment, such as a refrigerator). These two experiments used as the feed, medium that had been sterilized in the autoclave for 20 minutes at 121 - 125°C and, as already mentioned, it is likely that this medium did not contain all the necessary nutrients for effective dextransucrase secretion by the organism. Experiment CR50/14.11.88 was operated at a dilution rate of 0.2 h^{-1} throughout, but was fed with medium containing 20, 30 and 35 g/l sucrose, with the best results being recorded with the 30 g/l sucrose feed that is, a steady state activity of 12.7 DSU/cm^3 (0.61 U/cm^3), with a residual sucrose concentration of 0.099% w/v in the medium. (OD_{590} values were not available during this fermentation). Following this, the 30 g/l. sucrose feed was used, from which R^* salts had been accidentally omitted, and so there was very little growth or enzyme production taking place. The use of a 35 g/l sucrose feed failed to boost cell growth and the fermentation was terminated after 73.75 hours, with no incidence of contamination. Experiment CR51/21.11.88 was carried out with feed medium containing two levels of sucrose - 30 and 40 g/l. The steady-state results obtained from this, and the previous fermentation are shown in Table 8.2

Table 8.2.

Results Obtained from Continuous Fermentations in the MBR MiniBioreactor.

Run Number	OD ₅₉₀ Value	Activity (DSU/cm ³)	Residual Sucrose (% w/v)	Dilution Rate (h ⁻¹)	Duration (h)
CR50/14.11.88	-	12.7	0.099	0.2	20
Duration of total fermentation = 73.75 hours.					
CR51/21/11.88 (30 g/l Sucrose)	0.144	9.7	0.915	0.19	20
	0.206	10.5	-	0.26	5
(40g/l Sucrose)	0.249	10.1	-	0.26	16.25
	0.163	8.2	-	0.29	6.25
	0.175	3.7	-	0.32 *	16.75

Duration of total fermentation = 74 hours.

* Cell washout occurred.

Experiment CR51/21.11.88 produced several different incidences of steady-state, although the results obtained were not very consistent in that cell concentration and enzyme activity did not increase with increasing dilution rate. However, the results from these fermentations were all very poor and can be attributed to the Gistex and sterilization problems mentioned earlier.

Experiment CR54/12.12.88 was carried out with a new batch of Gistex yeast extract, with feed medium containing 30 g/l sucrose throughout. The medium was initially sterilized in the autoclave at 121 - 125°C for 20 minutes and at dilution rates of 0.25 and 0.27 h⁻¹ produced the steady-state results shown in Table 8.3. Following this, the sterilization cycle holding period was

continually monitored to ensure that the temperature was maintained between 115 and 119°C and the dilution rate was increased from 0.25 - 0.29 h⁻¹. This fermentation yielded a maximum activity of 28.8 DSU/cm³ (1.39 U/cm³) at a dilution rate of 0.27 h⁻¹, which was a considerable increase from the results obtained up to then. This boost in enzyme activity was most probably due to the fact that the sterilization cycle of the medium in the autoclave was more carefully controlled, where the maximum temperature did not exceed 119°C, although the holding time was still 20 minutes and the medium remained hot for many hours. Experiment CR56/9.1.89 was carried out in a similar manner to CR54/12.12.88 in that all the medium contained 30 g/l sucrose and all the feed was sterilized in the autoclave for 20 minutes between the temperatures of 116 - 119°C. The results from this experiment are shown in Table 8.3, along with those from CR54/12.12.88. These results show a maximum activity of 33.0 DSU/cm³ (15.89 U/cm³) obtained at a dilution rate of 0.21 h⁻¹, with the enzyme yield dropping slightly at increased dilution rates and cell washout occurring at a dilution rate of 0.3 h⁻¹. Figure 8.4 shows the combined OD₅₉₀ and activity results obtained from experiments CR54 and CR56 plotted against dilution rate, showing clearly the point at which cell washout occurs - this being at the point where the cells are growing at the maximum growth rate (μ_{\max}) possible under the particular conditions. These results do not appear to be very consistent, but as the enzyme activity values are so low, any discrepancies can easily be accounted for as having occurred through analytical error.

Table 8.3.
Results Obtained from Continuous Fermentations with Medium Prepared by Controlled Sterilization.

Run Number	Sucrose Concentration in Medium (g/l)	OD ₅₉₀ Value	Enzyme Activity (DSU/cm ³ /h)	Residual Sucrose (g/l)	Dilution Rate (h ⁻¹)	Duration of Dilution Rate (h)
CR54/12.12.88	30	0.209	27.4	5.3	0.25	16.75
		0.217	28.8	-	0.27	6
		-	18.9	2.83	0.29	24.75
Total Duration of Fermentation = 80 hours.						
CR56/9.1.89	30	0.151	32.2	4.0	0.17	18.75
		0.170	33.0	2.8	0.21	5.25
		0.170	20.8	3.0	0.27	4.5
		-	-	-	0.3 *	16.5

Total Duration of Fermentation = 72 hours.

* Cell Washout Occurred.

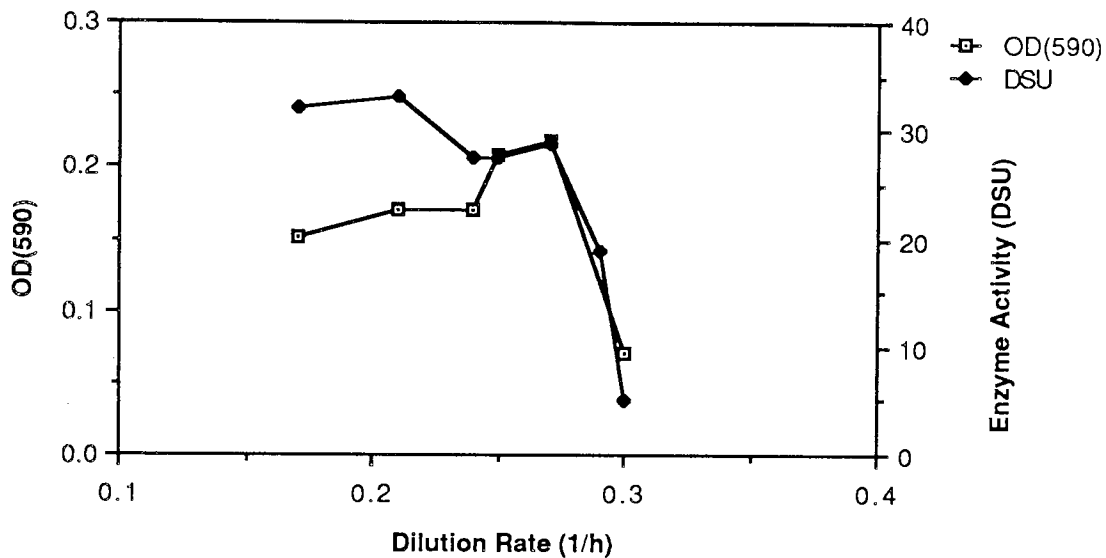


Figure 8.4: Dilution Rate Profiles from CR54 and CR56

Following these fermentations, experiment CR57/16.1.89 was carried out where the 30 g/l sucrose feed medium was again sterilized in the autoclave at 116 - 119°C for 20 minutes. This fermentation resulted in the highest activity so far achieved continuously of 37.8 DSU/cm³ (1.82 U/cm³) at a dilution rate of 0.25 h⁻¹, but after approximately 48 hours of the fermentation what appeared to be a contaminant organism was observed in the culture fluid. Attempts were made over the subsequent 26 hours to displace the contaminant with the hope that the *Leuconostoc mesenteroides* cells would outgrow it, but this did not occur, and after 74 hours of the experiment, no enzyme activity could be detected and the only growth recorded was that of the contaminant organism. This again emphasizes the importance of maintaining sterile conditions at all times and in taking great care with aseptic techniques, in particular the changing of feed bottles.

Table 8.4 shows the results obtained from the next group of continuous fermentations. With the exception of CR58/23.1.89, the medium in all the fermentations contained 35 g/l sucrose which had been sterilized in the autoclave at 116 - 119°C for 10 minutes in order to reduce any incidence of medium degradation which may have occurred as has already been discussed. Medium sterilized in this manner maintained its sterility for several days, although when attempts were made to reduce the total sterilization time still further by allowing no holding time whatsoever at 116°C, the medium

prepared did not remain sterile for very long, becoming contaminated after a few hours. It was therefore decided that a holding time of 10 minutes was sufficient to maintain sterility of the feed medium.

The data given in Table 8.4 shows that, in general, the results obtained from the continuous fermentation of *Leuconostoc mesenteroides* were improving, with activities in excess of 50 DSU/cm³ (2.4 U/cm³) being achieved on several occasions. This was still below the maximum activity achieved to date in the literature, of 70 DSU/cm³, (3.37 U/cm³), by Paul *et al* (19), but was nevertheless an increase on previous results.

Experiment CR58/23.1.89 was carried out at one dilution rate for the duration of the entire experiment. This resulted in the maximum enzyme activity so far measured under true steady-state conditions. Steady-state can be said to have occurred as the fermentation was operated continuously for 61 hours without any problems of leakage, contamination or cell starvation due to the depletion of the feed medium. The pH was also controlled accurately at a value of 6.65, the temperature maintained at 23.4°C and the agitation rate at 350 rpm. This was, to date, the most successful fermentation, in terms of both execution and enzyme yield.

Experiment CR/59.1.89 was carried out at two dilution rates of 0.27 and 0.3 h⁻¹ and improved on the results obtained in CR58/23.1.89 by approximately 25%. This can be immediately attributed to the fact that the feed medium contained of 35 g/l. sucrose, thus encouraging improved growth. However, despite the fact that 50.7 DSU/cm³ (2.44 U/cm³) was achieved with a dilution rate of 0.27 h⁻¹, increasing the dilution rate to a value of 0.3 h⁻¹ resulted in a decrease in cell growth - as indicated by the OD₅₉₀ value - and dextransucrase activity. This was unexpected and cannot be satisfactorily explained except to suggest that the medium feed rate was approaching the critical dilution rate at which washout was occurring.

Experiment CR60/6.2.89 was carried out at two dilution rates of 0.19 and 0.3 h⁻¹ and at the higher feed rate. An activity of 61.8 DSU/cm³ (2.98 U/cm³) was achieved, improving on the previous maximum results by approximately 20%. The HPLC analysis revealed only low levels of residual sucrose in the medium, showing that most of the substrate supplied to the cells was being utilized for either cell growth, maintenance or product formation. A problem

Table 8.4
Results Obtained from Further Continuous Fermentations.

Run Number	Sucrose Concentration in Medium (g/l)	OD ₅₉₀ Value	Enzyme Activity (DSU/cm ³ /h)	Residual Sucrose (g/l)	Dilution Rate (h ⁻¹)	Duration of Dilution Rate (h)	Duration of Fermentation (h)
CR58/23.1.89	30	0.265	41.0	2.7	0.27	61	74.75
CR59/30.1.89	35	0.350	50.7	1.2	0.27	49	
CR60/6.2.89	35	0.224	25.6	0	0.3	20	73.75
		0.282	30.0	0	0.19	25	
		0.295	61.8	1.5	0.3	48	89
CR61/13.2.89	35	0.315	49.0	1.14	0.32	18	75.5
CR62/20.2.89	35	0.187	39.3	10.2	0.32	18.5	
		0.160	26.8	15.7	0.28	15.5	
		0.234	24.1	15.3	0.28	15.5	74

that became evident with this fermentation was the slight pH increase that was occurring at high dilution rates. This was due to the fact that the cells were not growing at a sufficiently high growth rate to produce enough acid that would neutralise the alkaline medium which was being pumped into the vessel at a high rate. An increase in pH will not adversely affect the growth of *Leuconostoc mesenteroides*, but, as was discussed in Section 2.3.2, should the pH reach the dangerous neutral value of 7.0, an inversion in the pH gradient will occur, with the cell interior becoming acidic with respect to the exterior, resulting in a drastic reduction in dextransucrase secretion. This means that the incidence of the fermentation pH increasing at high dilution rates is a problem that should be resolved as soon as possible.

Experiment CR61/13.2.89 suffered from a pH rise to a value of 6.89 as it was operated at a high dilution rate of 0.32 h^{-1} . It was decided that, apart from using acid as a pH controlling fluid, the only method of reducing the pH and the high levels of sucrose that were building up within the fermenter (of 13 g/l) was to halt the feed to the vessel and allow the cells to grow batchwise for a time until the sucrose level in the fermenter had dropped, and the pH value had returned to the control point of 6.65. Unfortunately, during the course of this experiment, contamination occurred and the dextransucrase activity began to decrease. An attempt was made to remove the contaminant by returning the fermentation to the continuous mode, but this proved unsuccessful. The fermentation was terminated after 75.5 hours.

Experiment CR62/20.2.89 was operated continuously at dilution rates between 0.28 and 0.32 h^{-1} , but here too, the pH began to increase to values exceeding 6.80. The cells were also not growing quickly as there were unusually high levels of residual sucrose within the fermenter. Following a period of continuous operation, the feed to the vessel was terminated for a while to allow the cells to grow batchwise in order to utilize the residual sucrose and for the pH to decrease to a value of 6.65. Once this had been achieved, the feed was recommenced with a dilution rate of 0.28 h^{-1} . However, the pH slowly increased again and the fermentation was run batchwise once more to utilize the available sucrose and bring the pH back to the desired value. This fermentation was terminated after 74 hours, with no contamination having occurred, and with the problem of the increasing pH at high dilution rates still remaining to be resolved. The on-line readings from

this fermentation were also recorded and can in a way provide a picture of the overall fermentation process. Figure 8.5 shows the pH and dissolved oxygen concentration (DOC) profiles recorded throughout the fermentation, with the pH increases easily being distinguished, and the periods of batch growth being seen by an increase in the DOC value.

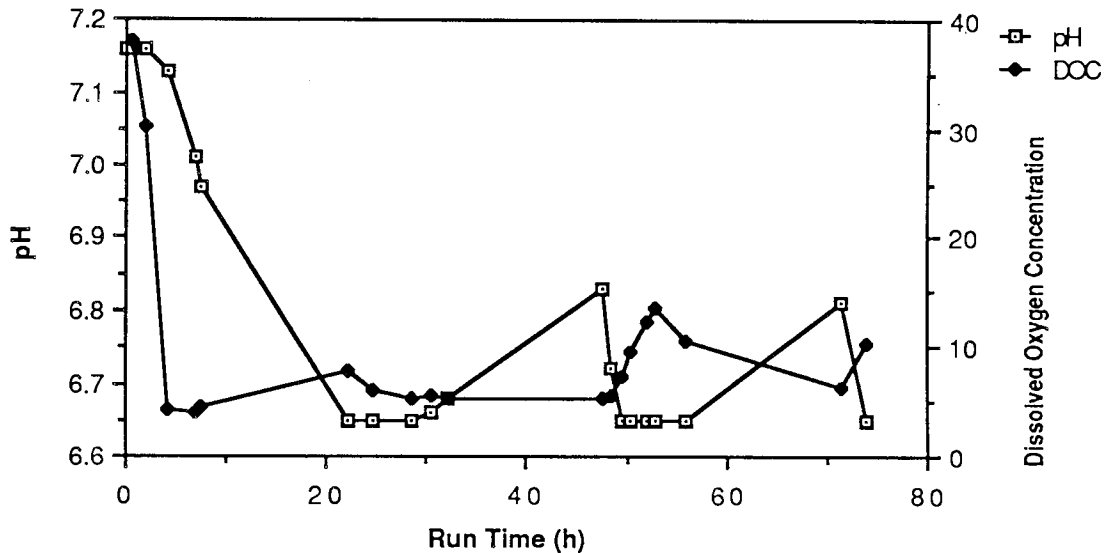


Figure 8.5: pH and DOC Profiles from CR62

Table 8.5 gives the results obtained from further continuous fermentations. The feed medium for all these fermentations was still being sterilized in the autoclave at 116 - 119°C for 10 minutes.

Experiment CR63/27.2.89 had a total duration of 264.25 hours, being the longest duration of any fermentation carried out to date, especially as this experiment was contaminant free. This fermentation suffered from similar problems to certain previous ones, with the pH of the medium increasing over a period of time at a high dilution rate and the residual sucrose in the medium not being consumed by the cells, leading to a build-up of substrate in the broth. This sucrose was again cleared by running the fermentation batchwise for a few hours, but whilst this was successful in reducing both the medium pH and the sucrose concentration in the broth, it did not allow for very long periods of steady-state. The maximum steady-state activity recorded during this section of the fermentation was 34.9 DSU/cm³ (1.68 U/cm³) at a slightly lower dilution rate of 0.31 h⁻¹. Following this period, the medium composition

was altered slightly - from containing 35 g/l sucrose, 40 g/l Gistex and 20 g/l K_2HPO_4 , it was changed to 35 g/l sucrose, 40 g/l Gistex and 20 g/l KH_2PO_4 . Potassium dihydrogen phosphate (KH_2PO_4) contains, as the name suggests, two hydrogen ions [H^+] compared to only one in dipotassium hydrogen phosphate (K_2HPO_4). As the concentration of H^+ ions in solution is directly related to the pH of that solution - the higher the value of [H^+], the more acidic the solution and the lower the pH. The medium containing 20 g/l KH_2PO_4 had a lower pH than the medium containing 20 g/l K_2HPO_4 , and as a result the problem of increased medium pH at high dilution rates did not occur as the pH of the incoming medium was already at a lower value than the set point of the broth and required the addition of alkali to restore the medium to the required pH. This fermentation was then continued with the newly formulated medium, at a constant dilution rate of $0.31\ h^{-1}$, resulting in a substantial increase in activity from $18.5\ DSU/cm^3$ ($0.89\ U/cm^3$) to $48.5\ DSU/cm^3$ ($2.33\ U/cm^3$). An increase in dilution rate to $0.34\ h^{-1}$ caused a further enhancement of activity, to $56.5\ DSU/cm^3$ ($2.72\ U/cm^3$) but at a slightly higher dilution rate of $0.35\ h^{-1}$, the cells utilized all the available substrate, as detected by the HPLC analysis of the residual sucrose, implying that the cells were growing at their maximum rate (μ_{max}), which was, in turn, equal to the critical dilution rate (D_{crit}) at which cell washout occurs. This can be observed from Table 8.5 as both OD_{590} value and enzyme activity decreased slightly at the dilution rate of $0.35\ h^{-1}$, implying that this point was very close to D_{crit} where cell washout occurred.

Experiment CR64/13.3.89 proved to be the most successful continuous fermentation carried out to date, surpassing the value of $70\ DSU/cm^3$ ($3.37\ U/cm^3$) achieved by Paul *et al* (19) in 1983. This experiment had a duration of 144 hours, with no contamination occurring, although by the end of the fermentation, the output tube from the vessel had become blocked, possibly due to the growth of cells from the output reservoir back up the output tube. However, until this point the fermentation had been operated continuously for over 111 hours and had reached periods of steady-state at dilution rates of 0.32 and $0.33\ h^{-1}$. The enzyme activity achieved was identical for both these flow rates which is not surprising as they were very similar. The OD_{590} values and residual sucrose concentrations recorded were also very consistent

Table 8.5
Results Obtained from Continuous Fermentations.

Run Number	Sucrose Concentration in Medium (g/l)	OD ₅₉₀ Value	Enzyme Activity (DSU/cm ³ /h)	Residual Sucrose (g/l)	Dilution Rate (h ⁻¹)	Duration of Dilution Rate (h)	Duration of Fermentation (h)
CR63/27.2.89	35	0.198	34.9	18.5	0.33	20.5	
		0.140	20.3	27.0	0.32	16.75	
		0.175	18.6	22.8	0.31	17.25	
		0.257	48.5	2.2	0.31	25	
		0.297	50.2	-	0.32	4.5	
		0.329	56.5	6.0	0.34	46.5	
		0.283	47.8	0	0.35	65.5	264.25
CR64/13.3.89	35	0.355	75.8	2.7	0.32	21.5	
		0.364	75.8	3.4	0.33	91	144
CR68/10.4.89	35	0.242	39.9	6.9	0.33	20	
		0.152	43.0	14.5	0.33	25.25	
		0.218	45.8	0	0.33	24.75	
		0.120	-	23.3	0.33	50	173.5

with each other, proving that steady-state conditions had indeed been attained. Figure 8.6 shows the pH and dissolved oxygen concentration profiles recorded during this fermentation.

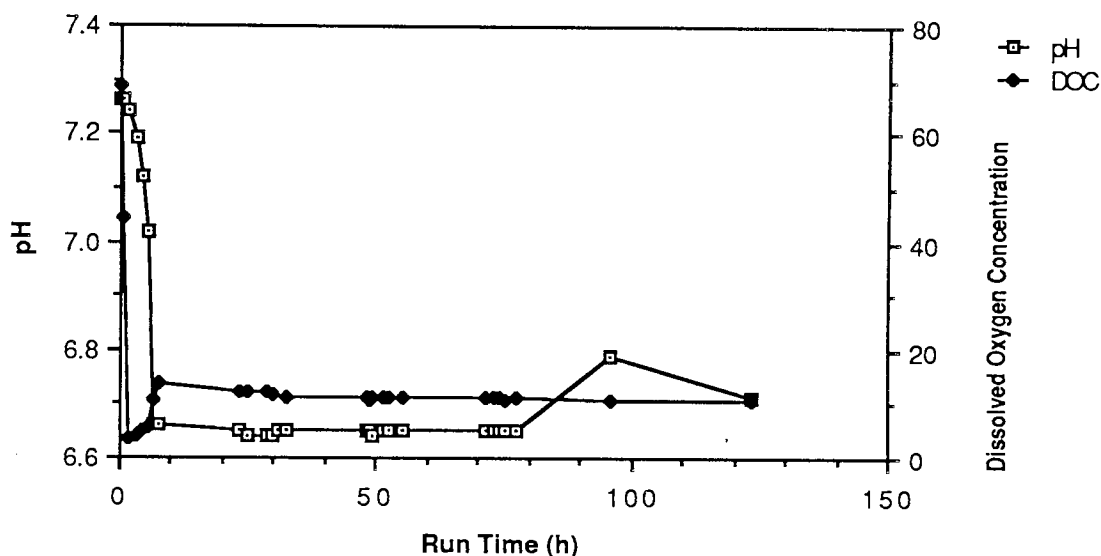


Figure 8.6: pH and DOC Profiles from CR64

Experiment CR68/10.4.89 was also operated for quite a long period of time (173.5 hours) without contamination. The medium for this fermentation was prepared with a new batch of Gistex yeast extract (batch number 8-36-09) along with 20 g/l of the alkaline K_2HPO_4 . At the dilution rate of 0.33 h^{-1} the medium pH soon began to increase to a value of 6.93 and the residual sucrose level in the culture fluid increased to a level of 14.5 g/l. The activity recorded at this point was approximately 43.0 DSU/cm^3 (2.07 U/cm^3) at steady state - with an OD_{590} value of 0.152. The feed medium composition was then changed slightly, with 5 cm^3 concentrated HCl being added to the medium before sterilization to reduce the pH of the medium. This did not appear to greatly affect the fermentation, with the activity remaining approximately constant at 45.8 DSU/cm^3 (2.21 U/cm^3), although the cell growth increased slightly. However, the pH soon began to slowly increase in value until finally a medium was prepared which contained 35 g/l sucrose, 40 g/l Gistex, 10 g/l K_2HPO_4 and 10 g/l KH_2PO_4 . The addition of this feed medium allowed the fermentation pH to remain at the set point, although it was not possible to enhance the cell growth or dextranucrase production rates, with OD_{590} values of approximately 0.116 being recorded. It is known

that once problems occur with a dextransucrase fermentation, they are very difficult to reverse as the cells often begin to utilize a different substrate (eg. fructose, mannitol) and the metabolism of the cells changes to such an extent that it cannot be altered. In such cases, the best form of action is to terminate the fermentation and commence a new one, using the information gained from the previous experiment.

Table 8.6 gives the results obtained from fermentations carried out with feed medium containing 10 g/l K_2HPO_4 and 10 g/l KH_2PO_4 , as in CR68/10.4.89.

Experiment CR69/24.4.89 again exceeds the results obtained by Paul *et al* (19) in surpassing their value of 70 DSU/cm³ (3.37 U/cm³). This fermentation used a slightly higher dilution rate of 0.35 h⁻¹ than the value of 0.33 h⁻¹ used in CR64/13.3.89, a rate at which cell washout was observed to be beginning in CR63/27.2.89. However, the different medium composition could well account for the apparent increase in the critical dilution rate observed from this fermentation. This experiment was successfully controlled, with the medium pH maintained at 6.65 ± 0.03 , the temperature at $23.4 \pm 0.1^\circ\text{C}$ and the agitation rate at 450 ± 10 rpm

Experiment CR70/8.5.89 produced slightly lower results at a slightly higher dilution rate in terms of both OD₅₉₀ value and enzyme activity. This could be due to the fact that the feed rate of 0.37 h⁻¹ was approaching the critical dilution rate, or that fluctuations in the pump flow rate, which must invariably occur, were causing slight variations in the dilution rate, resulting in it increasing slightly. However, the results obtained still showed an activity of approaching 70 DSU/cm³ (3.37 U/cm³) and the fermentations overall were becoming much more reliable and consistent with few problems occurring during the course of the experiment.

For experiment CR71/15.5.89, the dilution rate was increased still further to a value of 0.40 h⁻¹ - the point at which Paul and co-workers obtained their maximum activity of 70 DSU/cm³ (3.37 U/cm³). During this fermentation, a maximum activity of only 64.5 DSU/cm³ (3.11 U/cm³) was achieved, which indicated that the culture had still not reached the point where it would wash out of the vessel, although the critical dilution rate was being approached. The dilution rate was then pushed up to a value of 0.50 h⁻¹, at which, after

Table 8.6
Results Obtained from Continuous Fermentations with 10 g/l KH₂PO₄ and K₂HPO₄ Medium.

Run Number	Sucrose Concentration in Medium (g/l)	OD ₅₉₀ Value	Enzyme Activity (DSU/cm ³ /h)	Residual Sucrose (g/l)	Dilution Rate (h ⁻¹)	Duration of Dilution Rate (h)	Duration of Fermentation (h)
CR69/24.4.89	35	0.398	64.2	-	0.35	16.25	
		0.435	75.5	-	0.35	5.25	
		0.311	69.2	-	0.35	42.25	79.5
CR70/8.5.89	35	-	55.7	4.8	0.37	18	
		0.197	48.1	4.4	0.37	20.5	
		0.219	66.6	-	0.37	25.5	77.75
CR71/15.5.89	35	0.196	52.5	18.0	0.40		
		0.198	64.5	-	0.40	143.75	
		0.214	10.0	19.5	0.50 *	4.75	190

* Cell Washout Occurred.

only a few hours, the enzyme activity had dropped to only 10.0 DSU/cm³ (0.48 U/cm³) with complete cell washout occurring soon afterwards.

Figure 8.7 shows the OD₅₉₀ and activity values plotted against dilution rate for the fermentations carried out with the controlled sterilization of the feed medium in the Adelphi autoclave, with a sterilization temperature of 116 - 119°C along with a holding time of 10 minutes proving to be critical factors in the continuous production of dextransucrase.

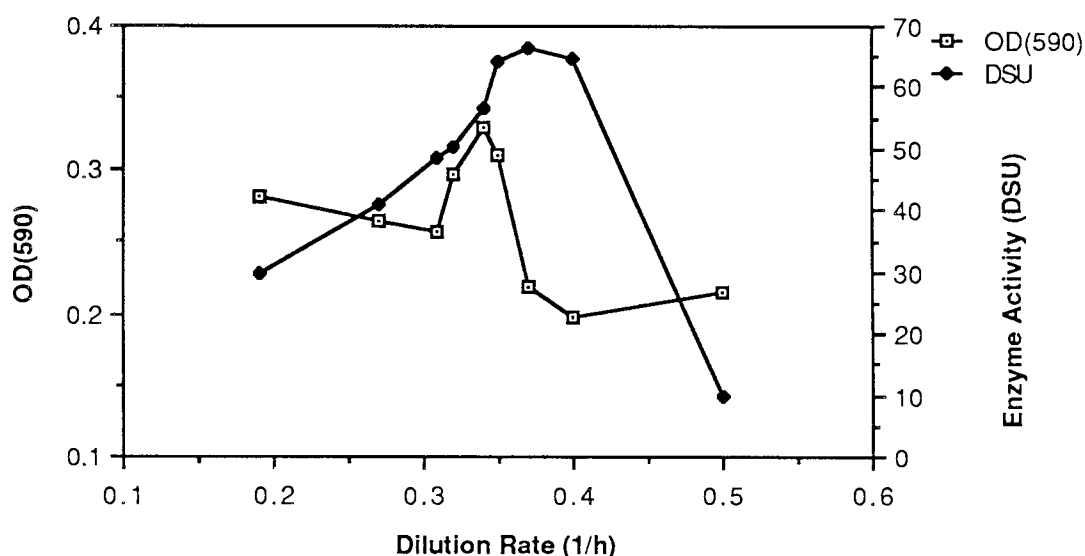


Figure 8.7: Results with Controlled Sterilization

However, from this point onwards, the sterilization procedure for the feed medium was changed quite considerably, as was described earlier in Section 5.3.6. The medium itself was sterilized in the New Brunswick fermenter at 116°C for 20 minutes and, as the medium was agitated throughout the sterilization process, the duration of the heating and cooling cycles were considerably reduced, with the cooling period especially being shortened. This was due to the fact that cooling water was passing through the heat exchanger, causing the temperature to decrease quite quickly. The sterile medium was then transferred aseptically to the empty aspirator bottles that had been sterilized in the autoclave. This procedure was much quicker which meant that, as the medium was not at a high temperature for very long, the probability of the yeast extract degrading was reduced, and also a larger volume of feed medium could be prepared in a shorter period of time.

8.2.3. Results Obtained with Different Yeast Extract Batches.

It has already been shown in Section 6.2.2.2, that the use of different types and batches of yeast extract can have a drastic effect on the subsequent enzyme activity. It was also found that Gistex paste was the best yeast extract to use for the fed-batch production of dextransucrase, and so Gistex was used as the medium component for all the continuous fermentations carried out over the course of this project.

Although a number of different batches of Gistex yeast extract were used during the continuous section of this project, the fermentations carried out and results obtained will not be discussed in detail in this section as they will be included in other parts of this Chapter. However, the results recorded with two specific batches of Gistex will be discussed here, in particular the results obtained with batch number 8-16-25. This yeast extract was supplied by Fisons plc, Pharmaceutical Division and was the batch that was used at Holmes Chapel in the medium of a fed-batch fermentation that had yielded 589 DSU/cm³ (28.36 U/cm³) of enzyme (100). The fermentation that had given the high enzyme activity had been carried out under conditions slightly different from those of a standard fed-batch fermentation, with the concentration of sucrose in the initial medium being increased by 33% and the concentration of NaOH in the sucrose/NaOH feed being reduced by 20%. The fermentation itself was run at an operating pH of 6.90, considerably higher than the value of 6.65 used for standard fermentations and under these conditions an activity of 589 DSU/cm³ (28.36 U/cm³) was attained. A continuous fermentation was therefore carried out using this yeast extract, and was compared to a fermentation carried out with the current Aston University batch, 8-36-09.

Table 8.7 shows the results obtained continuously with the two yeast extract batches, with the feed medium in both cases being sterilized in the New Brunswick vessel.

Table 8.7.

Results Obtained from Continuous Fermentations with Different Gistex Batches.

Run Number	Gistex Batch Number	Sucrose Concentration in Medium (g/l)	OD ₅₉₀ Value	Enzyme Activity (DSU/cm ³ /h)	Residual Sucrose (g/l)	Dilution Rate (h ⁻¹)	Duration of Dilution (h)	Duration of Fermentation (h)
266	8-16-25	35	0.255	59.0	-	0.35	19.5	
			0.259	50.5	-	0.35	43.25	
		40	0.289	72.6	3.0	0.35	124.75	
CR74/3.7.89	8-36-09	40	0.259	69.2	4.4	0.35	46.75	366.5
			0.116	58.3	-	0.35	42.5	
CR69/24.4.89	8-36-09	35	-	81.7	8.8	0.39	7	146
			0.398	64.2	-	0.35	16.25	
			0.435	75.5	-	0.35	5.25	
			0.311	69.2	-	0.35	42.25	79.5

Experiment CR73/5.6.89 was carried out with the Fisons yeast extract 8-16-25 as the nitrogen source. The dilution rate used for the entire fermentation was 0.35 h^{-1} , with this rate being maintained for 366.5 hours. The temperature of the cooling water during this fermentation was quite high as the external temperature at the time was high - exceeding 25°C at times. As the temperature of the cooling water was greater than the required fermentation temperature, it was not possible for the temperature set point to be reached and as a result, this fermentation was carried out at a temperature of approximately 25°C . This should have no effect on the cell growth other than to increase the growth rate slightly, but as dextransucrase begins to be broken down at 25°C , the fact that the operating temperature of the fermentation was above the value resulted in an overall decrease in the enzyme detected in the medium. The activity values recorded from CR73/5.6.89 varied between 50.5 (2.43) and 72.6 DSU/cm³ (3.50 U/cm³) over the duration of the experiment, although these variations can be attributed in some part to the temperature fluctuations that occurred. After 220 hours of the fermentation, when an activity of 72.6 DSU/cm³ (3.50 U/cm³) had been achieved, the feed medium was changed to that containing 40 g/l sucrose. With the temperature increasing to 25.1°C , an activity of 69.2 DSU/cm³ (3.33 U/cm³) was recorded, which coincided with the maximum activity values achieved in previous fermentations. Once the experiment had proceeded for approximately 312 hours, the appearance of a possible contaminant was observed in the fermenter and, although attempts were made to washout the contaminant, the enzyme activity began to slowly decrease until, by the time the fermentation was terminated after 366.5 hours, no dextransucrase activity could be detected in the broth. The OD₅₉₀ value at the time was measured at 0.190, although this was also recording the growth of the contaminant organism.

Experiment CR74/3.7.89 was carried out with the Aston Gistex - batch number 8-36-09 - in the medium, along with 40 g/l sucrose. Before this fermentation was started, a shutdown system was connected to the fermenter. It consisted of a level probe which was inserted through the top plate of the fermenter to a position slightly above the output weir in the vessel. From this time onwards, once the overflow tube becomes blocked, the broth level within the fermenter would rise and touch the probe, causing the shutdown system to be triggered,

shutting off both the alkaline and medium feed to the fermenter. This experiment was operated at dilution rates of 0.35 and 0.39 h⁻¹, resulting in activities of 58.3 (2.81) and 81.7 DSU/cm³ (3.93 U/cm³) respectively. The activity recorded at 0.35 h⁻¹ was lower than expected, but can be attributed to the high operating temperature. However, the increased dextransucrase yield at the high dilution rate of 0.39 h⁻¹ indicated that, with a sucrose composition of 40 g/l in the medium, the critical dilution rate was slightly higher than 0.39 h⁻¹, greater than that observed with 35 g/l sucrose medium. This fermentation was shutdown at some point between 122 and 146 hours, due to a blockage in the output tube.

These results can be compared to those obtained in CR69/24.4.89 which are also recorded in Table 8.6. They clearly show the achievement of 70 DSU/cm³ (3.37 U/cm³) from a continuous fermentation operated at a dilution rate of 0.35 h⁻¹, with feed medium sterilized in the autoclave at 116 - 119°C for 10 minutes. It would appear that the yeast extract supplied by Fisons plc, which had the capability of apparently producing 589 DSU/cm³ (28.36 U/cm³) of enzyme on the fed-batch scale, did not greatly improve the yields obtained continuously, with enzyme activities similar to those obtained with the Aston Gistex being recorded.

8.2.4. Results Obtained with Different Sucrose Concentrations in the Medium.

The fermentations already carried out have shown that increasing the sucrose concentration in the feed medium, leads to an increase in enzyme activity, OD₅₉₀ value and critical dilution rate. As the sucrose concentration in the medium increases, the cells respond with an increasing growth rate, utilizing the substrate as it enters the fermenter. It is possible to operate a continuous fermentation with a higher sucrose level in the medium than a batch fermentation, as in a continuous fermentation, the cells are at a growth rate where they can utilize all available substrate until the critical dilution rate is reached, when the substrate builds up within the vessel, washing out the cells. In a batch or fed-batch fermentation of dextransucrase, excess sucrose in the medium will be synthesized into dextran and fructose by the enzyme, a situation that is unwelcome with the production of dextransucrase. However, under continuous conditions, the sucrose level in the vessel can be increased to provide higher yields of enzyme, and this can be achieved by increasing the sucrose concentration in the feed medium and by increasing the dilution

rate of the fermentation. This section deals with the results obtained with increasing the medium sucrose concentration and the subsequent increases in dilution rate that occurred.

Table 8.8 shows the results recorded from a number of fermentations in which the sucrose concentration in the medium was 40 g/l. During these fermentations, the agitation rate was maintained at 450 rpm, which resulted in a working volume of 1,035 cm³.

The activities recorded from experiment CR75/10.7.89 appeared to decrease over the course of the fermentation. However, this was due to a period of shutdown of the fermentation. The activities recorded previous to the shutdown were approximately 70 DSU/cm³ (3.37 U/cm³), but at some point between 55 and 72.25 hours of the fermentation, a blockage occurred in the output of the fermenter, causing the vessel level to rise to the point of contact with the level probe, leading to the cessation of all feed to the fermenter. Once the feed had been restarted to the fermenter, an activity of approximately 60 DSU/cm³ (2.89 U/cm³) was achieved, but the previous value of 70 DSU/cm³ (3.37 U/cm³) could not be reached. However, an interesting new development occurred at this point in the project, with the activity of the collected broth being tested. The output from the fermenter was collected in an aspirator bottle, the contents of which were not controlled in any way and left to stand for approximately 24 hours before disposal. At the end of experiment CR75/10.7.89, this broth was found to have an enzyme activity of 90 DSU/cm³ (4.33 U/cm³) - far higher than that being achieved in the fermenter at that time. This enzyme enhancement was due to the fact that the cells continued to grow and secrete dextransucrase in the product reservoir, utilizing the sucrose that was still present in the fermentation broth. These results seemed to imply that the presence of a second fermenter in series with the current one would support greater cell growth and hence, enzyme formation. This phenomenon will be investigated in future fermentations.

Experiment CR76/17.7.89 suffered from the problem of a high fermentation temperature, with a large portion of the experiment being carried out at a temperature in excess of 26°C. This fermentation was operated at a similar dilution rate to CR75/10.7.89, and at first produced very similar results, with

Table 8.8.
Results Obtained from Continuous Fermentations.

Run Number	Sucrose Concentration in Medium (g/l)	OD ₅₉₀ Value	Enzyme Activity (DSU/cm ³ /h)	Residual Sucrose (g/l)	Dilution Rate (h ⁻¹)	Duration of Dilution Rate (h)	Duration of Fermentation (h)
CR75/10.7.89	40	0.126	78.4	3.4	0.37	18	
		0.099	67.6	5.7	0.37	24.25	
		0.396	68.9	4.9	0.37	19.25	78
CR76/17.7.89	40	0.439	67.2	1.31	0.36		
		0.373	68.1	12.7	0.36	16.5	
		0.383	45.3	1.9	0.36	25.25	
CR79/7.8.89	40	0.312	37.7	2.6	0.39		
		0.187	80.6	11.0	0.39		
		0.263	80.2	9.0	0.39	16.5	
		0.331	79.6	6.0	0.39	22.25	
		0.240	47.6	12.3	0.40	22	76.5

approximately 70 DSU/cm³ (3.37 U/cm³) of enzyme being obtained. However, as the temperature reached a value of 25.8°C, the activity was reduced sharply to approximately 45 DSU/cm³ (2.17 U/cm³), and with a further temperature increase to 26.5°C, the activity dropped to a value of 37.7 DSU/cm³ (1.82 U/cm³). The effect of temperature on dextransucrase formation is therefore clearly seen, confirming the information given by Alsop (50), that at a pH of 6.7, dextransucrase is inactivated extremely rapidly at temperatures above 24°C. He also reported that the optimum temperature for the growth of *Leuconostoc mesenteroides* is 30°C, and that this is an unfavourable condition for dextransucrase production. During this fermentation, the activity of the collected broth was also tested and the enhancement on hold-up factor was again observed, with medium that had contained enzyme with an activity of approximately 45 DSU/cm³ (2.17 U/cm³) within the fermenter, containing 84.2 DSU/cm³ (4.05 U/cm³) of enzyme after 24 hours. This was an increase of almost 100% on the enzyme formed within the fermenter. However, the broth that was tested once the experiment had been terminated gave only 33.4 DSU/cm³ (1.6 U/cm³), which again reflected the temperature under which the broth was standing.

Experiment CR79/7.8.89 was operated initially at a dilution rate of 0.39 h⁻¹ from which the high activity of approximately 80 DSU/cm³ (3.85 U/cm³) was obtained, although increasing the dilution rate to 0.40 h⁻¹, resulted in a sharp decrease in activity and build-up of residual sucrose within the fermenter, indicating that the cells were by then growing at the maximum growth rate possible under those conditions and were unable to utilize all the available substrate. It would appear that the critical dilution rate of the process at a medium sucrose concentration of 40 g/l is at the value of 0.40 h⁻¹, which is the dilution rate at which Paul *et al* (19) achieved their maximum enzyme activity.

Experiment CR80/14.8.89 was carried out under the same conditions as experiment CR79/7.8.89, in that the medium feed contained 40 g/l sucrose and the fermentation was run at a temperature of approximately 24°C, pH of 6.65 and agitation rate of 450 rpm. At a dilution rate of 0.42 h⁻¹, the cells washed out of the fermenter and there was very little product formation. It would appear therefore that a dilution rate of between 0.40 and 0.42 h⁻¹ is actually the critical dilution rate of the fermentation under these conditions, with the maximum activity being achieved under these conditions being

approximately 80 DSU/cm³ (3.85 U/cm³). The results obtained from this set of experiments are shown in Figure 8.8.

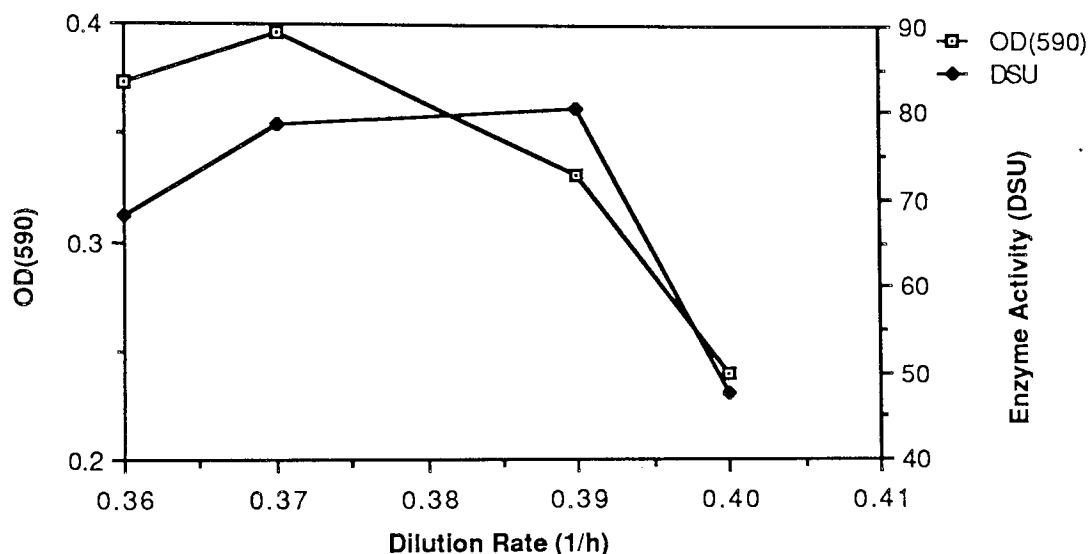


Figure 8.8: Results obtained from CR75/76/79

8.2.5. Results Obtained with Varying Agitation Rates.

Previous to this point, it was thought that operation of the fermentation at high agitation rates would cause a high degree of shear damage to the microorganisms and enzyme, leading to a loss of productivity, with respect to the secretion of dextransucrase. It was also generally believed that shear effects were a major cause of protein denaturation in solution and of enzyme deactivation. However, Thomas (79) and co-workers showed that the effects of shear on fermentations were not as severe as was previously believed, provided that the proteins were subjected to shear alone. They found that it was necessary for there to be high levels of both agitation and aeration in the fermentation vessel for shear damage to occur. They claimed it should be possible to eliminate "shear damage" to proteins in unaerated stirred tanks, as is the case with this project. It was therefore decided to increase the agitation rate of the present continuous fermentations, with the aim of increasing the circulation of the cells throughout the medium, to facilitate the mixing into the medium of both feed and NaOH - hence avoiding stagnant zones. This led to an increase in the availability of the nutrients to the cells, thereby increasing the maximum growth rate of the cells, the critical dilution rate of the

fermentation and the rate of product formation of the cells - dextransucrase being growth-related.

The agitation rate of the fermentation was therefore increased to levels of 700 and 850 rpm, resulting in decreases in the working volumes of the fermentation to 950 and 925 cm³ respectively. The results obtained from this first set of continuous fermentations with the increased agitation rates are shown in Table 8.9.

Experiment CR81/2.10.89 was the first fermentation to be carried out at the increased agitation rate of 700 rpm, although the results achieved were not significantly greater than those previously obtained with an agitation rate of 450 rpm. At a dilution rate of 0.39 h⁻¹, an activity of 74.7 DSU/cm³ (3.60 U/cm³) was achieved, which was similar to the results obtained from experiment CR79/7.8.89, with the same dilution rate and feed medium sucrose composition. Experiment CR82/16.10.89 was operated in a similar manner to the previous one, except that the feed medium now contained 45 g/l of sucrose. This fermentation yielded a result of 73.4 DSU/cm³ (3.53 U/cm³) with a dilution rate of 0.39 h⁻¹, very similar to the results achieved from CR81/2.10.89. Both of these fermentations were carried out successfully, with no incidence of contamination in either case. Figures 8.9a and 8.9b show the pH and DOC profiles obtained from these two fermentations, with their close similarities being observed from these graphical outputs.

Table 8.9
Results Obtained from Continuous Fermentations.

Run Number	Sucrose Concentration in Medium (g/l)	Agitation Rate (rpm)	OD ₅₉₀ Value	Enzyme Activity (DSU/cm ³ /h)	Residual Sucrose (g/l)	Dilution Rate (h ⁻¹)	Duration of Dilution Rate (h)	Duration of Fermentation (h)
CR81/2.10.89	40	700	0.383	58.0	2.1	0.34	14.75	79.5
			0.405	59.0	1.9	0.36	24.75	
			0.406	74.7	-	0.39	22	
CR82/16.10.89	45	700	0.351	48.4	3.7	0.17	20	82.5
			0.378	55.6	1.1	0.38	22	
			0.425	73.4	-	0.39	28.75	
CR83/23.10.89	45	700	0.382	61.7	9.3	0.41	48	221
			0.360	83.2	-	0.43	41	
			0.359	108.7	-	0.47	47.75	
CR84/6.11.89	45	850	0.341	34.2	-	0.50	26.75	74
CR/85/13.11.89	45	850	0.293	39.0	14.8	0.50	48	78.5

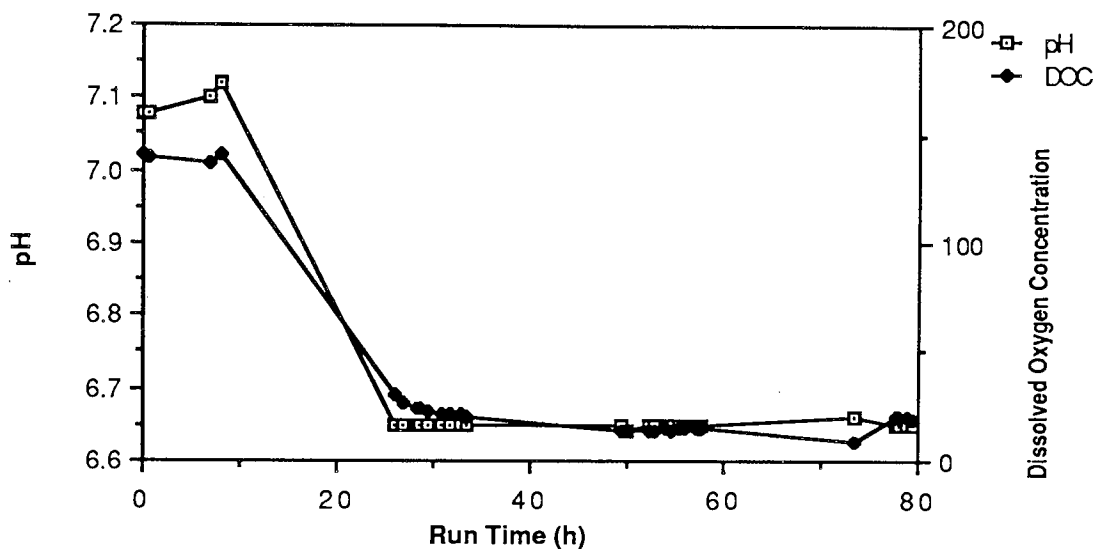


Figure 8.9a: pH and DOC Profiles from CR81

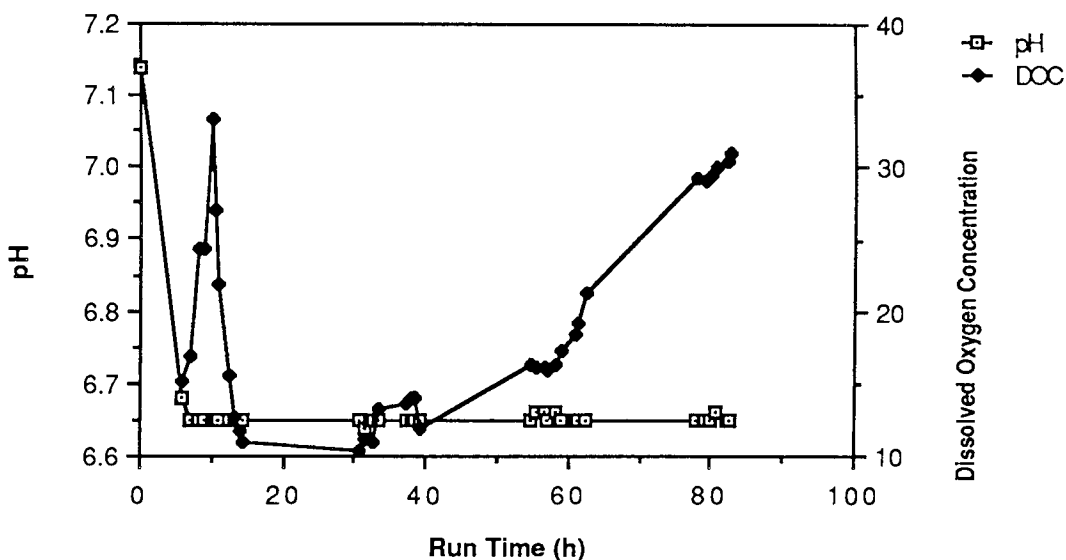


Figure 8.9b: pH and DOC Profiles from CR82

During experiment CR83/23.10.89 the dilution rate was pushed to levels higher than had previously been used without washout occurring. An initial dilution rate of 0.41 h^{-1} , at which cell washout would have taken place with a fermentation operated at 450 rpm, produced dextransucrase with an activity of approximately 61.7 DSU/cm^3 (2.97 U/cm^3) - somewhat lower than expected. However, with an increased dilution rate of 0.43 h^{-1} , the activity of the fermentation broth also increased, to a value of 83.2 DSU/cm^3 (4.01 U/cm^3) -

thus equalling the previous best results so far obtained during the course of this project (see experiment CR79/7.8.89 - Table 8.8). Following this, the dilution rate of the fermentation was increased still further, to a value of 0.47 h^{-1} , and at this point, dextransucrase with an activity of approximately 108.7 DSU/cm^3 (5.23 U/cm^3) was detected - a value which greatly surpassed all other results so far achieved, both from these studies and from the work of others. The fermentation was operated at steady-state under this dilution rate for a period of 47.75 hours, until the dilution rate was increased yet again to a value of 0.50 h^{-1} . At this level the activity dropped very slightly to a value of 105.9 DSU/cm^3 (5.10 U/cm^3), whilst the OD_{590} value decreased from a level of 0.359 at the dilution rate of 0.47 h^{-1} to 0.266 at 0.50 h^{-1} . It would appear that, under the operating conditions of this particular fermentation, the dilution rate of 0.50 h^{-1} was near the critical dilution rate as it seemed that a slight amount of cell washout was taking place. This fermentation was operated under completely sterile conditions, for its complete duration of 221 hours, with no problems occurring, either in temperature or pH control, with the temperature being maintained at a value of 23.4°C throughout the entire experiment. The pH of the broth was controlled at a level of 6.65 ± 0.01 at all times as can be seen from the pH profile shown in Figure 8.10. The DOC profile is also given in Figure 8.10, showing that a level of approximately 30 units was achieved at all times, a value considerably higher than in many other unaerated continuous fermentations. The activity of the broth collected during this experiment was tested at the point where the dilution rate of the fermentation was only 0.43 h^{-1} - and hence the activity being 83.2 DSU/cm^3 (4.0 U/cm^3) - and was found to have a value of 155.7 DSU/cm^3 (7.50 U/cm^3). This was an enhancement of the original enzyme activity of almost 100%, supporting the findings of previous fermentations where enhancement on hold-up had been detected (see CR76/17.7.89). Figure 8.11 shows the variation in activity and OD_{590} value with dilution rate under the conditions of 700 rpm agitation and 45 g/l sucrose in the feed medium. This diagram clearly shows that the critical dilution rate had not yet been achieved under these conditions, even at a rate of 0.50 h^{-1} , which was probably approaching the critical value. It would appear that increasing the agitation rate of the fermentation increases the growth rate of the cells, leading to a higher critical dilution rate, as $\mu_{\text{max}} = D_{\text{crit}}$. It was therefore possible that a further increase in agitation rate would serve to continue this trend.

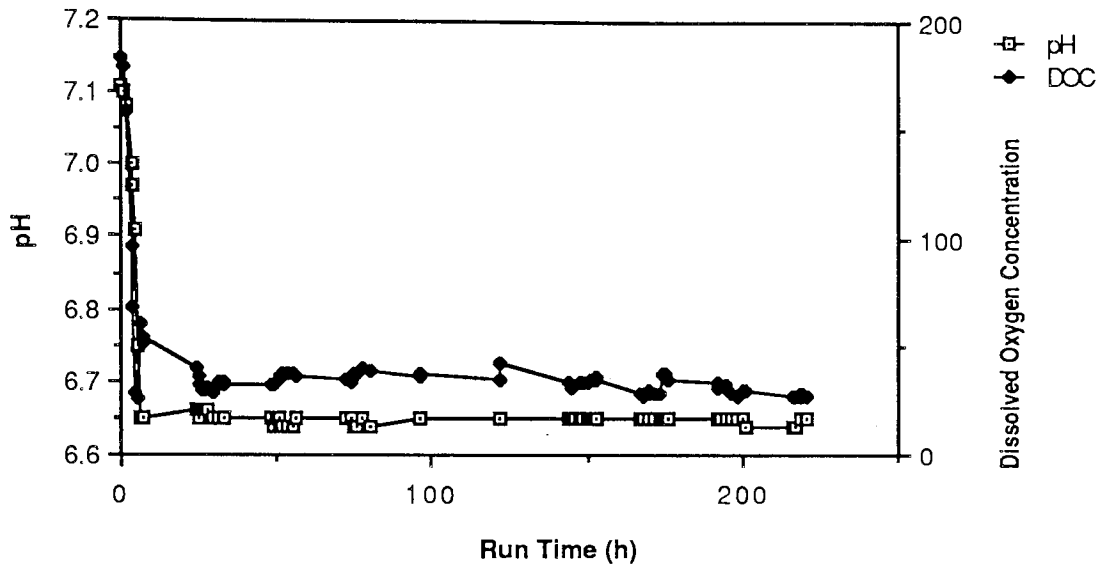


Figure 8.10: pH and DOC Profiles from CR83

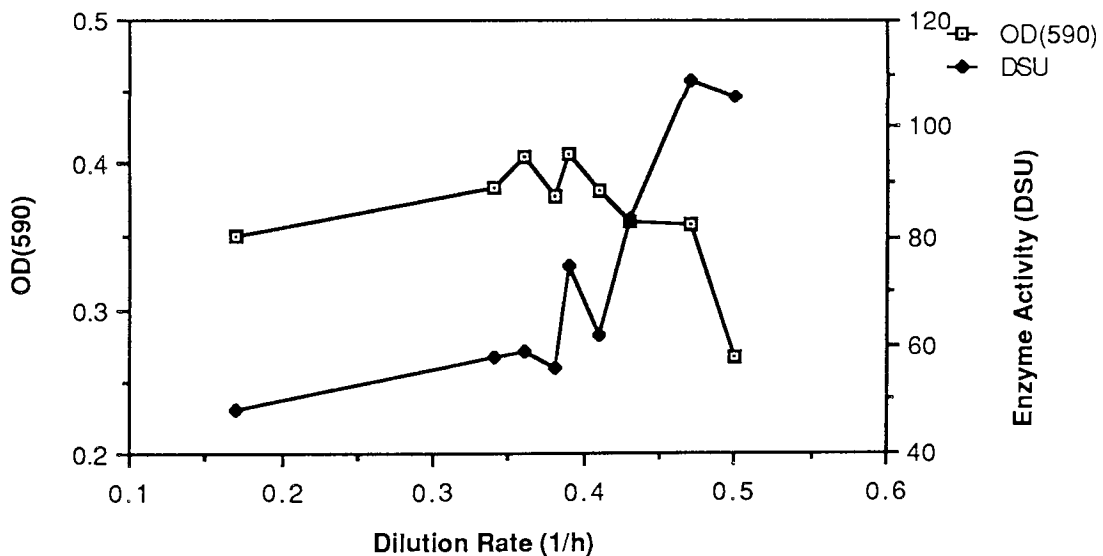


Figure 8.11: Results obtained from CR81/82/83

Experiment CR84/6.11.89 was operated at an agitation rate of 850 rpm for a steady-state period of 26.75 hours, although it was only possible to achieve an activity of approximately 34.2 DSU/cm³ (1.65 U/cm³) at the previously successful dilution rate of 0.50 h⁻¹. The duration of steady-state for this experiment was considerably shorter than the duration of the entire fermentation due to the fact that the high amount of foam generated by the broth at the high agitation rate of 850 rpm touched the level probe inserted

into the head plate of the vessel, shutting down the fermentation. This problem was solved by pulling the level probe further out of the vessel and then repeating the experiment. However, CR85/13.11.89 produced almost identical results to CR84/6.11.89 under the same conditions, with an activity of 39.0 DSU/cm³ (1.88 U/cm³) being achieved at steady-state. It would therefore appear that the agitation rate of 850 rpm caused some damage, probably to the cells rather than the enzyme, resulting in enzyme yields considerably lower than those achieved with the agitation set at 700 rpm.

From this point, a number of fermentations were carried out in order to reproduce the results obtained from experiment CR83/23.10.89, although this proved to be somewhat more difficult than was at first thought, with the subsequent fermentations being unable to yield high levels of dextransucrase. The results from these fermentations are summarized briefly in Table 8.10.

It can be clearly seen from Table 8.10 that it was not possible to achieve the high activities that had been reached during previous fermentations. Indeed, it was only possible to reach a value of 55.0 DSU/cm³ (2.65 U/cm³) from experiment CR97/6.2.90, a yield of enzyme still somewhat lower than the previous maximum value of 70 DSU/cm³ (3.37 U/cm³) (19) that had once been achieved very consistently. The reason for these poor results was not fully understood as, with the exception of CR88/4.12.89, all of the fermentations were carried out very successfully and with no contamination problems. Prior to the commencement of CR19/15.1.90, the pH probe used in the fermentations was regenerated by immersing it in a HCIF solution for one minute. The probe was then recalibrated, and was found to have been reading a value of 7.6 with a pH buffer of 7.0. It was thought that this action of recalibrating the pH probe would serve to improve results, but this was not the case. This fermentation, however, did exhibit some enhancement on hold-up, with an enzyme activity of 52.6 DSU/cm³ (2.53 U/cm³) being detected in the broth, 24 hours after an activity of 34.4 DSU/cm³ (1.66 U/cm³) had been recorded in the vessel. Experiment CR94/28.1.90 used, for its inoculum, slopes of *Leuconostoc mesenteroides* that were freshly prepared from a new vial of culture supplied by Fisons plc, Pharmaceutical Division. It was thought possible that some mutations might have occurred with the old culture,

Table 8.10
Results Obtained from Continuous Fermentations.

Run Number	Sucrose Concentration in Medium (g/l)	OD ₅₉₀ Value	Enzyme Activity (DSU/cm ³ /h)	Residual Sucrose (g/l)	Dilution Rate (h ⁻¹)	Duration of Dilution (h)	Duration of Fermentation (h)
CR86/20.11.89	45	0.355	43.6	6.0	0.47	70.25	77.75
CR88/4.12.89	45	0.276	28.5	26.3	0.50	90.25	98.25 (*)
CR89/11.12.89	45	0.43	38.4	-	0.45	51.25	195
CR91/15.1.90	45	0.33	34.4	-	0.46	68.25	76
CR94/28.1.90	45	0.385	37.3	-	0.46	48.75	73.5
CR97/6.2.90	45	0.426	55.0	-	0.43	23.75	53
CR98/12.2.90	45	0.245	35.1	-	0.49	69.3	79
CR100/19.2.90	45	0.226	47.4	15.4	0.50	27	
		0.262	53.7	9.7	0.52	308.5	389
CR103/12.3.90	50	0.271	55.9	-	0.45	120.5	
		0.490	49.3	-	0.46	92	244

* Fermentation Contaminated.

rendering it less viable than before, but as the results obtained from the fermentation using the fresh slopes did not improve upon previous results, this theory was also deemed to be inaccurate. The medium for experiment CR97/6.2.90 was prepared with a new batch of Gistex yeast extract, namely batch number DG4-04 as used in R96/5.2.90 and CR99/13.2.90 (see Table 6.8). This Gistex was known to be capable of producing dextransucrase with an activity of approximately 400 DSU/cm³ (19.26 U/cm³) using the New Brunswick fermenter in fed-batch mode. The use of this Gistex did appear to increase enzyme activity slightly, from the low levels that were currently being achieved, although activities in excess of 70 DSU/cm³ (3.37 U/cm³) were still not obtained. Experiments CR98/12.2.90 and CR100/19.2.90 were also unsuccessful with regard to achieving high levels of dextransucrase, although CR100/19.2.90 was operated for a considerable period of time at a dilution rate of 0.52 h⁻¹ with no cell washout occurring. Indeed, CR100/19.2.90 was run for a duration of 389 hours with no contamination or other problems occurring. This experiment again showed that it is possible to operate a dextransucrase fermentation continually for long periods of time without any problems, a positive factor that should be taken into consideration when considering scale-up of the process.

As can be seen from Table 8.10, the feed medium for Experiment CR103/12.3.90 contained 50 g/l sucrose, instead of 45 g/l as with the previous experiments in this section. It was hoped that this extra substrate would serve to boost the cell growth and subsequent enzyme production. However, activities of approximately 50 DSU/cm³ (2.41 U/cm³) were recorded from this fermentation, which equalled the standard set by the previous experiment but were still only 50% of the maximum results so far achieved. The exit gas from this experiment was also analysed using a mass spectrometer to determine the levels of N₂, O₂ and CO₂ in the output gas from the fermenter. Table 8.11 gives the analyses of the exit gas from experiment CR103/12.3.90.

Table 8.11.
Exit Gas Analyses from Experiment CR103/12.3.90.

Sample Number	% v/v N ₂	% v/v O ₂	% v/v CO ₂
1330A/23.3.90	75	18	7
1330B/23.3.90	69	27	4
1400C/23.3.90	68	26	6
1400D/23.3.90	76	18	6

It can be seen therefore that it did not seem possible to repeat the achievement of 100 DSU/cm³ (4.81 U/cm³) of enzyme that had occurred in experiment CR83/23.10.89, despite altering various parameters of the fermentation, such as Gistex batch, culture slopes and sucrose concentration in the feed. The pH probe was also regenerated and recalibrated, but to no avail. It was then decided to check the actual pH of the broth during the course of experiment CR103/12.3.90 and it was found to have a value of 6.00, which was well below the pH for optimum dextransucrase secretion. It was realised at this point that the probe had reached the end of its useful life - being approximately 22 months old - and so a new Ingold pH probe was purchased and used during experiments CR104/23.4.90 and CR105/21.5.90. The steady-state results obtained from these fermentations are shown in Table 8.12. From the data given, it can be seen that, under the conditions of the fermentation, i.e. temperature = 23.4°C, pH = 6.65 ± 0.01, agitation rate = 700 rpm, an activity of approximately 97.6 DSU/cm³ (4.70 U/cm³) was achieved at a dilution rate of 0.48 h⁻¹, a result that was very similar to that obtained in experiment CR83/23.10.89. The data shows that the enzyme activity increased steadily with dilution rate to this point, but as the dilution rate reached the value of 0.50 h⁻¹ the activity dropped dramatically, indicating that the critical dilution rate of this fermentation lay between the values of 0.48 and 0.50 h⁻¹. The OD₅₉₀ values decreased with increasing dilution rate, but they dropped substantially above a dilution rate of 0.50 h⁻¹. The results from these fermentations demonstrated the importance of not operating the process too close to the critical dilution rate, as this point can easily be surpassed, leading

to cell washout and, hence, almost complete loss of product. Figure 8.12 shows the steady-state results obtained from experiments CR104 and CR105. These fermentations reproduced the results obtained from experiment CR83/23.10.89 proving that it is possible to produce dextransucrase with an activity in excess of 100 DSU/cm³ (4.81 U/cm³) from a one-stage continuous fermentation of *Leuconostoc mesenteroides*, with this value being boosted to at least 155 DSU/cm³ (7.46 U/cm³) on hold-up. This implies that a second fermenter in series with the first would dramatically enhance the final enzyme activity, allowing the growth rate of the cells to increase beyond the level set by the critical dilution rate.

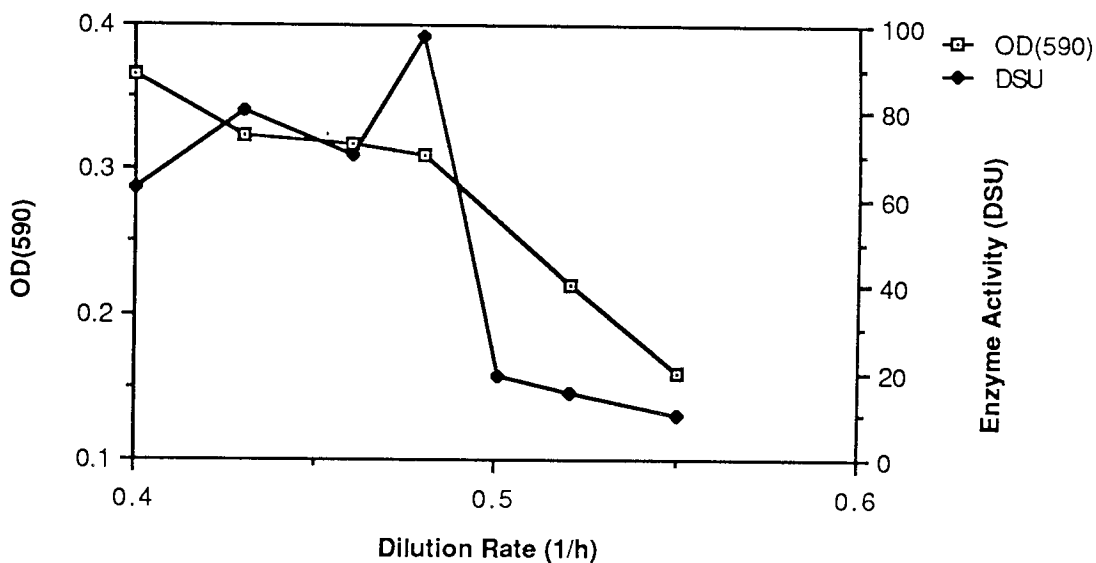


Figure 8.12: Results obtained from CR104/CR105

Table 8.12.

Steady-State Results from Experiment CR104/23.4.90 and CR105/21.5.90.

Sucrose Concentration in Medium (g/l)	OD₅₉₀ Value	Enzyme Activity (DSU/cm³)	Dilution Rate (h⁻¹)
45	0.365	62.5	0.40
	0.324	80.1	0.43
	0.318	70.3	0.46
	0.310	97.6	0.48
	-	19.25	0.50
	0.221	15.0	0.52
	0.159	10.1	0.55

9. DISCUSSION OF CONTINUOUS FERMENTATION RESULTS AND MODELLING.

9.1 Continuous Fermentations.

The results obtained from these experiments are described in Chapter 8 of this thesis, with the experiments having been carried out in two vessels - the New Brunswick BioFlo C30 model and the MBR MiniBioreactor model. The experiments carried out in the New Brunswick vessel were merely preliminary fermentations and will not be discussed in greater detail here. This section will instead deal with the continuous fermentations that were carried out in the MBR vessel.

9.1.1. Effects of Sterilization Procedures on Product Formation.

It was necessary to produce high volumes of sterile medium to use as feed for the continuous fermentations. There were two distinct methods of producing this feed : the first by sterilizing 5 litre aspirator bottles of medium in the Adelphi autoclave ; and the second by sterilizing the medium in the New Brunswick fermenter and aseptically transferring it to the aspirator bottles that had previously been sterilized, empty, in the autoclave. The Adelphi autoclave was very efficient in sterilizing additional fermentation equipment, such as tubings, hyperdermic needles and inoculum containers, but problems soon arose when it came to the sterilization of 5 litres of feed medium. As there was no agitation, the medium was heated to sterilization temperature, with the external medium reaching high temperatures well before the central medium. The reverse was the situation during the cooling of the medium following sterilization, with the cooling period being of several hours duration. The overall result of this was that the feed medium remained at high temperatures for long periods of time. It was thought that such higher temperatures for prolonged periods would degrade the medium and in particular the yeast extract component of the medium. This degradation could in fact be observed visually by the colour of the medium following sterilization. A light brown medium indicated little degradation, whereas a dark brown, almost black medium indicated various degrees of yeast extract decay. Various sterilization holding times were used with the Adelphi autoclave, ranging from 20 minutes to zero minutes. It was found that a sterilization time of less than ten minutes was insufficient to maintain sterility within the feed medium, and so a holding time of ten minutes was selected for the sterilization cycle. The sterilization temperature at which the autoclave was operated was also

important. When the autoclave was set at a temperature of 121°C, it controlled the sterilization between the limits of 117 and 125°C - values which would contribute to any degradation of the yeast extract. It was therefore decided to manually control the sterilization cycle of the autoclave at 116 ± 1°C, a factor which greatly reduced medium destruction. However, this controlled sterilization did not serve to reduce the time period at which the feed medium was at high temperatures, particularly during the cooling cycle. A number of fermentations were carried out with the feed medium having undergone various degrees of sterilization. For example, the fermentations shown in Table 8.2 utilized medium that had been sterilized with very little control with regards to either temperature or time. These fermentations yielded a maximum steady state activity of approximately 12 DSU/cm³ (0.58 U/cm³). Table 8.3 shows the results obtained from two fermentations where the feed medium had been sterilized at 116 - 119°C for 20 minutes, instead of between the temperatures of 121 - 125°C. The feed for experiment CR54/12.12.88 underwent varying degrees of sterilization, with the medium fed at the dilution rates of 0.25 and 0.27 h⁻¹ not having undergone any degree of controlled sterilization and the medium used at the higher dilution rate of 0.29 h⁻¹ having been sterilized at a controlled temperature of 116 - 119°C for 20 minutes. The overall activity did not increase at this point, although the cells consumed a greater amount of sucrose from the latter medium. It is probable, though, that the critical dilution rate was approaching, leading to a subsequent decrease in overall activity. The overall activities recorded from CR56/9.1.89, where the feed medium was sterilized at 116 - 119°C for 20 minutes were higher than those of CR54/12.12.88, with a maximum activity of 33.0 DSU/cm³ (1.59 U/cm³) being recorded at a dilution rate of 0.21 h⁻¹. Above this point, the activity decreased slightly with a corresponding increase in residual sucrose, indicating that the cells were not growing at a rate sufficient to utilize all the input sucrose, until the dilution rate of 0.30 h⁻¹ was reached, at which point almost total cell washout occurred.

Table 8.4 gives the results recorded from a set of fermentations with which the feed medium had been sterilized at 116 - 119°C for 10 minutes only. The activities yielded by these experiments were generally greater than the results obtained from previous fermentations, with experiments CR59/30.1.89 and CR61/13.2.89 in particular producing dextransucrase with an activity of at

least 50 DSU/cm³ (2.41 U/cm³). There was some doubt as to the value of the critical dilution rate for these fermentations, with CR59/30.1.89 experiencing a significant drop in activity and OD₅₉₀ value at a dilution rate of 0.30 h⁻¹, whereas CR60/6.2.89 and CR61/13.2.89 experienced their maximum activities at dilution rates of 0.30 and 0.32 h⁻¹ respectively. These fermentations used feed medium with a sucrose concentration of 35 g/l - slightly higher than the concentrations employed during previous fermentations. This factor, however, of varying medium sucrose composition, has been discussed in greater detail in Section 8.2.3.

The fermentations recorded in Table 8.4 were all operated at high dilution rates and, as such, suffered from the problem of increasing pH during the actual fermentation. This was due to the fact that the alkaline feed medium was entering the fermenter at such a high rate that the cells were unable to produce enough acid, through growth, to maintain the fermentation at the required pH set point of 6.65. In certain cases, such as with experiment CR61/13.2.89, the pH increased to a value of 6.89, corresponding to a build-up of residual sucrose to levels of approximately 13 g/l within the fermenter. This implied poor cell growth, along with reduced dextransucrase formation and, as a result, the culture was allowed to grow batchwise for a period of time in order to utilize excess sucrose and reduce the fermentation pH. An excess of sucrose in the medium could lead to unwanted dextran formation and so it was necessary to reduce the residual substrate levels, although the periods of operating the fermentations batchwise were very undesirable as this reduced the lengths of time at which the experiments were held in steady state continuous operation.

Table 8.5 gives the results obtained from further fermentations carried out with controlled medium sterilization in the Adelphi autoclave. It is significant to note that these fermentations were operated for considerably longer periods of time than previous fermentations, with particular note being made of experiment CR63/27.2.89 which had an overall duration of over 260 hours, without there being any incidence of contamination. This fermentation was also successful in that a maximum activity of 56.5 DSU/cm³ (2.72 U/cm³) was achieved at the higher dilution rate of 0.34 h⁻¹, with slight cell washout beginning to occur at a dilution rate of 0.35 h⁻¹. This fermentation did not suffer from increased pH throughout its duration as the feed medium was prepared with 20 g/l of KH₂PO₄ which was of a much lower pH than the

K_2HPO_4 usually used, as it contained an extra H^+ ion. The medium formulation was altered at a point during the fermentation that can be easily detected from the column concerning residual sucrose composition in Table 8.5. When the medium contained K_2HPO_4 , there was a build-up of residual sucrose that exceeded 20 g/l, whereas when the phosphate type was altered to KH_2PO_4 , the residual sucrose levels decreased considerably, indicating significantly improved cell growth. Experiment CR64/13.3.89 was the most successful by then, with an activity of approximately 75 DSU/cm³ (3.61 U/cm³) being achieved at a dilution rate of between 0.32 and 0.33 h⁻¹. The feed medium used for this fermentation was also prepared with 20 g/l KH_2PO_4 , as was the case with the previous experiment and the results from this fermentation exceeded the maximum previously achieved in the literature - of 70 DSU/cm³ (3.37 U/cm³) by Paul *et al* (19) in 1983. This fermentation was operated, without contamination, for 144 hours.

Experiment CR68/10.4.89 was again carried out with 20 g/l K_2HPO_4 in the medium and, again, there was considerable residual sucrose build-up that indicated poor cell growth during this fermentation. As a result, the overall activities achieved were somewhat lower than those obtained from experiment CR64/13.3.89. The fermentations recorded in Table 8.5, however, show that it is possible to operate a dextransucrase fermentation continuously for several days and weeks without any incidence of contamination, provided that great care is taken in performing any aseptic operations, with an example being the changing of feed reservoirs.

The fermentations recorded in Table 8.6 were carried out with feed medium that contained 10 g/l K_2HPO_4 and 10 g/l KH_2PO_4 . Unfortunately, it was not possible to determine residual sucrose concentrations during experiments CR69/24.4.89 and CR70/8.5.89 and, therefore, valuable data concerning the growth of culture was unavailable during these fermentations. These two fermentations yielded dextransucrase with activities of approximately 70 DSU/cm³ (3.37 U/cm³) at dilution rates of 0.35 and 0.37 h⁻¹. When Paul *et al* (19) achieved their maximum activity of 70 DSU/cm³ (3.37 U/cm³), they did so at a dilution rate of 0.40 h⁻¹, and so a fermentation was subsequently carried out at a rate of 0.40 h⁻¹. This experiment, CR71/15.5.89, yielded approximately 65 DSU/cm³ (3.13 U/cm³) of dextransucrase at the dilution rate of 0.40 h⁻¹ and although the OD₅₉₀ value was low at 0.198, washout did not appear to be occurring. However, there was some build-up of residual

sucrose during this fermentation, indicating that the cells were not growing very efficiently. Indeed, the low OD₅₉₀ value and high activity would imply that the sucrose was being consumed for enzyme production, with reduced cell growth. This altered cell metabolism could be the result of some cell starvation that occurred prior to the fermentation entering the continuous mode of operation. However, the culture appeared to recover from this sufficiently to produce 64.5 DSU/cm³ (3.11 U/cm³) of enzyme, although had this change in cell metabolism not occurred, it is possible that a higher enzyme yield would have been achieved. The dilution rate of CR71/15.5.89 was then increased to a value of 0.50 h⁻¹, at which point the activity quickly decreased to a level of only 10 DSU/cm³ (0.48 U/cm³), indicating cell washout. The OD₅₉₀ values from this fermentation are not consistent, due, in the main, to the starvation problems encountered during the first few hours of the fermentation. This experiment shows that the critical dilution rate of the process under these particular conditions lies between values of 0.40 and 0.50 h⁻¹.

From this point onwards, the sterilization procedure was altered to the second method described at the beginning of this section, whereby the actual medium was quickly heated to the sterilization temperature in the New Brunswick vessel, held at that value for 20 minutes, then quickly cooled to room temperature, before transfer to the sterile aspirator bottles. This method was much more convenient than autoclave sterilization and provided a much quicker process of producing large volumes of sterile feed medium, a factor that was particularly advantageous for fermentations of longer duration.

9.1.2. Effects of Different Gistex Batch on Product Formation.

It has already been discussed during Section 7.3.5 that subtle differences in the composition of various batches of Gistex can result in widely differing final activities obtained from fed-batch fermentations. A set of fermentations were therefore carried out using different Gistex batches in the respective media. Table 8.7 shows the results obtained from these fermentations. Gistex batch 8-16-25, one of the batches analysed and discussed in Section 7.3.5, was supplied by Fisons and was reported to have yielded 589 DSU/cm³ (28.36 U/cm³) from a non-standard fed-batch fermentation. Gistex batch 8-36-09 was the current Aston University batch that had been used in all the continuous fermentations carried out by then. These fermentations were carried out with varying compositions of sucrose in the feed medium of

between 35 and 40 g/l. However, the results achieved from these experiments were all very similar, with activities ranging from 65 to 80 DSU/cm³ (3.13 to 3.85 U/cm³) recorded in all cases. However, experiment CR74/3.7.89 yielded the most promising result, with an activity of 81.7 DSU/cm³ (3.93 U/cm³) recorded at a dilution rate of 0.39 h⁻¹. The residual sucrose level within the fermenter at this point was very low at 0.88 g/l, indicating that the cells were consuming almost all available sucrose for growth, maintenance and product formation. Two fed-batch fermentations were carried out with these two Gistex batches, in the MBR vessel, the results of which are given in Table 9.1. It can be seen that the activity values obtained from these fermentations were very similar to each other, confirming the findings from the two continuous fermentations.

Table 9.1.
Fed-Batch Fermentations in MBR Vessel with Different Gistex Batches.

Parameter	Gistex 8-36-09	Gistex 7-40-07
OD ₅₉₀	0.888	0.688
Activity (DSU/cm ³)	266.3	276.5
Sucrose Used (g)	136.2	73.2
Growth Rate (h ⁻¹)	0.24	-
Duration (h)	17	17.5
Temperature (°C)	26.4 ± 0.1	23.6 ± 0.2

The major differences between these two fermentations are the values relating to OD₅₉₀ and sucrose consumed, with this data being much higher for Gistex 8-36-09 than for Gistex 7-40-07. However, this was due to the fact that the operating temperature was much higher for the Gistex 8-36-09 fermentation than with the Gistex 7-40-07 run, resulting in increased cell growth and correspondingly higher sucrose usage.

These fermentations, along with those recorded in Table 8.7, indicate that Gistex batch is less critical a factor for these continuous fermentations than with the fed-batch fermentations carried out in the New Brunswick vessel. As the MBR vessel does not appear to be capable of producing high yields of

dextranucrase with any Gistex batch, the Gistex batches become less critical and are less likely to be the cause of widely varying results from fermentations - both batch and continuous.

9.1.3. Effects of Varying Medium Sucrose Composition on Product Formation.

The fermentations carried out to this point in the research programme utilized feed medium with sucrose concentrations of between 10 and 40 g/l. The maximum enzyme activities have been achieved thus far with the medium sucrose compositions of 35 and 40 g/l, with experiment CR74/3.7.89 yielding approximately 80 DSU/cm³ (3.85 U/cm³) at a dilution rate of 0.39 h⁻¹, with a sucrose composition of 40 g/l in the feed medium. The data presented in Table 7.8 show the results obtained from a set of fermentations in which the feed medium contained 40 g/l sucrose. The results obtained from experiment CR74/3.7.89 were repeated in CR75/10.7.89 and CR79/7.8.89, with the achievement of approximately 80 DSU/cm³ (3.85 U/cm³) of dextranucrase, at dilution rates of between 0.37 and 0.39 h⁻¹. The repeated production of this enzyme yield surpasses the maximum activity achieved previously by Paul *et al* (19), although it was suspected that the yield could be increased still further. The residual sucrose levels in experiment CR79/7.8.89 were quite high, with almost 25% of the sucrose entering the fermenter in the feed not being consumed by the cells. This alone implied that the fermentation results could be improved. High residual sucrose levels should be avoided as they can lead to excess dextran formation, which in turn can lead to problems with purification of the enzyme. However, the presence of this excess sucrose in the fermentation was the cause of an interesting phenomenon that was detected at this point. The fermentation broth leaves the vessel through the overflow tube and is collected in a 20 litre aspirator bottle. This reservoir is completely uncontrolled in any way, regarding pH, agitation and temperature. The pH of the collected broth would, in fact, decrease steadily to a value as low as 5.2, whilst the temperature would remain at room temperature. There is no mixing within this container. However, when the product collected over a period of approximately 24 hours was shaken and sampled, it was found that its activity was considerably higher than that obtained within the actual fermenter over the period of collection of the enzyme. For example, during the final 20 hours of experiment CR75/10.7.89, the enzyme activity detected within the fermenter, at steady state, was approximately 58 DSU/cm³ (2.79 U/cm³), whereas the enzyme collected over this period had an average activity of approximately 90 DSU/cm³ (4.33 U/cm³). It would appear that the

sucrose remaining in the broth as it leaves the vessel enables the fermentation to proceed by being consumed by the cells to produce more dextransucrase. It would therefore be probable that the greater the residual sucrose level, the greater would be the subsequent enzyme enhancement on hold-up. This phenomenon occurred again during the course of experiment R76/7.7.89 whereby the enzyme collected at an activity of 43.5 DSU/cm³ (2.09 U/cm³) was boosted to a value of 84.2 DSU/cm³ (4.05 U/cm³) following a 24 hour collection period. This factor could prove to be crucial in the course of events regarding the optimization of the continuous production of dextransucrase. It would seem from these results that, if a very basic, uncontrolled container, acting as a second vessel in series with the original fermenter, could cause the enhancement of the resultant enzyme activity by factors such as those mentioned above, then a second, controlled, continuously stirred vessel in series with the first, would boost the enzyme activity by an even greater extent. This factor will be discussed still further, with the collected enzyme being assayed during the subsequent continuous fermentations.

The fermentations listed in Table 8.8 suffered from a problem which occurred as a result of the external environmental conditions of the time. Experiments CR76/17.7.89 and CR79/7.8.89 were operated with a temperature set point of 23.4°C, although the actual operating temperatures were much higher - between values of 24.2 and 26.6°C. Referring back to Section 8.1.2.8, where the effects of high temperatures on fed-batch fermentations were discussed, it is clear that an increase in fermentation temperature would result in an increase in cell growth rate, along with a slight decrease in enzyme activity. This could be the case with the experiments mentioned above which were carried out at high operating temperatures due to the warm external weather conditions that prevailed at the time. The cooling water used for these fermentations was stored under the roof of the Chemical Engineering building at Aston University and was not chilled in any way before passing through the cooling finger of the fermenter. Therefore, high external temperatures would result in a warming of the cooling water such that it would be unable to reduce the temperature of the fermentation to the required value. This was a problem that will occur annually during the warmer periods of the year, unless a chiller unit were to be fitted to the fermenter, to reduce the temperature of the cooling water to a value below the fermentation set point. This would require a certain

amount of capital investment, which would be necessary so that accurate results could be obtained during these fermentations.

The point during a continuous fermentation where the critical dilution rate is approached is an extremely sensitive one. The fermentations can be operated at a particular dilution rate, slightly below the critical point, with maximum enzyme yields being obtained. However, a slight variation in medium flow rate to increase the dilution rate could cause the critical dilution rate to be reached quickly, resulting in cell washout and loss of product. This occurrence has been illustrated by experiment CR79/7.8.89, where the increase in dilution rate from 0.39 h^{-1} to 0.40 h^{-1} caused a drop in enzyme activity from 79.6 to 47.6 DSU/cm^3 (3.83 to 2.29 U/cm^3). The cell growth also decreased somewhat, with OD_{590} values dropping from 0.331 to 0.240 . The residual sucrose level also increased by 100% at this point, indicating that the input of medium to the fermenter was at a rate faster than that at which the cells were capable of growing. The results obtained from this fermentation provide a value for the critical dilution rate of 0.40 h^{-1} . However, during experiment CR80/14.8.89, a repeat of CR79/7.8.89, an activity in excess of 70 DSU/cm^3 (3.33 U/cm^3) was achieved at a dilution rate of 0.41 h^{-1} , whereas at a dilution rate of 0.42 h^{-1} , quite significant cell washout was observed. It can therefore be claimed that under the conditions specified by these particular fermentations, at an agitation rate of 450 rpm and medium composition of $20 \text{ g/l KH}_2\text{PO}_4$ and 40 g/l sucrose , the critical dilution rate lies between values of 0.40 and 0.42 h^{-1} . It should be noted that Paul and co-workers (19) obtained their maximum enzyme yield of 70 DSU/cm^3 (3.37 U/cm^3) at a dilution rate of 0.40 h^{-1} . The combined results obtained from this set of experiments can be seen in Figure 8.8, with the average point at which cell washout occurred being clearly shown to be at a dilution rate of 0.39 h^{-1} .

This section concerned the effects of varying sucrose composition on product formation. Prior to the achievement of approximately 80 DSU/cm^3 (3.85 U/cm^3) of enzyme with a medium sucrose composition of 40 g/l , activities of between 70 and 75 DSU/cm^3 (3.37 and 3.61 U/cm^3) were obtained with a medium sucrose composition of 35 g/l . The residual sucrose levels monitored from these various fermentations have been recorded in Tables 8.5 - 8.8, and it was noticed that, with the exception of the first half of experiment CR63/27.2.89 and the whole of CR68/10.4.89, where the feed medium

contained the alkaline K_2HPO_4 , the residual sucrose levels of the fermentations operated with 35 g/l sucrose medium were considerably lower than those obtained with 40 g/l sucrose medium. For example, experiment CR70/8.5.89, recorded in Table 8.6, which was carried out with a medium sucrose composition of 35 g/l, consumed almost all the available sucrose - with the exception of approximately 4.6 g/l residual sucrose per hour. During this fermentation, the working volume was 1,035 cm³ and with a dilution rate of 0.37 h⁻¹, the volume of medium passing into the fermenter each hour was 383 cm³. The amount of sucrose fed into the vessel was approximately 13.4 g. With the residual sucrose concentration averaging a value of 4.6 g/l during this fermentation, the sucrose level in the broth leaving the fermenter was approximately 1.8 g. Therefore, a total amount of 11.6 g of sucrose was being consumed by this fermentation in the period of one hour. This can be compared to the figures obtained from experiment CR71/15.5.89 (Table 8.6), where cell washout occurred at a dilution rate of 0.50 h⁻¹. The amount of sucrose being passed into the vessel at this point was $(1.035 \times 0.5 \times 35) = 18.1$ g. The residual sucrose level at this time was 19.5 g/l, so that the amount of unconsumed sucrose leaving the fermenter with the enzyme was $(1.035 \times 0.5 \times 19.5) = 10.1$ g. The quantity of sucrose consumed by the cells in this case was only 8 g, considerably less than that consumed at a dilution rate of 0.37 h⁻¹. This again shows that cell washout is occurring, with the lower rate of sucrose consumption indicating a lower cell growth rate. The rate of sucrose consumption can be determined for every dilution rate for which residual sucrose levels have been determined. When the medium sucrose composition was increased to a level of 40 g/l, as with experiments shown in Tables 8.7 and 8.8, further calculations were carried out, with the results given in Table 9.2.

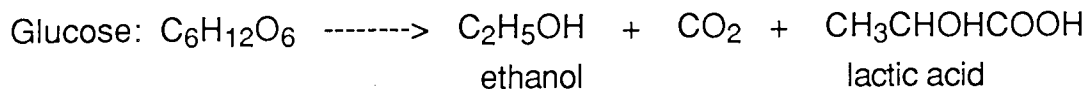
Table 9.2.
Rates of Sucrose Consumption with 40 g/l Sucrose Medium.

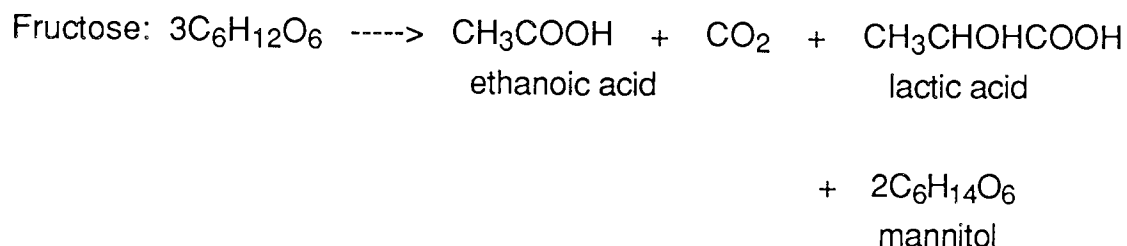
Run Number	Dilution Rate (h ⁻¹)	Sucrose in (g)	Sucrose out (g)	Sucrose consumed (g)
CR73/5.6.89	0.35	14.5	1.6	12.9
CR74/3.7.89	0.39	16.1	3.6	12.5
CR75/10.7.89	0.37	15.3	1.3	14.0
		15.3	2.2	13.1
CR76/17.7.89	0.36	14.9	4.7	10.2
		14.9	0.7	14.2
CR79/7.8.89	0.39	16.1	4.4	11.7
		16.1	2.4	13.7
	0.40	16.6	5.1	11.5

The quantity of unutilized sucrose leaving the fermenter is indicative of the growth rate of the cells, with low levels of sucrose leaving the fermenter being the results of either good cell growth, good product formation, or both.

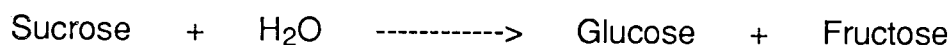
It would be interesting to carry out a simple mass balance at this point to attempt to show the overall reaction that is taking place during the continuous fermentations.

Hetero-fermentative lactic acid bacteria, such as *Leuconostoc mesenteroides*, utilize glucose and fructose as follows:-

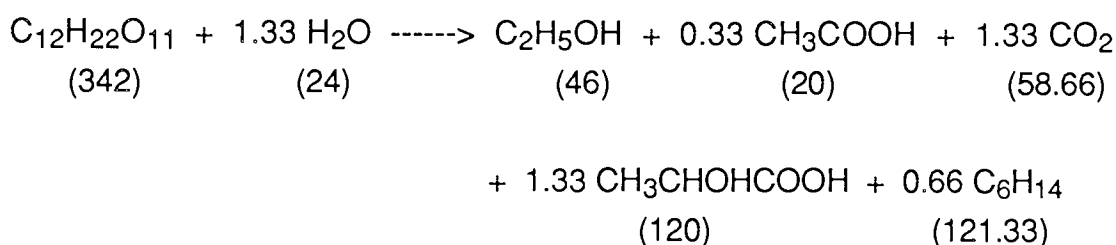




Assuming that sucrose undergoes the following reaction:-

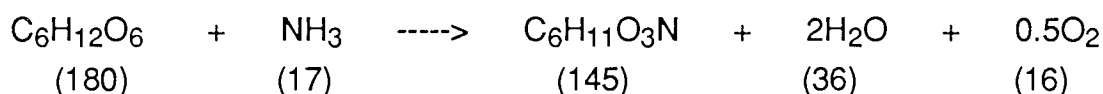


the following balanced equation can be written:-



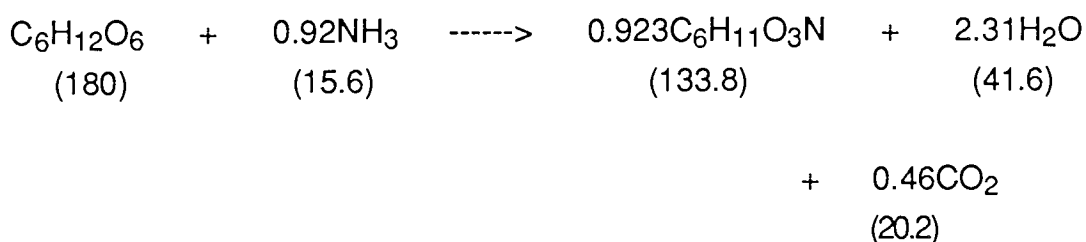
which can provide an estimate of the metabolic spectrum of this particular fermentation.

Under anaerobic fermentation conditions, approximately 10 to 20% of the carbon source is converted to biomass as shown below:-



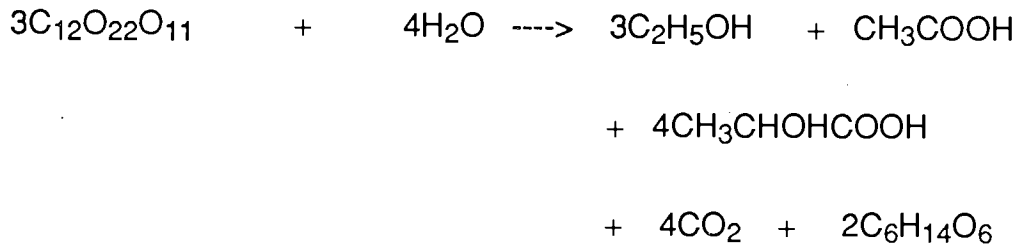
It should be noted here that to obtain 0.5O₂ on the product side is unlikely, but is included as it completes the elemental balance.

However, an alternative method is to suppose that combustion of the biomass takes place, which leads to:-



An assumption can be made that for every six molecules of sucrose that are utilized by the cells for the production of metabolic products, one is utilized for new cell material along with the required product enzyme (108).

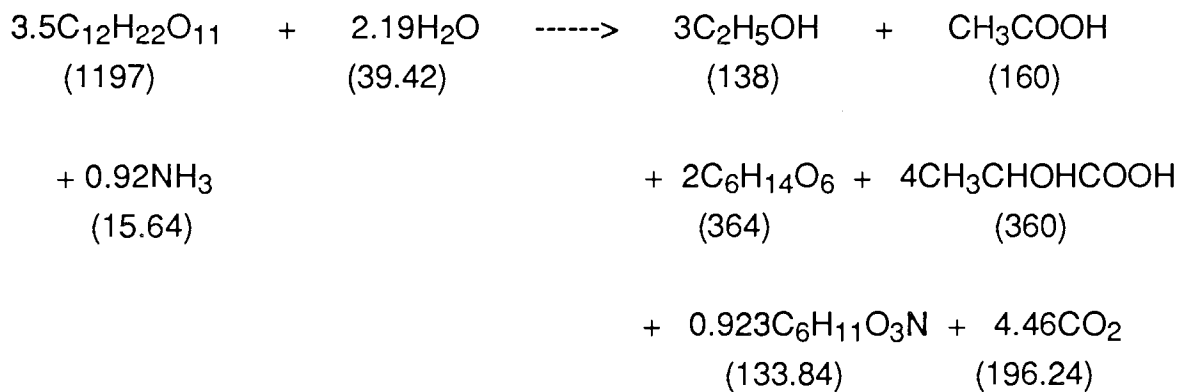
Therefore Metabolic Products:-



And New Cell Material:-



This leads to an overall reaction equation of:-



It should be noted here that dextransucrase production could also be included with both metabolic products and new cellular material, although in this case, the enzyme has been included with the new cell material.

9.1.4. Effects of Varying Agitation Rate on Product Formation.

The data shown in Table 9.2 indicates that, with high sucrose compositions in the feed medium, there are still reasonably high levels of non-utilized sucrose leaving the fermenter, this being one of the reasons for the occurrence of the enzyme enhancement on hold-up phenomenon. It is necessary for there to be some sucrose remaining in the fermentation broth outlet stream because, if the cells were to consume all the available sucrose it is probable that some unwanted cell starvation would occur. It was decided to increase the rate of mixing of the fermentation, by increasing the agitation rate, in order to encourage the cells to grow at higher rates, to consume greater levels of sucrose and, therefore, increase the critical dilution rate of the process. There was though some concern as to whether higher agitation rates would cause shear damage to the cells and product enzyme. However, following the work carried out by Thomas (82) where it was found that, whilst a combination of high levels of agitation and aeration would indeed cause shear damage to the enzyme, it was thought that high agitation rates alone were less likely to cause any damage to the product. The results given in Table 8.9 show the cell growth and dextransucrase activities obtained from a number of fermentations carried out with agitation rates of 700 and 850 rpm. Experiments CR81/2.10.89 and CR82/16.10.89 produced very similar results, with activities of 74.7 and 73.4 DSU/cm³ (3.60 and 3.53 U/cm³) and OD₅₉₀ values of 0.406 and 0.425 being obtained at a dilution rate of 0.39 h⁻¹. In the fermentations previous to this set, which were carried out at agitation rates of approximately 450 rpm, it was found that cell washout usually occurred at, or near, this dilution rate. However, in the case of experiment CR83/23.10.89, the sucrose concentration in the medium was increased to 45 g/l and the dilution rate was increased to levels beyond the previous critical dilution rate of approximately 0.40 h⁻¹. The working volume of the process at the higher agitation rate of 700 rpm was reduced to 950 cm³. This fermentation yielded 108.7 DSU/cm³ (5.23 U/cm³) at a dilution rate of 0.47 h⁻¹ and 105.9 DSU/cm³ (5.10 U/cm³) at a rate of 0.50 h⁻¹. The OD₅₉₀ values recorded at these dilution rates dropped from 0.359 to 0.266 as the medium feed input rate increased, implying that the critical dilution rate was approaching. This was the first incidence of the continuous production of greater than 100 DSU/cm³ (4.81 U/cm³) of enzyme. The results obtained from this, and the previous fermentation, provide a complete range of data and dilution rates between 0.17 and 0.50 h⁻¹ and these results are shown graphically in Figure

8.11. There was also some enzyme enhancement that occurred following a hold-up period of approximately 24 hours, with dextransucrase having its activity boosted from 83.2 DSU/cm³ (4.01 U/cm³) to 155.7 DSU/cm³ (7.50 U/cm³) - an enhancement of almost 100%. This would indicate that there was still some sucrose remaining in the outlet stream from the fermenter that would enable the cells to secrete further quantities of dextransucrase.

The results obtained from this fermentation led to the belief that increasing the agitation rate still further - this also being an increase in the degree of mixing of the fermentation would increase still further the rate of enzyme formation by the cells. However, increasing the agitation rate to a value of 850 rpm resulted in a very severe reduction in enzyme activity, with only 34.2 DSU/cm³ (1.65 U/cm³) and 39.0 DSU/cm³ (1.88 U/cm³) being produced from experiments CR84/6.11.89 and CR85/13.11.89, both at dilution rates of 0.50 h⁻¹. These results contradict somewhat the findings of Thomas (82) as it is only the enzyme activity that is significantly reduced at the higher agitation rate, with the cell growth being reasonably consistent with those obtained at the agitation rate of 700 rpm. It would appear therefore that shear damage would occur to the enzyme at very high agitation rates. It is necessary to compare the tip speeds and power input to the fermenter at the different agitation rates of 450, 700 and 850 rpm and this will be discussed in the next section.

After this point, a number of fermentations were carried out in an attempt to reproduce the results obtained from experiment CR83/23.10.89, with the data recorded from these fermentations shown in Table 8.10. These experiments failed to produce the high enzyme yields that had been expected, although the reasons for this are not completely understood. There could have been some problems with the quality of the culture. As in many of the fermentations, the OD₅₉₀ values were reasonably high, yet the resultant activities were very poor. This could have been caused by some mutation of the culture during storage. However, previous to the sterilization of the fermenter for experiment CR91/15.1.90, the pH probe was regenerated by immersing it in a solution of HCIF for one minute. The probe was then recalibrated using fresh buffers, and was found to have previously been giving high values, of approximately 0.6 of a pH unit too high. This would mean that, during a number of fermentations, the actual operating pH was approximately 6.0 - a value too low for efficient dextransucrase production,

favouring instead dextran synthesis, which has an optimum pH of 5.2. However, it was not until experiment CR103/12.3.90, that the fermentation pH was checked using a manual pH probe, with the actual pH being found to be, again, only 6.0. It was realised at this point that the pH probe had reached the end of its useful life and so a new probe was purchased and used during experiment CR104/23.4.90 and all following fermentations. The results obtained from this fermentation are shown in Table 8.12 and Figure 8.12, and provide a good profile of the process between dilution rates of 0.40 and 0.55 h⁻¹. The results obtained at dilution rates above 0.50 h⁻¹ were, in fact, from a separate fermentation to CR104/23.4.90, carried out by Mr. P. Zanganas in conjunction with the author and are included in Table 8.12 and Figure 8.12 to demonstrate cell washout. It was possible to obtain an enzyme activity of 97.6 DSU/cm³ (4.70 U/cm³) from experiment CR104/23.4.90 at a dilution rate of 0.48 h⁻¹, results very similar to those obtained from experiment CR83/23.10.89. CR104/23.4.90 also shows that the critical dilution rate of the fermentation under the conditions specified, i.e. agitation rate of 700 rpm, lies at a value of approximately 0.50 h⁻¹ as indicated by the dramatic reduction in enzyme yield at this point.

There now follows a discussion concerning the tip speeds of the agitator at the various stirrer speeds used, along with the power inputs to the fermenter at these points, in order to determine the effects these parameters could have on cell growth and production formation from the fermentation. The various scale-up parameters have already been discussed in Section 7.1.2.6, in which the importance of such factors as tip speed, power number, power exerted per unit volume and the ratio of impeller turbulent shear stress to impeller pumping power were mentioned. Table 7.4 lists the results of these various parameters with respect to the MBR vessel when operated at agitation rates of 450 and 700 rpm. These results were compared to the values obtained from the New Brunswick vessel in order to determine why it did not appear possible to achieve an enzyme activity of at least 450 DSU/cm³ (21.67 U/cm³) from a fed-batch fermentation in the MBR vessel. Table 9.3 extends the results of Table 7.4 to determine the parameters from the MBR fermenter when operated at an agitation rate of 850 rpm - where dextransucrase production was severely reduced.

Table 9.3
Scale-Down Parameters for MBR Vessel.

Parameter	450 rpm	700 rpm	850 rpm
Impeller Diameter, D	0.0572 m	0.0572 m	0.0572 m
Agitation Rate N (rps)	7.5	11.67	14.17
Tip Speed (ND)	0.429	0.67	0.81
P (kW)	0.077	0.082	0.084
Po	0.30	0.08	0.05
N/D	131.1	204.0	247.7
P/V	0.074	0.086	0.091

The tip speeds of the agitator at the various speeds used are shown in Table 9.3, with the tip speed of the impeller at 850 rpm being almost twice that at 450 rpm. All three values are much greater than the tip speed of the New Brunswick fermenter, indicating some shear damage to the organism. This can also be applied solely to the MBR vessel, with possible shear damage to the organism increasing with increasing agitation rate. Although this is only to be expected, it could be possible that the tip speed at 850 rpm is sufficient to significantly damage the cells such that they would produce considerably less enzyme.

The P/V values at the various agitation rates used are given in Table 9.3 and are slightly higher than that calculated for the New Brunswick vessel. As the P/V value is representative of the aeration capacity of the fermenter, it can be assumed that even at the lower agitation rate of 450 rpm, the MBR vessel is transferring a greater amount of oxygen to the cells than is the New Brunswick fermenter under conditions designed to produce optimum enzyme yield. As this particular fermentation is an unaerated process, this is obviously undesirable, leading to more aerated conditions in the MBR vessel and, hence, lower enzyme yield.

The power numbers calculated for the vessel at the various agitation rates decrease with increasing impeller speed. It has already been stated that the higher the power number, the less turbulent the culture fluid within the fermenter, implying that at the highest agitation rate the solution would be in a state of greater turbulence than at the lower rates. Again this factor appears obvious but, as with the tip speeds, could indicate that sufficient shear damage was occurring at the high agitation rate of 850 rpm to prevent high enzyme yields.

The N/D values for each agitation rate are also shown in Table 9.3, with values increasing with increasing impeller speed, again as would be expected. The higher the N/D value, the lower the DOC of the fermentation at a given aeration rate, with lower oxygen levels within the fermenter being preferred for the production of high yields of dextransucrase. Theoretically, therefore, when operated at the higher agitation rate, there would be less oxygen transfer to the cells. However, under conditions of no aeration this parameter becomes less important.

All of the various scale-down/up parameters shown in Table 9.3 are very similar to each other, with the main differences occurring with respect to the tip speed and power number. The tip speed varies as $450 < 700 < 850$ rpm, whilst the power number varies as $450 > 700 > 850$ rpm. It would seem that, generally, the higher tip speeds and lower power numbers obtained with the MBR fermenter would account for the lower enzyme yields from the MBR vessel when compared to the New Brunswick vessel. The lower enzyme yields with the MBR vessel are probably due to some cell shear damage occurring in the smaller vessel. It would appear, therefore, that the enzyme yield at 700 rpm should be less than at 450 rpm due to increased shear damage to the cells. However, the increased mixing occurring throughout the medium as observed by the increase in critical dilution rate from approximately 0.40 h^{-1} at 450 rpm to approximately 0.50 h^{-1} at 700 rpm, would compensate for any extra shear damage that would occur at the higher agitation rate. However, at an agitation rate of 850 rpm, it would appear that any improvement in the mixing capacity of the fermenter is eliminated by increased shear damage to the cells caused by the higher tip speed. This phenomenon is not obvious from the results of the scale-down calculations shown in Table 9.3 and can only be deduced from the experimental results obtained.

9.2. Modelling of the Continuous Fermentation.

The continuous process was modelled using the ISIM Interactive Simulation Language, which is copyright of Simulation Sciences (1983), Worsley, Manchester, and is marketed by Salford University Research Ltd. The basic ISIM structure is in three regions; the INITIAL, DYNAMIC and TERMINAL regions, along with an optional CONTROL region. The INITIAL region is used for setting the various initial values and constants. The DYNAMIC region contains the model equations, and the TERMINAL region is used for end of run calculations.

9.2.1. Determination of Model Equations.

The model equations used for the simulation were determined from carrying out material balances across the fermenter with respect to cell, product and substrate concentrations. It was important here to ensure that each balance equation was linearly independent of the others, such that no balance equation could be formed by adding together any combination of the others. Each balance equation was written using the basic model given below:-

$$\begin{array}{rclcl} \text{Rate of} & & \text{Rate of} & & \text{Rate of} \\ \text{accumulation} & = & \text{input to} & - & \text{output to} \\ \text{in vessel} & & \text{vessel} & & \text{vessel} \end{array} \quad 9.1$$

Input terms consisted generally of inlet flow and generation factors, whilst output terms were usually outlet flow and consumption factors. The rate of accumulation of a component, e.g. cells - denoted by X - can be written very simply as $d(VX)/dt$ - where V is the volume of the fermenter. This term describes how the concentration of cells within the vessel changes with time, although in the case of this continuous process, the working volume is constant. The volume term can then be removed from the differential term, giving

$$\frac{VdX}{dt} = \text{cell accumulation}$$

This can be similarly applied for substrate, S, and product, P, accumulation. The balance equations can be determined for each separate factor of the fermentation.

(i) Cells.

$$\begin{aligned} \text{Input terms :} & \quad \text{Flow in} = F_i X_i \\ & \quad \text{Generation} = r_x V \\ \text{Output terms :} & \quad \text{Flow out} = F_o X_o \\ \text{giving} \quad \frac{VdX}{dt} & = F_i X_i + r_x V - F_o X_o \end{aligned} \quad 9.2$$

where $F_{i,o}$ = Medium flowrate into/out of the vessel
 r_x = rate of cell growth, $\text{kgm}^{-3}\text{h}^{-1}$.

(ii) Substrate.

$$\begin{aligned} \text{Input terms :} & \quad \text{Flow in} = F_i S_i \\ \text{Output terms :} & \quad \text{Flow out} = F_o S_o \\ \text{Consumption} & = r_{sx} V \\ \text{giving} \quad \frac{VdS}{dt} & = F_i S_i - F_o S_o - R_{sx} V \end{aligned} \quad 9.3$$

where r_{sx} = rate of substrate consumption, $\text{kgm}^{-3}\text{h}^{-1}$.

(iii) Product.

$$\begin{aligned} \text{Input terms :} & \quad \text{Flow in} = F_i P_i \\ & \quad \text{Generation} = r_p V \\ \text{Output terms :} & \quad \text{Flow out} = F_o P_o \\ \text{giving} \quad \frac{VdP}{dt} & = F_i P_i - r_p V - F_o P_o \end{aligned} \quad 9.4$$

where r_p = rate of product formation, $\text{kgm}^{-3}\text{h}^{-1}$.

These equations can all be simplified as inlet and outlet flows are all equal (i.e. $F_i = F_o$) and the inlet streams contain no cells or product (i.e. $X_o = P_o = 0$). Equations 9.2 - 9.4 therefore become :-

$$\frac{dX}{dt} = r_x - \frac{FX}{V} \quad 9.5$$

$$\frac{dS}{dt} = \frac{F(S_i - S)}{V} - r_{sx} \quad 9.6$$

$$\frac{dP}{dt} = r_p - \frac{FP}{V} \quad 9.7$$

As the vessel is assumed to be perfectly mixed, the outlet stream has the same composition as the vessel contents, so that $X_o = X$, $S_o = S$ and $P_o = P$.

The dilution rate, D , is defined as F/V , so that these equations become :-

$$\frac{dX}{dt} = r_x - DX \quad 9.8$$

$$\frac{dS}{dt} = D(S_i - S) - r_{sx} \quad 9.9$$

$$\frac{dP}{dt} = r_p - DP \quad 9.10$$

The rate terms, r_x , r_{sx} and r_p can all be expressed in terms of growth rate, μ , and specific rate coefficients $Y_{x/s}$ and $Y_{p/x}$, where $Y_{x/s}$ is (kg cells/kg substrate) and $Y_{p/x}$ is (DSU enzyme/kg cells). Therefore :-

$$r_x = \mu X \quad 9.11$$

$$r_{sx} = \frac{\mu X}{Y_{x/s}} \quad 9.12$$

$$r_p = \mu X Y_{p/x} + K_p X \quad 9.13$$

Equation 9.13 is an example of a Leudeking-Piret equation, whereby the rate of product formation is a function of both cell growth and cell concentration. This kinetic expression has proved extremely useful and versatile in fitting product formation data from many different fermentations. This has been found to be an expected kinetic form when the product is the result of an energy yielding metabolism as in several anaerobic fermentations. In the case of dextransucrase production, the value of K_p , the Leudeking-Piret Constant, is not known, and so values of between -0.1 and -2.0 have been tested in the model. The results from these simulations are shown in Appendix B.

During a dextransucrase fermentation, the cell growth is assumed to follow simple Monod kinetics in which there is no inhibition and where the substrate, sucrose, is the only limiting component. Therefore, μ is found using:-

$$\mu = \frac{\mu_{\max}S}{K_s + S} \quad 9.14$$

where K_s is the substrate saturation constant.

As already mentioned:-

$$D = \frac{F}{V} \quad 9.15$$

Equations 9.8 - 9.15 are those used in the model to simulate the continuous fermentation of dextransucrase. The model will calculate the values of the various parameters as they change with time until, as is the case with continuous fermentations, the point of steady state is reached, at which:-

$$\frac{dX}{dt} = \frac{dS}{dt} = \frac{dP}{dt} = 0$$

The results obtained will, therefore, remain constant throughout the rest of the fermentation.

The method of determining the values of the kinetic parameters, K_s , $Y_{p/x}$ and $Y_{x/s}$ is discussed in the following section. A sample program, together with simulation results, is shown in Appendix B.

9.2.2. Determination of Kinetic Parameters.

A number of plots were made using the results obtained from fed-batch fermentations carried out during the course of this project. Previous to this point, the majority of the results that have been presented graphically have been plotted with respect either to time or dilution rate. In this section, the different yields have been plotted against each other in order to determine the values of $Y_{p/x}$ and $Y_{x/s}$ as used in the model.

A plot of enzyme activity, P, against total cell concentration, X, at any point is shown in Figure 9.1. This graph produces a straight line with a gradient of 98.05, which becomes the value of $Y_{p/x}$. However, the results shown in Figure 9.1 were obtained from fed-batch fermentations in which there were higher yields of both enzyme and cells. During the fed-batch experiments, the cells are in an exponential state of growth, producing high quantities of enzyme. During continuous fermentations, the cells grow at very high rates,

with μ values up to 0.50 h^{-1} , although due to the high dilution rates used, the cells do not remain in the vessel for long periods of time and, therefore, do not have the opportunity to secrete large amounts of enzyme. During the continuous section of this project, the highest activity achieved was 108.7 DSU/cm^3 (5.23 U/cm^3), which corresponded to an OD_{590} value of 0.359, or a dry cell weight of 3.08 g/l . These results give a $Y_{p/x}$ value of only 35.3 - considerably lower than the value determined from the fed-batch experiments. It would be interesting to compare the results obtained from the model using these two values of $Y_{p/x}$, with the greater $Y_{p/x}$ value producing theoretical results and the lower $Y_{p/x}$ value producing results that would be more likely to be achieved continuously in this particular vessel. It has already been discussed that the MBR vessel appears to be incapable of producing high yields of dextransucrase when compared to the New Brunswick vessel.

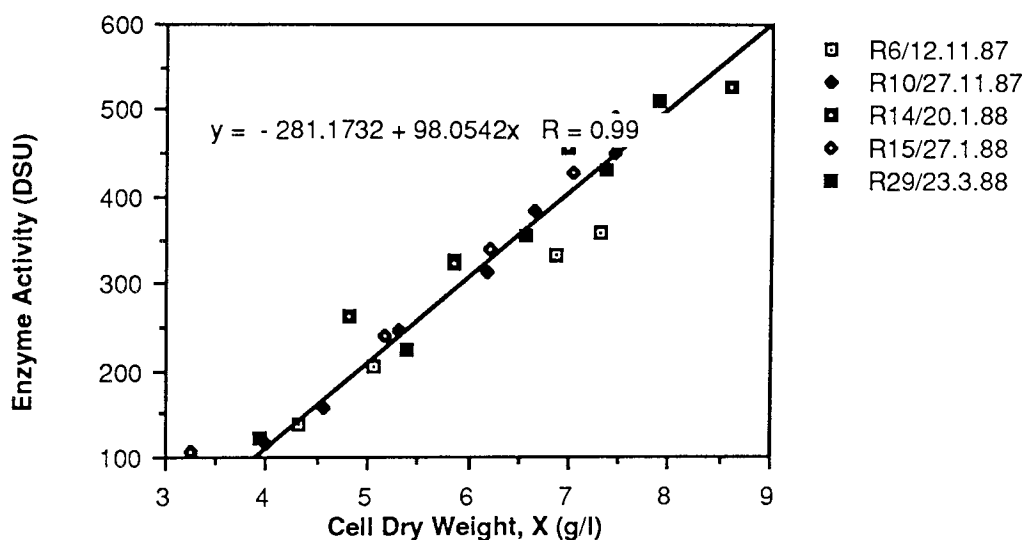


Figure 9.1: Graph to determine $Y_{p/x}$ value (from 6 litre fed-batch fermentations)

The value for $Y_{x/s}$ as used in the model was determined using experimental results obtained from fed-batch fermentations carried out in the New Brunswick vessel. This constant compared the total cell dry weight obtained from the fermentation with the total sucrose consumed during the fermentation. This takes into account the fact that the working volume of the fermentation increases quite considerably during a fed-batch process. During a successful fermentation where enzyme activities in excess of 450 DSU/cm³ (21.67 U/cm³) were achieved, experimental results indicated that a maximum cell dry weight of approximately 9 g/l was achieved. However, as the working volume of the fermentation itself had increased at this point - from 6 litres at the start of the process, to approximately 8 litres by the end - the total cell dry weight was determined to be (9 x 8 =) 72 g of cellular material. This can be divided by the total sucrose consumed during the fermentation, which again can be determined from experimental data. During unaerated fermentations, the total sucrose consumed was quite high, with values up to 1000 g being recorded, and so that value will be used for the purposes of this calculation. It should be noted here that this is indeed only an assumption used for this purpose and, as such, could be a cause of some error in the model. These figures give a $Y_{x/s}$ value of (72/1000 =) 0.072, which is used in the model. These values can be compared to those obtained earlier from the mass balance that was carried out over the process and where 1197 g of sucrose produced 134 g of cells - giving a $Y_{x/s}$ value of (134/1197 =) 0.11 - which agrees quite well with the experimentally produced value for $Y_{x/s}$.

The determination of the K_s value for the system proved to be somewhat difficult in this case. As previously defined, K_s is the substrate saturation constant or, in other words, the concentration of the limiting substrate in the medium that will produce a cell growth rate, μ , equal to half the maximum growth rate, μ_{max} . Ajongwen-Numfor⁽¹¹⁾ claimed that μ_{max} for *Leuconostoc mesenteroides* was $0.34 \pm 0.08 \text{ h}^{-1}$. However, the process has been operated continuously at a dilution rate of approximately 0.50 h^{-1} and, as during a continuous fermentation, $D_{crit} = \mu_{max}$, this value has been taken as the maximum growth rate of the culture. The value used by Ajongwen-Numfor in his model of the fed-batch process was $K_s = 0.11$ and, in the absence of other data, this value will also be used in the model of the continuous process.

The model presented in the Appendix is intended to be only a very preliminary attempt to simulate the process and does not take into account certain factors such as the phenomenon of the enzyme being mainly produced during the linear growth period of the cells, with very little being produced during the initial hours of the fermentation. This could alter the value of $Y_{P/X}$ somewhat. The tailing off of activity at the end of a fed-batch fermentation has not been discussed in detail either, although the negative value of the Leudeking-Piret constant appears to point to this. This model requires more extensive work to enable it to more accurately predict experimental results.

The working volume used in this model was 0.95 l, which was the case when the fermenter was operated at an agitation rate of 700 rpm and under which conditions the maximum enzyme yields were obtained.

The results from this model at various values of K_p are given in graphical form in Appendix B2, together with some actual experimental results included for comparison.

10. Conclusions and Recommendations for Future Work.

10.1. Conclusions.

The following conclusions can be drawn from the results of this research work:-

10.1.1. Fed-Batch Fermentations.

1. The findings of Ajongwen-Numfor (11), that higher yields of dextransucrase could be produced under unaerated conditions compared to the highly aerated systems of Schneider (10,12) *et al*, were confirmed. Enzyme yields of between 450 and 500 DSU/cm³ (21.66 and 24.07 U/cm³) were regularly produced under unaerated conditions, whereas activities of only 235 to 255 DSU/cm³ (11.31 and 12.27 U/cm³) were achieved with high levels of aeration. However, cell growth continued to increase for several hours following the achievement of maximum enzyme activity. It is possible that dextransucrase production is inhibited in some way by high dextransucrase and oxygen levels.

2. Different yeast extract types, such as DCL, Orhly, Gistex powder and Gistex paste, were found to significantly affect the fermentation results, with Gistex paste supporting the highest levels of cell growth and enzyme formation. A number of peptones, such as Bacteriological Peptone, Peptone P, Special Peptone and Casein Hydrolysate, were also used as the medium nitrogen source instead of the Gistex yeast extract, but all failed to support good cell growth and enzyme production.

3. A large variability was found between Gistex batches for dextransucrase fermentations, with certain batches producing only 270 DSU/cm³ (13.00 U/cm³). A detailed analysis was carried out of various Gistex batches with respect to their concentration of trace vitamins, amino acids and carbohydrates. However, it proved to be very difficult to accurately pinpoint the specific factors that were either limiting cell growth or enzyme formation, or which had the ability to enhance fermentation performance, as there were a large number of differences between the compositions of the various batches.

4. Different stirrer speeds were used during fed-batch fermentations to determine the effect this would have on overall enzyme production. Increased agitation from 100 rpm to 450 rpm did not cause a significant reduction in final

dextranucrase activity with the higher agitation rate still producing in excess of 415 DSU/cm³ (19.98 U/cm³) of enzyme.

5. A number of fermentations, both aerated and unaerated, were carried out with the addition of approximately 2 cm³ of a silicone antifoaming agent to the medium before sterilization. Aerobic fermentations with low levels of aeration and agitation were largely unaffected by antifoam addition as there was insufficient mixing to adequately disperse the antifoam throughout the medium. However, under conditions of higher aeration, the antifoam was much more thoroughly mixed throughout the medium and had the effect of reducing the oxygen transfer capability of the fermentation, thus increasing the resultant enzyme activity. During unaerated fermentations, where there were low levels of agitation, there was again insufficient mixing to disperse the antifoam throughout the medium, so there was little effect on the overall fermentation performance. However, with higher agitation conditions, the antifoam became well mixed into the medium and served to drastically reduce the final enzyme activities attained, by somehow preventing enzyme formation or secretion.

6. A series of fermentations were carried out in which either 0.003 g/l or 0.0206 g/l folic acid was added to the medium to determine whether any enhancement or inhibition of enzyme activity would occur. The final results depended very much on the Gistex batch used in the various media, but in general, with a Gistex batch capable of producing in excess of 450 DSU/cm³ (21.67 U/cm³) of enzyme, folic acid addition of either concentration mentioned served to reduce the final enzyme yield. However, with a Gistex batch that appeared incapable of producing high enzyme yields, the addition of folic acid seemed to slightly enhance enzyme activity, with the higher level of folic acid producing slightly greater enzyme enhancement than the lower level of folic acid. It is possible that folic acid is a limiting component in dextranucrase fermentations, although it can cause inhibition in high concentrations.

7. A number of Industrial Scale fermentations were carried out in two fermenters - a 1,000 litre seed vessel located at Holmes Chapel, and 800 litre BioLafitte vessel at Birmingham University. It was shown that it was possible to successfully produce approximately 450 DSU/cm³ (21.67 U/cm³) of enzyme using the 1,000 litre vessel, with this enzyme immediately being transferred to a 50,000 litre vessel to produce clinical dextran of a similar

quality to that currently being manufactured using the whole-cell method. The cost savings using this cell-free method were determined to be at least £637 per batch of dextran. However, it has not yet been possible to consistently produce these high levels of dextransucrase in the 1,000 litre vessel. The 800 litre vessel at Birmingham University, yielded only 130 DSU/cm³ (6.26 U/cm³), but subsequent scale-up calculations have indicated that it should be possible to produce high enzyme yields in this vessel providing there is sufficient mixing.

8. The process was also scaled down to a 2.5 litre MBR vessel, which was found to be incapable of producing high enzyme yields. These findings were confirmed by the scale-down calculations where high agitator tip-speeds appeared to be causing some shear damage to the cells.

9. A small number of fermentations were carried out in both the New Brunswick 16 litre and MBR 2.5 litre vessels, using the more acidic KH₂PO₄ as opposed to the alkaline K₂HPO₄ which was used in the media to produce high enzyme yields. With both vessels, the use of KH₂PO₄ served to reduce the final enzyme activity by approximately 140 DSU/cm³ (6.74 U/cm³) and was therefore not used for fed-batch fermentations.

10.1.2. Continuous Fermentations.

1. The sterilization procedure for the preparation of the feed medium was found to be a crucial aspect for the continuous fermentation of dextransucrase. The long, uncontrolled sterilization cycle of a 5 litre aspirator bottle of medium in the Adelphi autoclave, where the temperature reached a value of 125°C, was found to result in severe medium degradation due, in the main, to destruction of the yeast extract. The more controlled method of manually controlling the sterilization temperature between 115 and 117°C, and maintaining this value for only 10 minutes, greatly improved medium quality and led to increased enzyme activities being produced. However, this sterilization technique was still very time consuming, with long cool-down periods being observed. A more rapid sterilization procedure was then developed, where the medium was sterilized in the New Brunswick vessel and then quickly cooled before transfer to the aspirator bottles which had been sterilized empty in the autoclave. This eliminated the long cool-down period, improved still further the quality of the medium and allowed a greater quantity of medium to be prepared in a shorter period of time.

2. During experiments operated at high dilution rates, in excess of 0.30 h^{-1} , it was not possible for the cells to produce sufficient acid during growth to compensate for the high volumes of alkaline medium being added and still maintain the operating pH at, or below 6.65. The K_2HPO_4 used as the phosphate source in the medium was then substituted with the more acidic KH_2PO_4 . This change resulted in a significant lowering of the feed medium pH, allowing the fermentation broth to be controlled at a pH of 6.65 with the addition of 5M NaOH. The final enzyme activities were also significantly improved with the use of KH_2PO_4 in the medium, thus contradicting the findings of the fed-batch fermentations.

3. The sucrose concentration in the feed medium was increased from a level of 20 g/l to 45 g/l, with each increase resulting in a subsequent improvement in overall enzyme yield. Sucrose levels of 40 g/l in the medium gave enzyme yields of approximately $80\text{ DSU/cm}^3/\text{h}$ ($3.85\text{ U/cm}^3/\text{h}$) at dilution rates of between 0.37 and 0.39 h^{-1} . These results surpassed the previous best found in the literature by approximately $10\text{ DSU/cm}^3/\text{h}$ ($0.48\text{ U/cm}^3/\text{h}$).

4. The Gistex batch used in the medium was altered to determine its effects on the overall productivity of the fermentations. The various batches tested did not appear to affect the enzyme yield as greatly as they had affected the fed-batch fermentations. This was due to the fact that the enzyme yields from the continuous process were considerably lower than those from the fed-batch process, such that a slight alteration in enzyme yield from the continuous mode would be less noticeable than from the fed-batch. Therefore, Gistex quality is a less critical factor for continuous fermentations than for fed-batch.

5. The agitation rate of the fermentation was increased from 450 rpm, where $80\text{ DSU/cm}^3/\text{h}$ ($3.85\text{ U/cm}^3/\text{h}$) of enzyme had been produced at dilution rates of $0.37 - 0.39\text{ h}^{-1}$, to 700 rpm, where $105\text{ DSU/cm}^3/\text{h}$ ($5.06\text{ U/cm}^3/\text{h}$) of enzyme were produced at dilution rates of between 0.47 and 0.50 h^{-1} . The higher agitation rate, despite causing more shear damage to the cells than the 450 rpm rate, significantly improved the mixing of the fermenter, thus utilizing greater amounts of sucrose. Increasing the agitation rate still further caused more shear damage to the cells, which was not compensated for by improved mixing conditions. Therefore, a critical dilution rate of approximately 0.50 h^{-1}

was determined with an agitation rate, corresponding to a maximum theoretical growth rate value, μ_{\max} , also of 0.50 h⁻¹.

6. During continuous operation, the enzyme was collected in a reservoir, where it remained standing for approximately 24 hours. It was noticed that the cells continued to grow, even under these static and uncontrolled conditions, producing more enzyme. The cells consumed any residual sucrose remaining in the medium, with the improved enzyme yield greatly surpassing the activity being produced within the vessel. For example, enzyme which had an activity of approximately 90 DSU/cm³/h (4.33 U./cm³/h) during continuous operation, had its activity boosted to 155.7 DSU/cm³ (7.50 U/cm³) following 24 hours of hold-up.

7. It was found possible to operate a fermentation continuously, whilst maintaining sterility at all times, for periods of up to 360 hours. This shows that it is perfectly possible to continuously produce high yields of dextransucrase for long periods, providing great care is taken to maintain complete sterility, thus eliminating inefficient down-times which occur regularly during fed-batch fermentations when the fermenter is emptied, cleaned and re-sterilized. A continuous fermentation of 360 hours duration producing 100 DSU/cm³/h (4.81 U/cm³/h) of enzyme would give a total enzyme yield of 36,000 DSU/cm³ (1,733.3 U/cm³) in the MBR vessel. During the same time period, approximately 15 fed-batch fermentations could take place - assuming 16 hour fermentation and 8 hour turnaround time - which would give only 6,750 DSU/cm³ (325.0 U/cm³) of dextransucrase, assuming each fermentation yielded 450 DSU/cm³ (21.67 U/cm³). Therefore, a successfully operated continuous fermentation should be much more efficient than a number of successfully operated fed-batch fermentations.

10.2. Recommendations for Future Work.

The following recommendations were made for future work on this project:-

1. Carry out further analyses of Gistex batches used during fed-batch operation in order to accurately pinpoint the specific limiting nutrients for the growth of *Leuconostoc mesenteroides* and production of dextransucrase.

2. Carry out fermentations using peptones, followed by broth analysis to determine the various components that had not been utilized during the fermentation.
3. Carry out further large-scale fermentations to improve the consistency of obtaining high enzyme yields. In particular, it would be useful to repeat the fermentation using the highly controlled BioLafitte vessel at Birmingham Univeristy which should be capable of producing high enzyme yields.
4. Carry out further continuous experiments with increased sucrose concentrations. If necessary, with sucrose levels of above 60 g/l, the sucrose should be sterilized separately from the other medium components.
5. Carry out continuous fermentations under aerobic conditions to investigate the results thus obtained.
6. Carry out continuous fermentations with two vessels in series which should allow enzyme enhancement on a more scientific basis than has been reported in this thesis. This should allow for enzyme yields of at least 150 - 200 DSU/cm³/h (7.22 - 9.63 U/cm³/h).
7. Carry out continuous experiments with cell recycle back to the fermenter. This will increase the cell concentration in the culture fluid, leading to improved enzyme production in the fermenter.
8. Attempt to scale-up the continuous process to a fermenter capable of producing higher yields of enzyme. This will result in larger volumes of dextransucrase being produced in specific periods of time. This should therefore reduce the number of Industrial Scale dextransucrase fermentations that would need to be carried out in order to produce sufficient enzyme for the manufacture of the required amount of clinical dextran, thus reducing still further the overall cost of commercial dextran production.

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NOMENCLATURE

a	Gas liquid interface area/unit volume	(cm ² /cm ³)
ADP	Adenosine diphosphate	
AMP	Adenosine monophosphate	
ATP	Adenosine triphosphate	
C _{1,3,6}	1st, 3rd, 6th carbon of carbohydrate molecule	
C _L	Dissolved oxygen concentration in the culture	(mmole/l)
C*	Saturated dissolved oxygen concentration	(mmole/l)
d	Dilution factor of enzyme	
D	Impellor diameter	(m)
D	Decimal reduction time	(h)
D	Dilution rate	(h ⁻¹)
D _{crit,c}	Critical dilution rate	(h ⁻¹)
DEAE	Diethyl amino ethyl group	
DNA	Deoxyribonucleic acid	
DOC	Dissolved oxygen concentration	
DSU	Dextranucrase unit	
E	Activation energy	
ES	Enzyme - substrate complex	
E _T	Total enzyme concentration	
F	Faraday's constant	
F	Known cell number	
F _{i,o}	Medium flowrate into and out of vessel	
FAD	Flavin adenin dinucleotide	
FMN	Flavin mononucleotide	
ΔG	Free energy change	

GLC	Gas liquid chromatography	
GPC	Gas permeation chromatography	
G-1-P	Glucose-1-phosphate	
HPLC	High performance liquid chromatography	
K	Specific death rate of the organism	(h ⁻¹)
K _L	Mass transfer coefficient	(cm/h)
K _m	Michaelis-Menton constant	
K _S	Saturation or Monod constant	(g/l)
K _{1,-1,2}	Enzyme reaction rates	
K _{La}	Volumetric oxygen transfer rate	
L	Fermenter Height (m)	
K _m	Maintenance coefficient	
M _w	Molecular weight	
n	Flow behaviour index	
n	Number of organism doublings	
n	Number of log cycles during sterilization	
N	Number of viable organisms	
N _{o,t}	Number of viable organisms at start and time t of sterilization cycle	
N	Impellor speed	
NAD	Nicotinamide adenine nucleotide	
NADH	Nicotinamide adenine nucleotide reduced form	
NADP	Nicotinamide adenine dinucleotide phosphate	
N _{Re}	Reynolds number	
OD ₅₃₀	Optical density at 530 nm	
OD ₅₉₀	Optical density at 590 nm	
P	Product concentration	(g/l or DSU)

P	Productivity	
P	Degree of reductance of product	
P	Power exerted by the fermenter	
Pg/V	Volumetric power input	
Po	Power number	
Px	Biomass productivity	(g/l/h)
Δp	Proton motive force	(mV)
ΔpH	Change in pH	
q _p	Product formation rate	(h ⁻¹)
q _s	Substrate utilization rate	(h ⁻¹)
R	Gas constant	
Re	Reynolds number	
RNA	Ribonucleic acid	
R _p	Rate of product formation	(kgm ⁻³ h ⁻¹)
rpm	Revolutions per minute	
R _{sx}	Rate of substrate consumption	(kgm ⁻³ h ⁻¹)
R _x	Rate of cell growth	(kgm ⁻³ h ⁻¹)
S	Residual substrate concentration	(g/l)
S and S'	Substrate concentrations into and out of the culture	(g/l/h)
\bar{S}	Substrate concentration at steady state	(g/l)
S _R	Original substrate concentration	(g/l)
stp	Standard temperature and pressure	
t	Time	(h)
T	Temperature	(°C or K)
T	Fermenter Width (m)	
t _d	Cell doubling time	(h)

U	SI unit of enzyme activity	
UV	Ultra violet	
V	Rate of enzyme reaction	
V	Volume of culture	(l)
V _o	Original volume	(l)
v/v	Volume per unit volume	
vvm	Volume of air per volume of medium per minute	
V _{max}	Maximum rate of enzyme reaction	
w/v	Weight per unit volume	(g/l)
X	Dry cell weight	(g/l)
\bar{X}	Total biomass in the culture	(g)
\bar{X}	Cell dry weight at steady-state	(g/l)
X _o	Number of cells at inoculation	
X _t	Number of cells at time t	
Y	Yield factor	
Y _{p/s}	Product yield coefficient (g product/g substrate)	
Y _{x/s}	Biomass yield coefficient (g cell dry weight/ g substrate)	
Z	Carbon fraction of substrate used for product formation	
Z	(2.3 RT/F)	(mV)

Greek Symbols.

α	Specific death rate	(h ⁻¹)
β	Rate of product denaturation	(h ⁻¹)
γ	Shear rate	
ϵ_p	Number of ATPs generated by product formation	

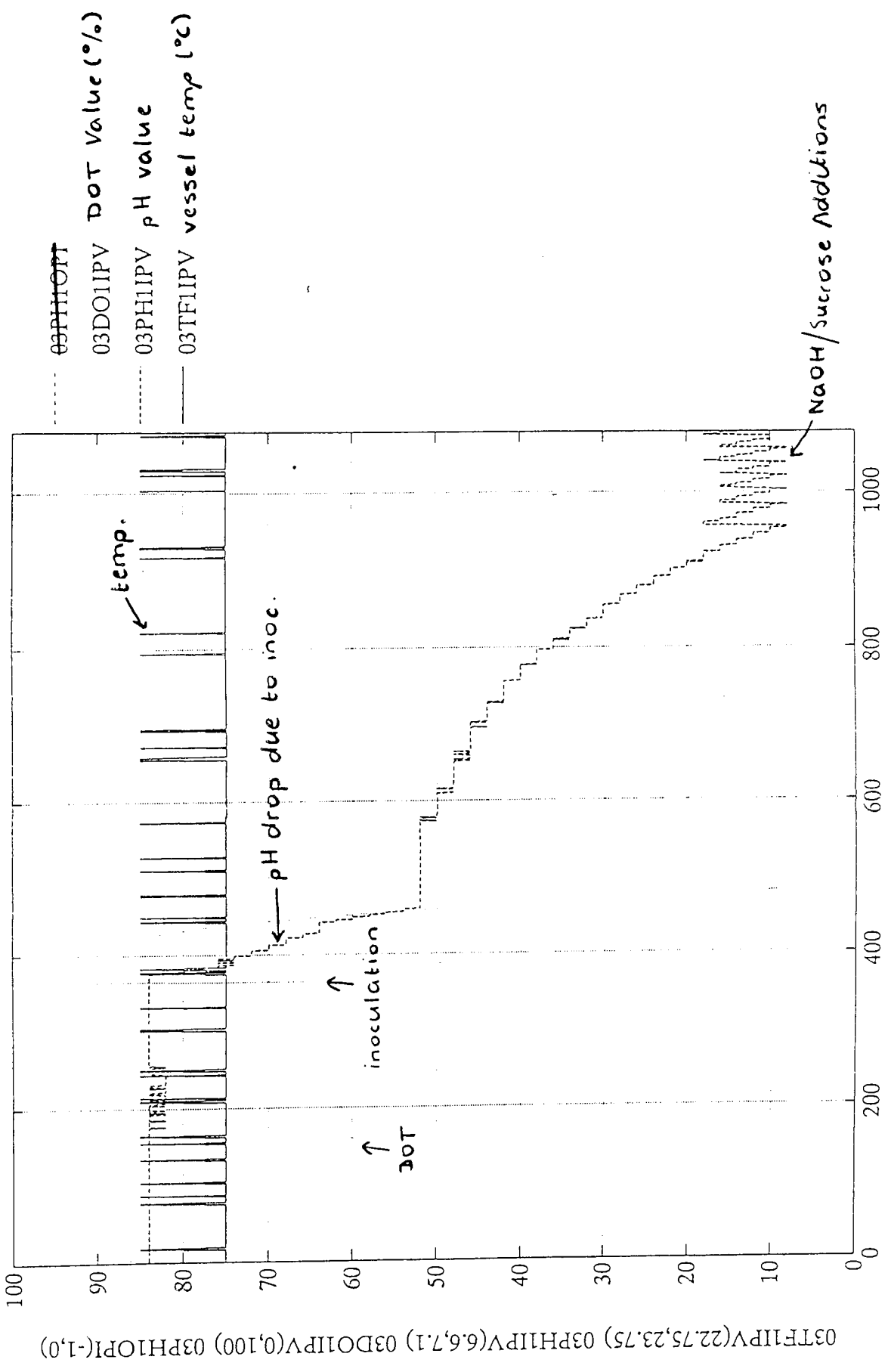
ϵ_s	Number of substrate level phosphorylations per carbon mole	
τ	Shear stress	
τ_y	Yield stress	
ρ	Liquid density	
μ	Cell growth rate	
μ	Viscosity	
μ_a	Apparent viscosity	
μ_{max}	Maximum cell growth rate	(h ⁻¹)
$\Delta\mu_{H^+}$	Difference in electrochemical potential of proteins across cytoplasmic membrane	(Kcal)
$\Delta\phi$	Membrane potential	
πND_i	Impeller tip speed	

APPENDICES

hour
file
Profile

A1

03ASTI PHILIP 25-JUL-90 05:59:00



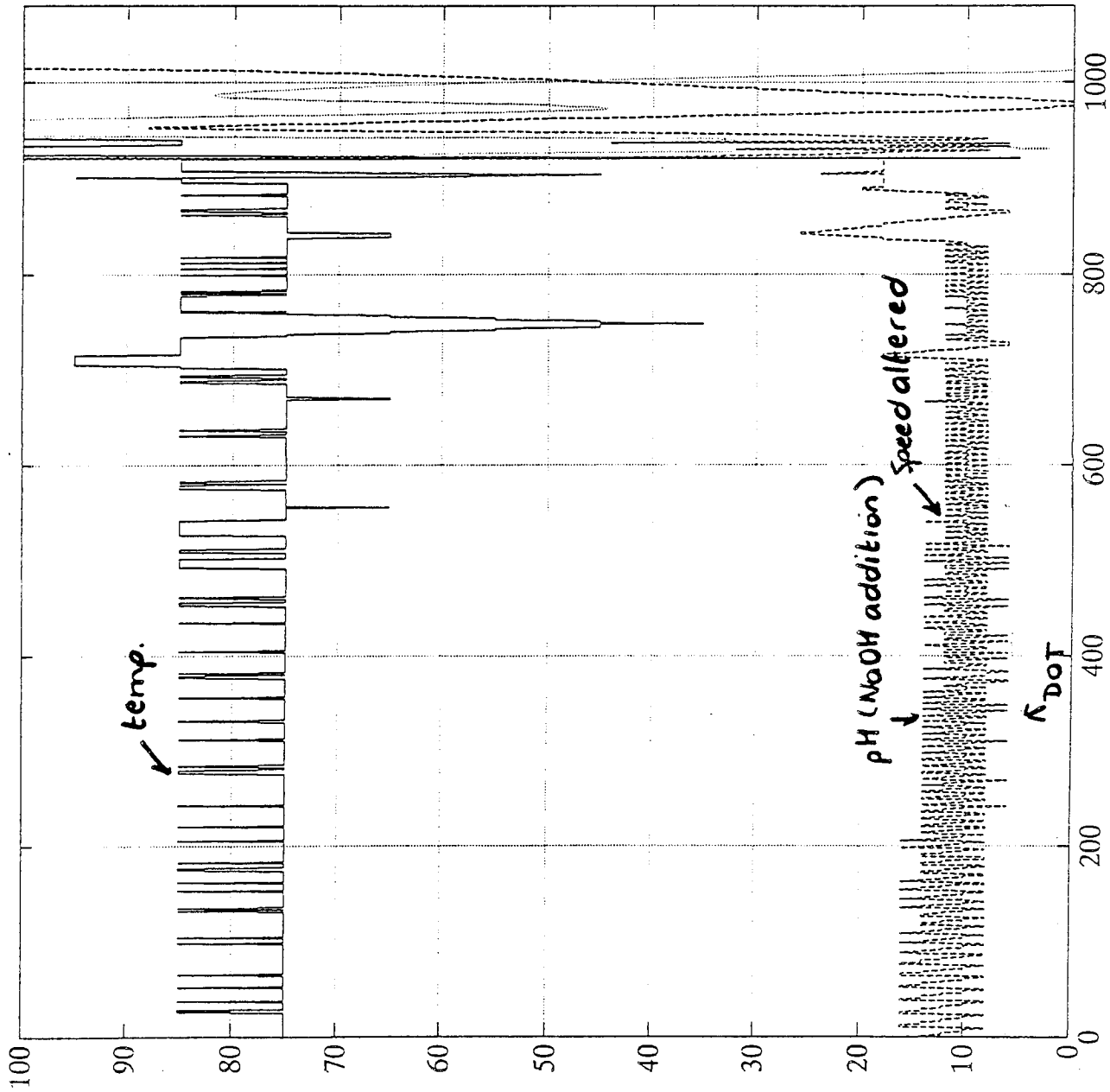
23.59
25/7/90

Time (mins) since 05:59:00

05:59
25/7/90

03AST1 PHILIP 26-JUL-90 00:00:00

03TF1IPV(22.75,23.75) 03PH1IPV(6.6,7.1) 03DO1IPV(0,100) 03PH1OP1(-1,0)



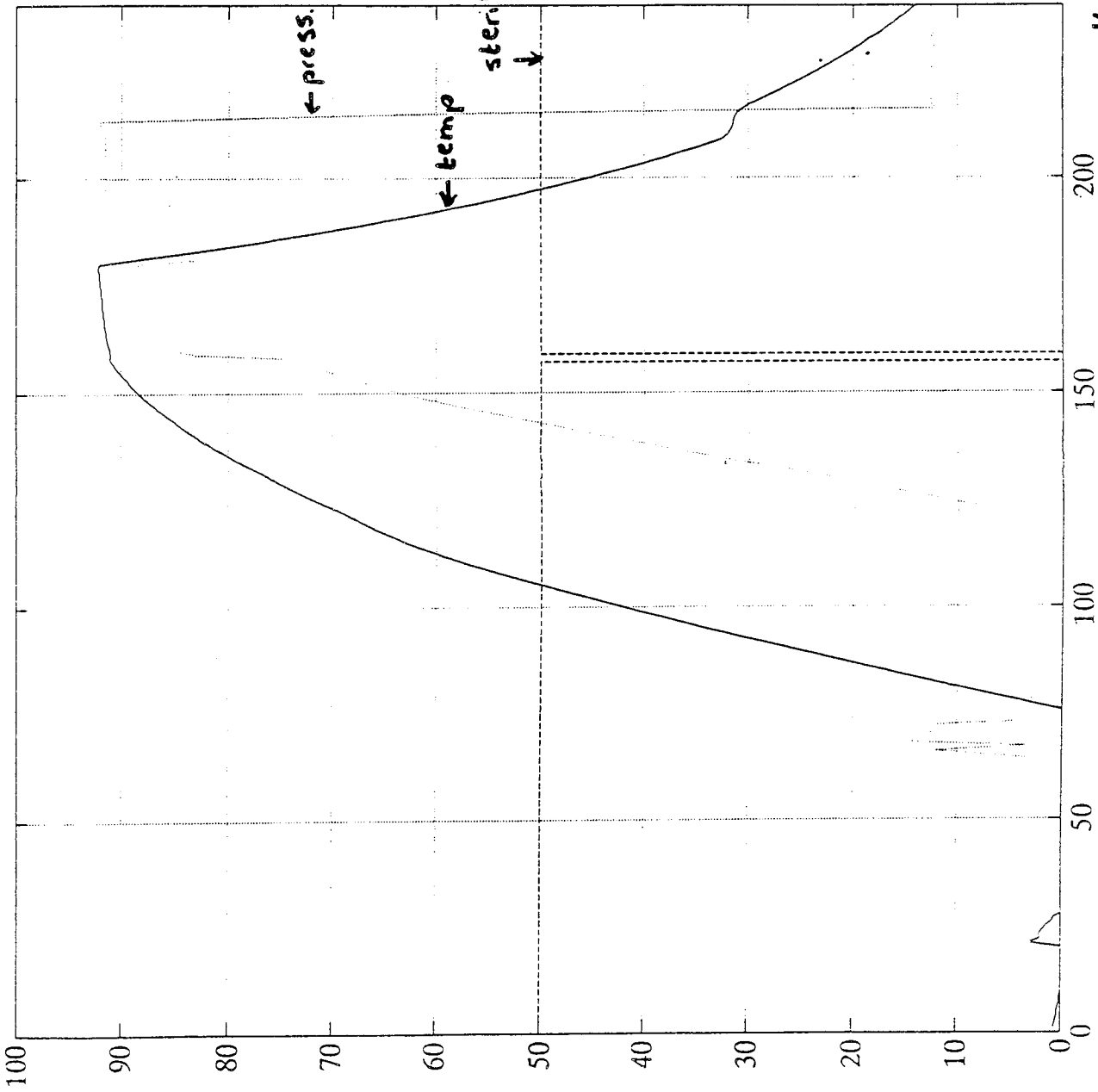
13:00
26/7/90

Time (mins) since 00:00:00

00:00
26/7/90

12
hour
file

03ASTI PHILIP 24-JUL-90 12:00:00



16:00
24/7/90

Time (mins) since 12:00:00

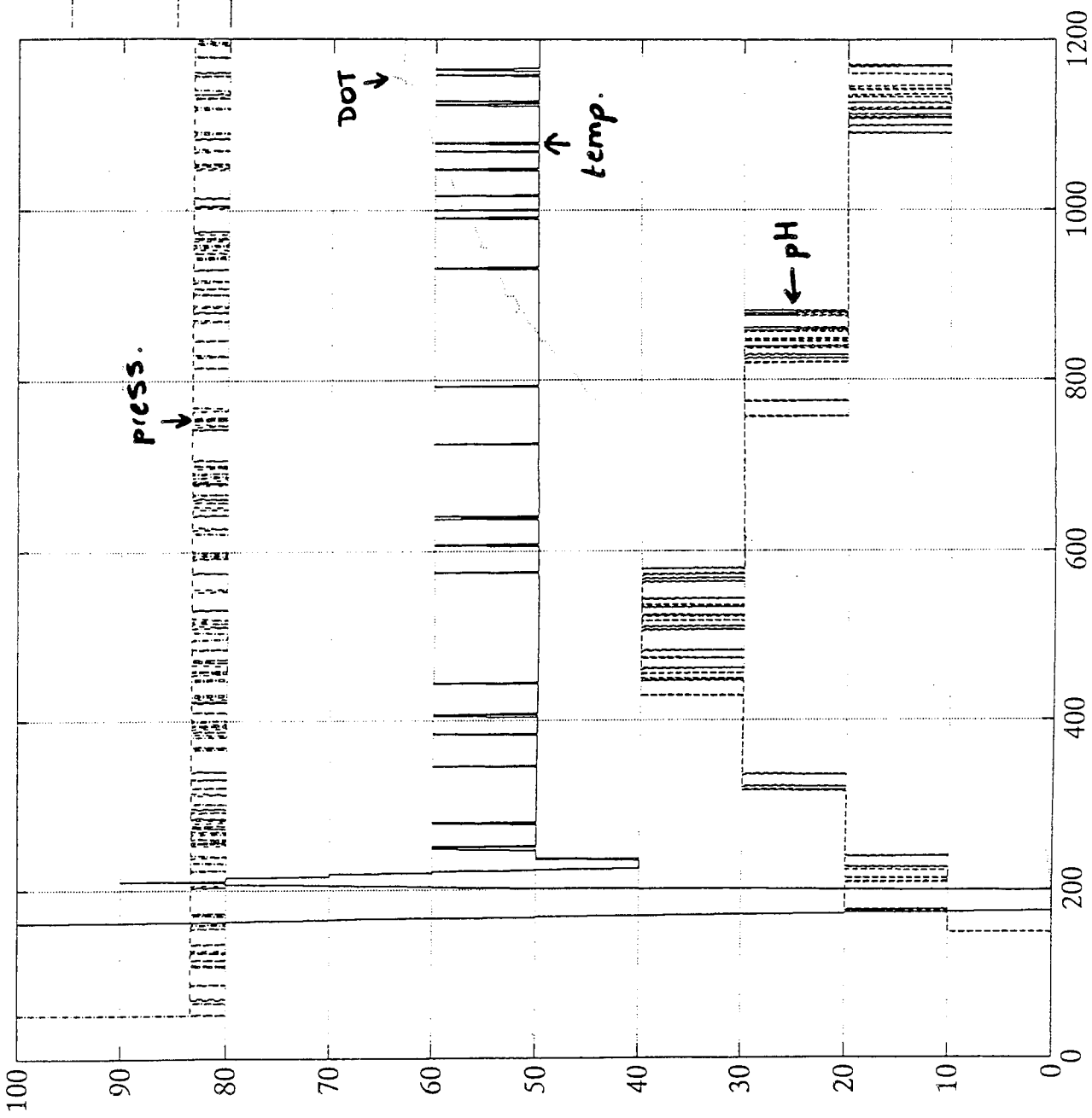
1200
24/7/90

- 19CP1MAS
- 03PR1IPV (Pressure - bar)
- 03TS1SLV (Sterilization s.p.)
- 03TF1IPV (vessel temp. °C)

sterilization temp. set point

← press.

← temp



03TF1IPV(23,24) 03PH1IPV(7,7.1) 03DO1IPV(0,100) 03PR1IPV(0,0.3)

14:45 24/7/90

Time (mins) since 14:45:00

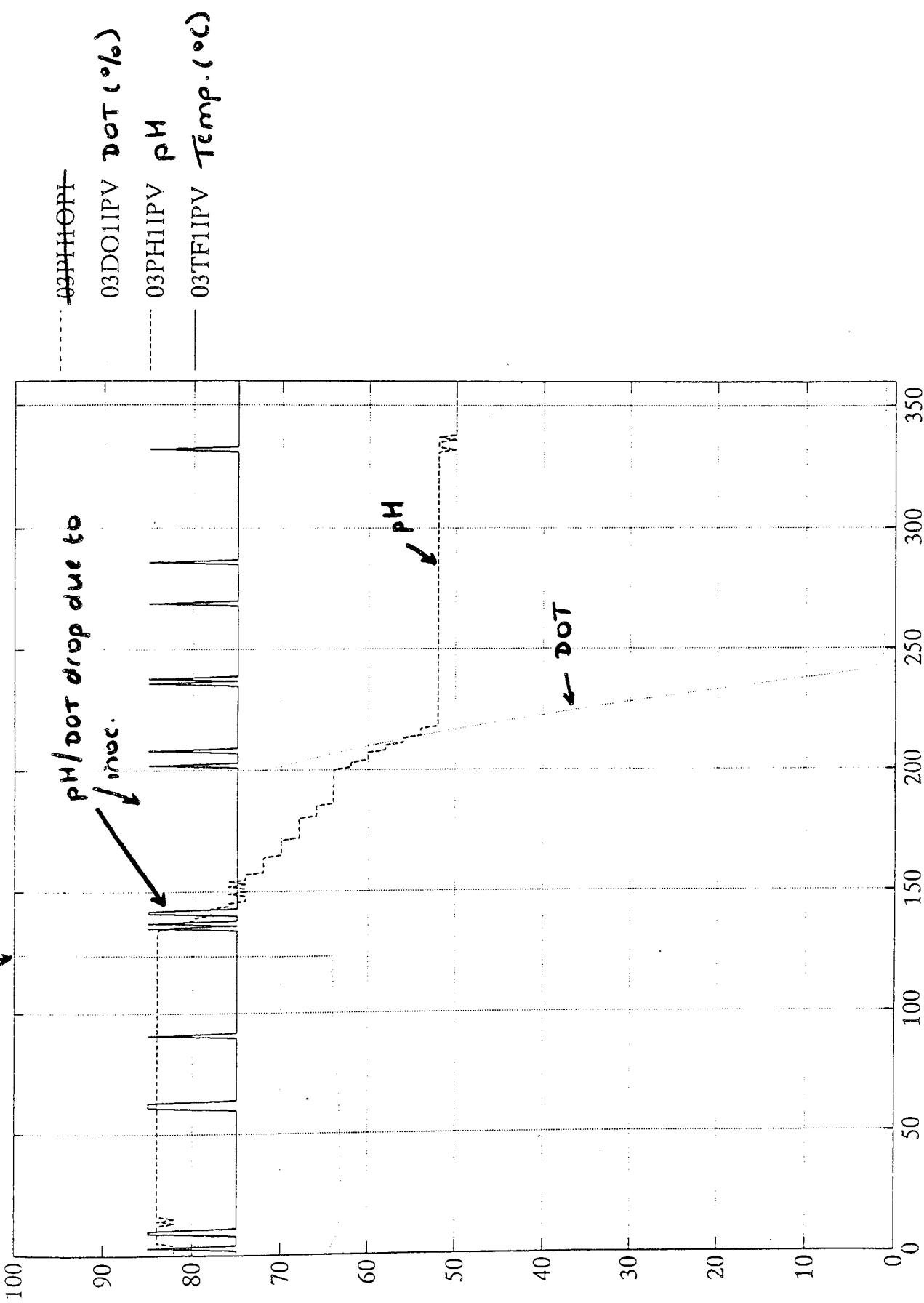
10:45 25/7/90

PHILIP 25-JUL-90 10:00:00

03ASTI

5

03TF1IPV(2.75,23.75) 03PH1IPV(6.6,7.1) 03DO1IPV(0,100) 03PH1OPV(0,0)

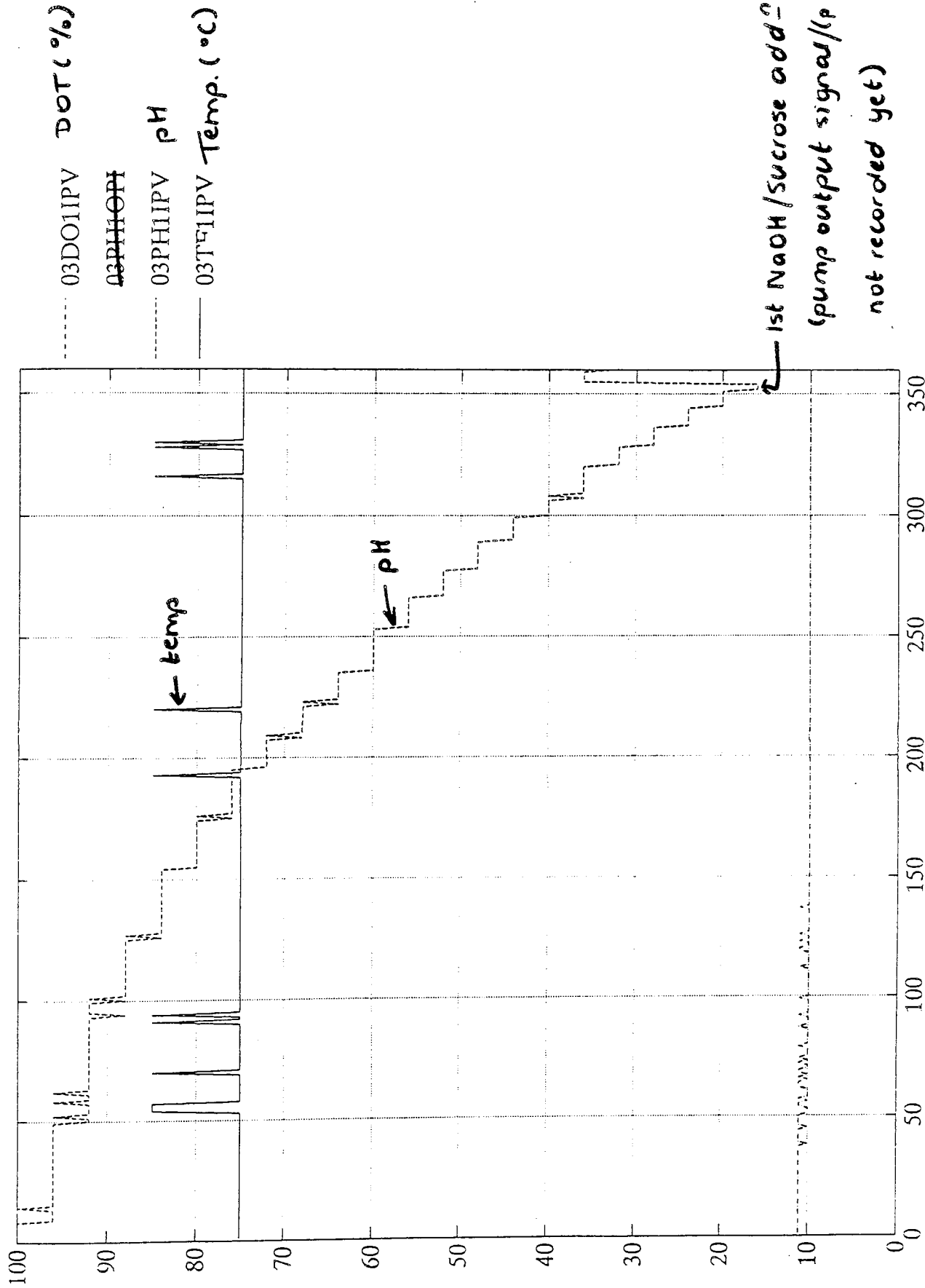


16:00
25/7/90

Time (mins) since 10:00:00

10:00
25/7/90

03TF1IPV(22.75,23.75) 03PH1IPV(6.6,6.85) 03PH1OP1(0.500) 03DO1IPV(0.10)



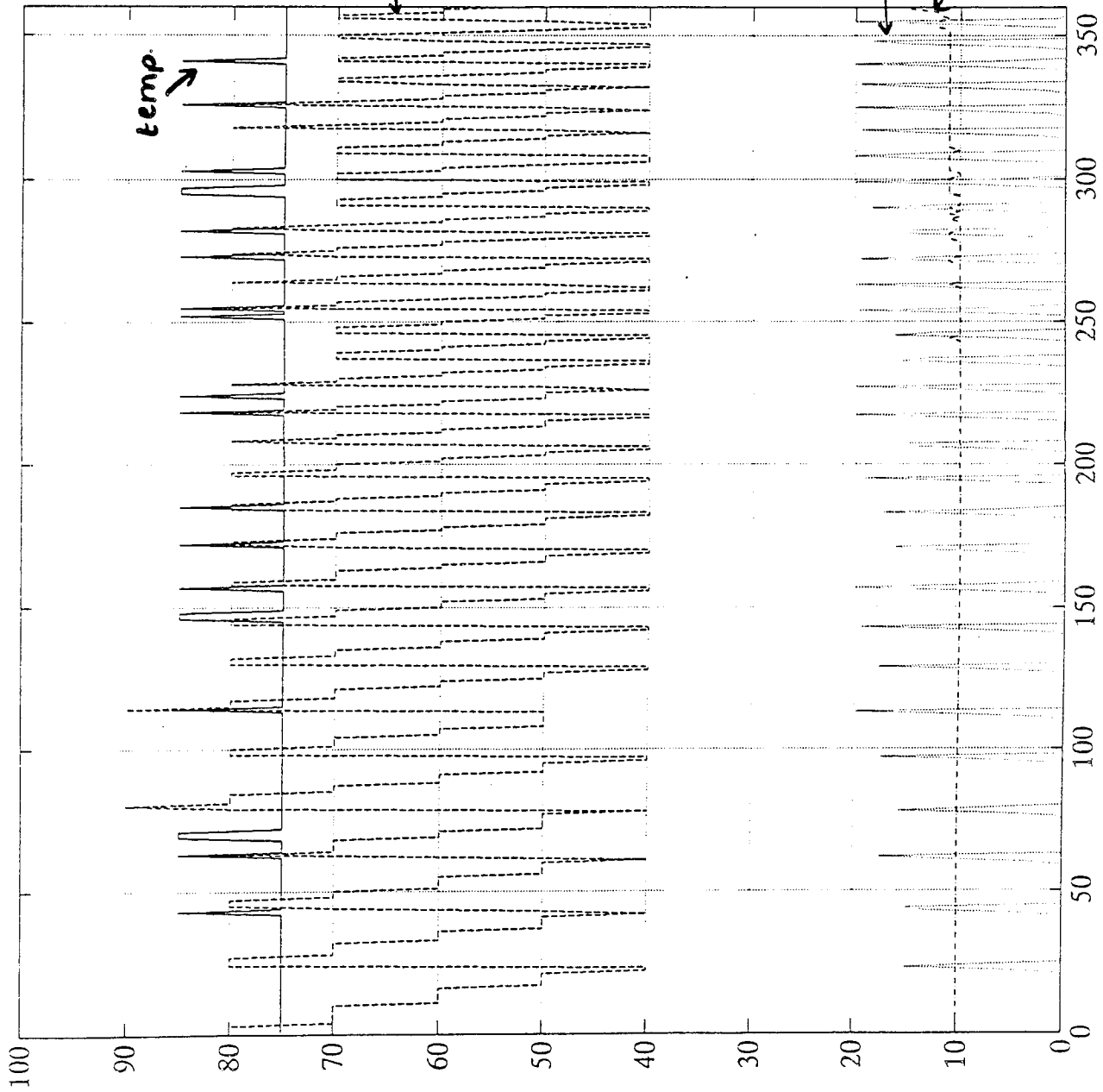
1st NaOH/Sucrose add
(pump output signal (pH1OP1)
not recorded yet)

16:00
25/7/90

Time (mins) since 16:00:00

16:00
25/7/90

03AST1 PHILIP 25-JUL-90 22:00:00



03TF1IPV(22.75,23.75) 03PH1IPV(6.6,6.7) 03PH1OPI(0.500) 03DO1IPV(0.10)

- 03DO1IPV (DOT value)
- _____ 03PH1OPI (pH control output)
- 03PH1IPV (pH value)
- _____ 03TF1IPV (temp. value)

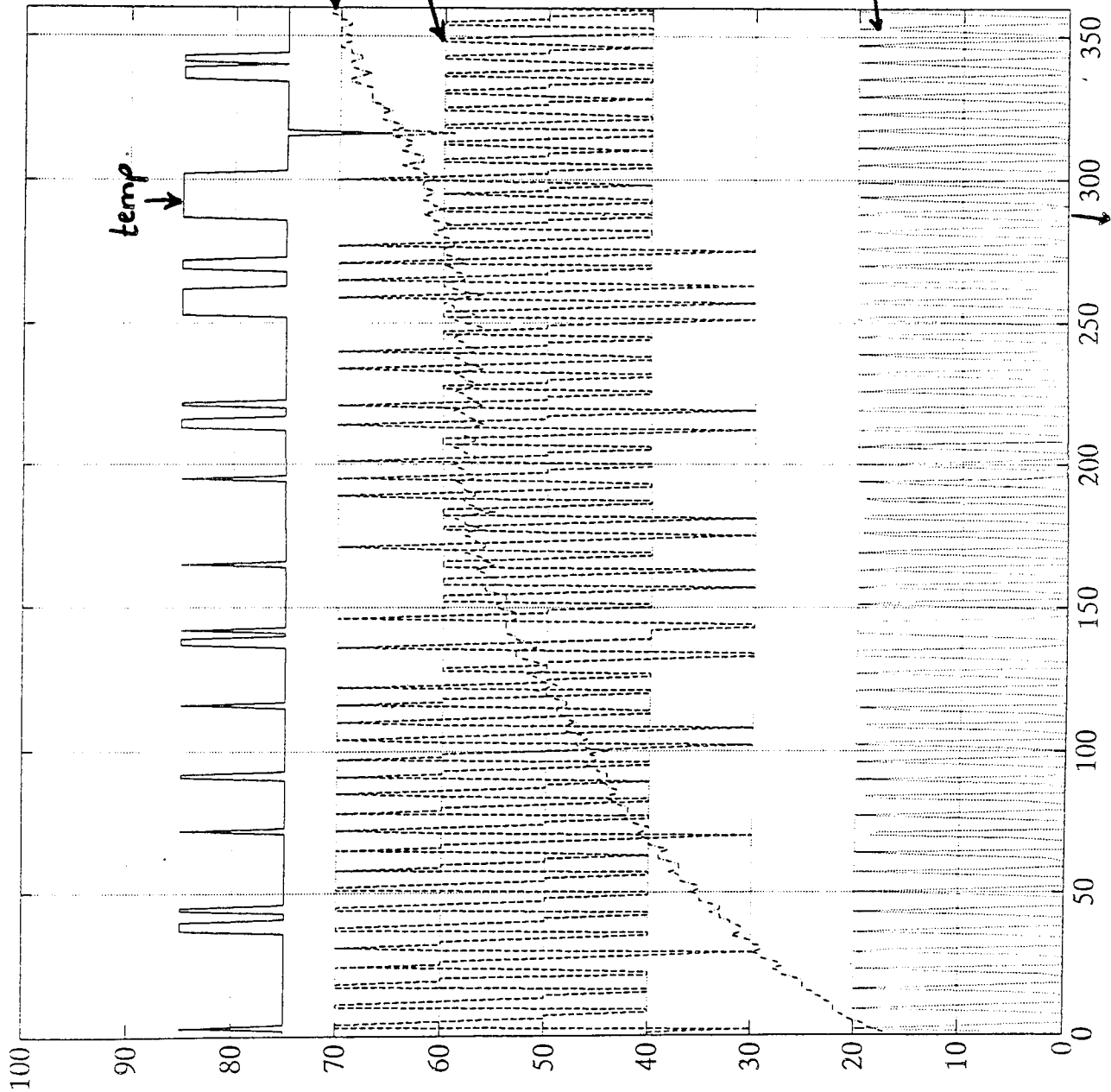
22:00 04:00
 25/7/90 26/7/90
 Time (mins) since 22:00:00

26-JUL-99 06:40:00

PHILIP

03ASTI

03TF1IPV(22.75,23.75) 03PH1IPV(6.6,6.7) 03PH1OPI(0.500) 03DO1IPV(0.10)



03DO1IPV (DOT %)
 03PH1OPI (alkali addition)
 03PH1IPV (pH, 0-14)
 03TF1IPV (temp - °C)

alkali addition, every
 peak: slug of NaOH/sucrose
 301s.

10:00
 26/7/90

08:00

Time (mins) since 04:00:00

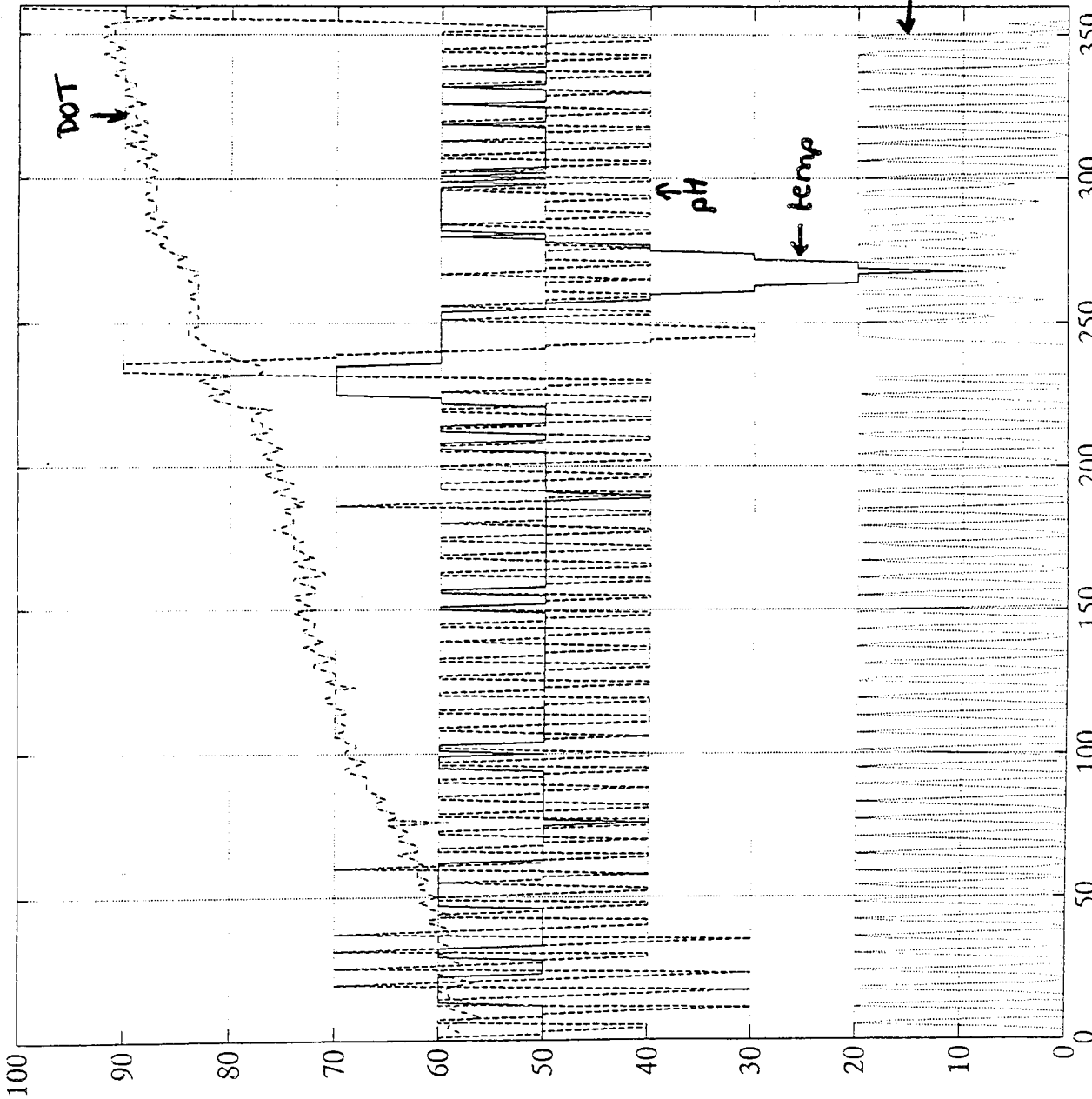
04:00

26/7/90

26-JUL-90 08:00:00

PHILIP

03ASTI



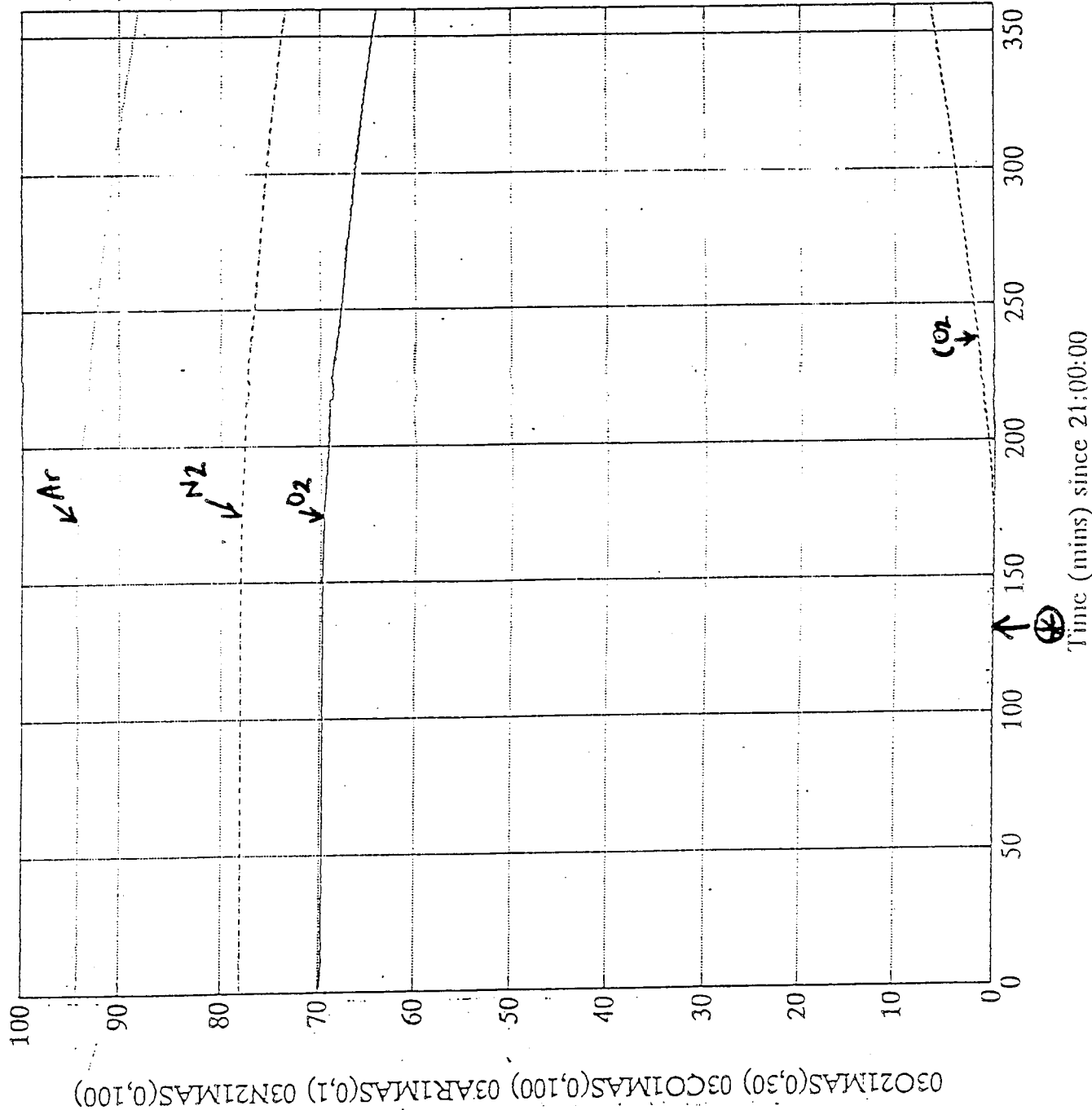
03DO1IPV (DOT value)
 03PH1OPI (pH control signal)
 03PH1IPV (pH value)
 03TF1IPV (temp. value)

03TF1IPV(23,24) 03PH1IPV(6,6,7) 03PH1OPI(0,500) 03DO1IPV(0,10)

14:00
 26/7/90
 END OF RUN

Time (mins) since 08:00:00

08:00
 26/7/90



Mass Spectrometer (UG 890)
 readings
 03N21MAS (N₂ value) } inerts,
 03AR1MAS (Ar value) } for
 03CO1MAS (CO₂ value) } calibr.
 03O21MAS (O₂ value)

all readings are %

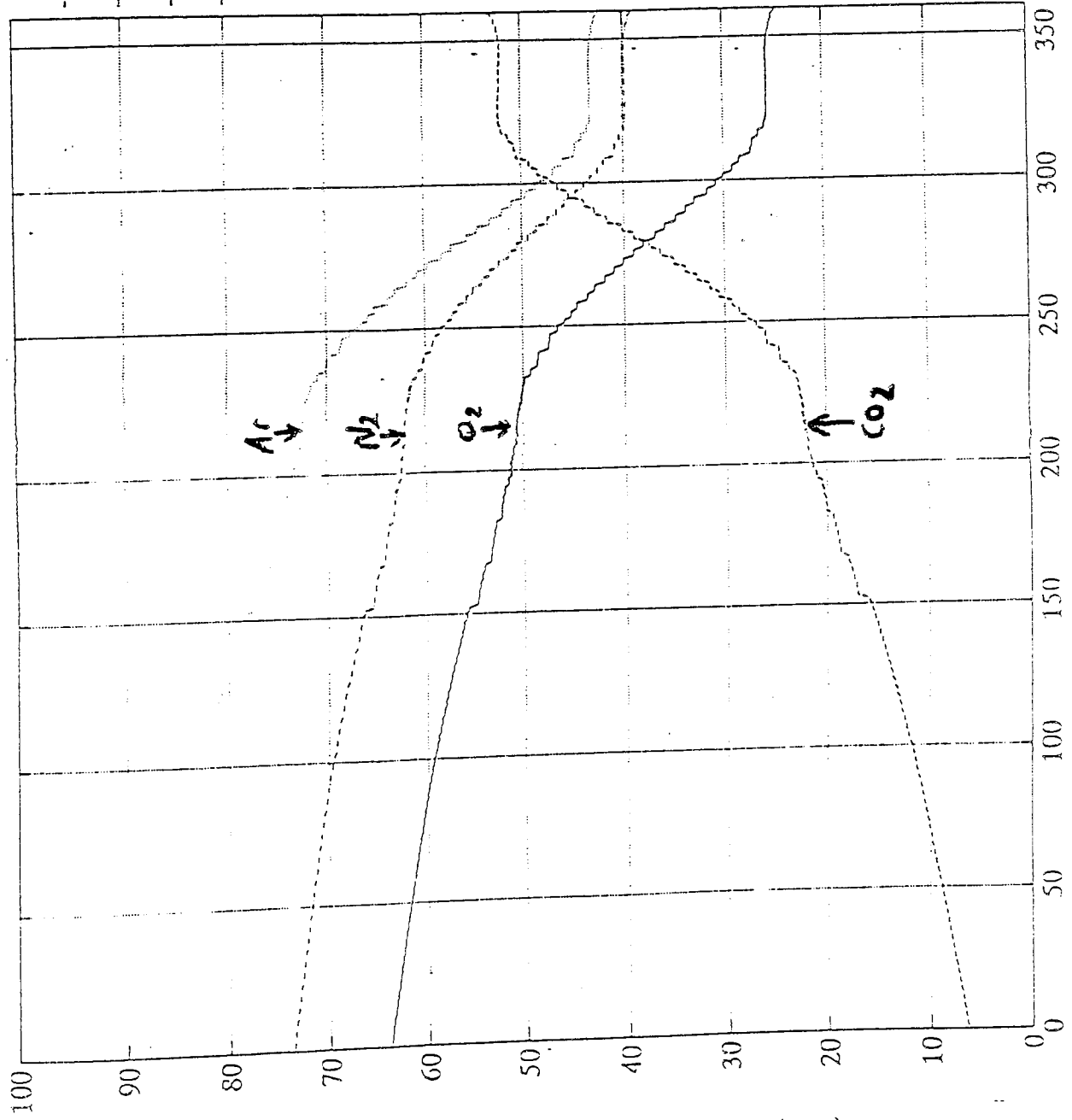
Note

Headspace gas introduced from inoculation until 11:10pm on 25/7/90 (from 12:00pm, midday, 25/7/90).

This is indicated by **B** at 130 mins along the y-axis. Afterwards, the CO₂ evolution was used to control the vessel overpressure, and explains the drop in all gases except CO₂, which rises to 28% at 5/3. Between 12 midday on 25/7/90 and 21:00 25/7/90, the N₂, O₂, O₂ and Ar results did not alter enough to warrant plotting.

03:00am
 21:00 pm

03ASFI PHILIP 26-JUL-90 03:00:00



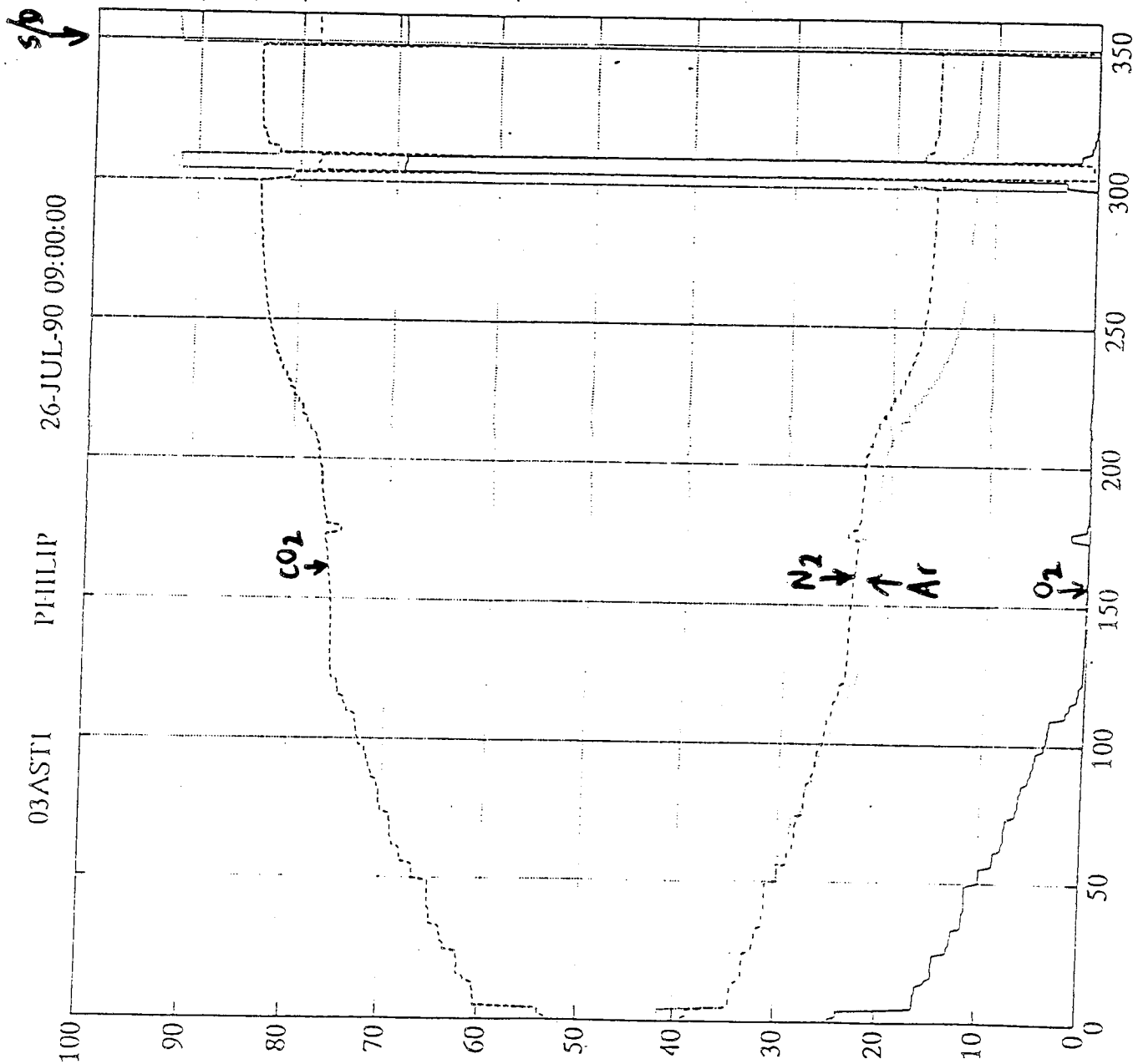
03N21MAS (N₂, 0-100%)
 03AR1MAS (Ac, 0-1%)
 03CO1MAS (CO₂, 0-100%)
 03O21MAS (O₂, 0-30%)

03O21MAS(0,30) 03CO1MAS(0,100) 03AR1MAS(0,1) 03N21MAS(0,100)

Time (mins) since 02:00:00 15:00:00

ALZ

03O2IMAS(0,30) 03CO1MAS(0,100) 03AR1MAS(0,1) 03N21MAS(0,100)

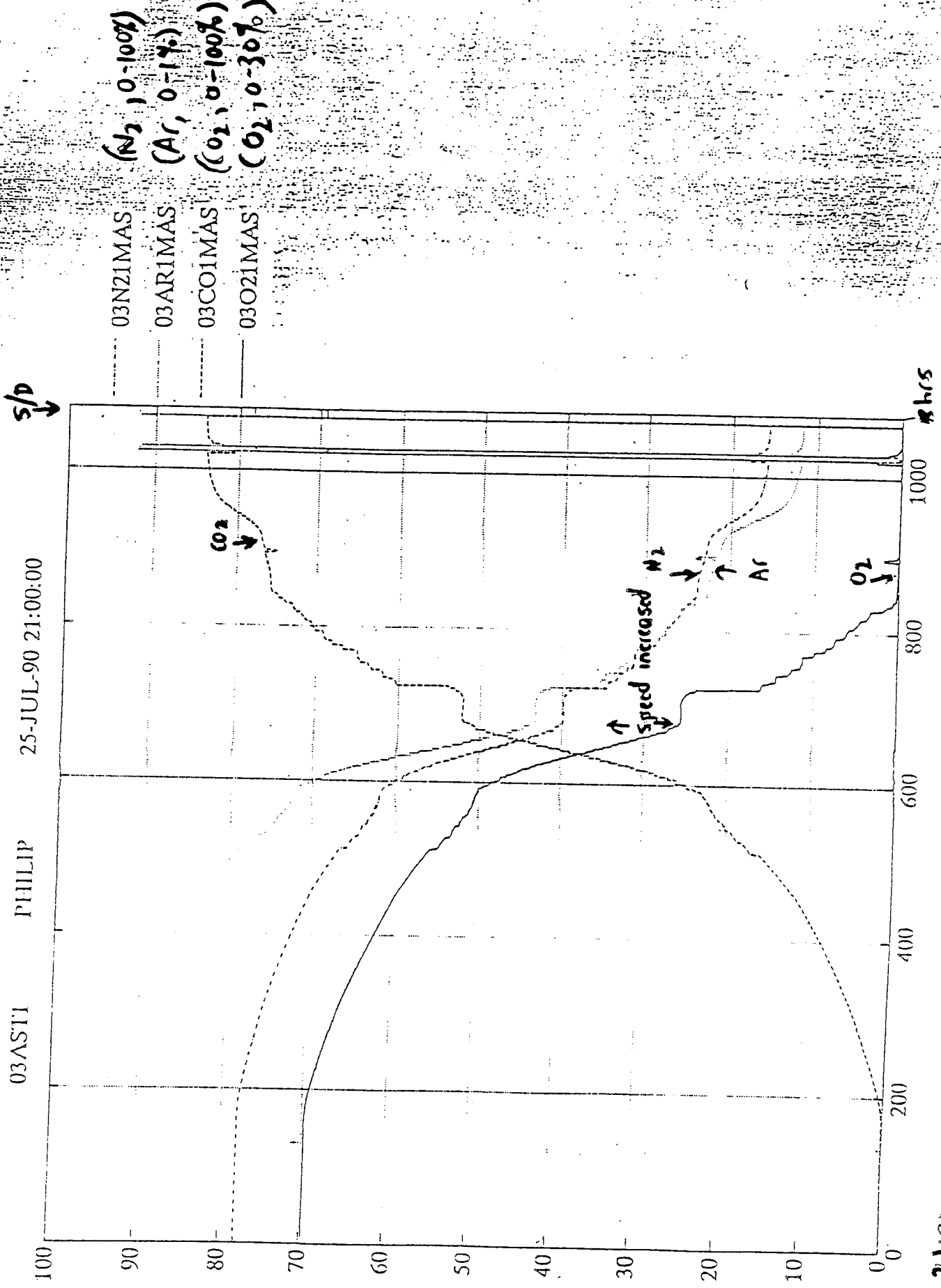


Time (mins) since 09:00:00

09:00AM

15:00PM

18 hour plot, indicating all salient gas results until shutdown (S/D)



03O2IMAS(0,50) 03CO1MAS(0,100) 03AR1MAS(0,1) 03N2IMAS(0,100)

03N21MAS (N₂, 0-100%)
 03AR1MAS (Ar, 0-1%)
 03CO1MAS (CO₂, 0-100%)
 03O21MAS (O₂, 0-30%)

21:00pm
 25/7/90
 Time (mins) since 21:00:00
 15:00pm

APPENDIX B.1

ISIM INTERACTIVE SIMULATOR

```
1 : PROGRAM FOR SIMULATING CONTINUOUS FERMENTATIONS
2 :
3 : CONSTANTS FOR DIFFERENTIAL EQUATIONS
4 :
5 : CONSTANT UMAX=0.50, KS=0.11, YPX=35.3, YXS=0.07
6 : CONSTANT V=0.95, F=0.35
7 : CONSTANT KP=-1.0
8 : CONSTANT SI=40
9 :
10 : CONTROL PARAMETERS FOR THE SIMULATION
11 :
12 : CONSTANT CINT=5.0, T=0, TFIN=50.0
13 :
14 : INITIAL CONDITIONS
15 :
16 : INITIAL
17 : S=40
18 : P=0
19 : X=0.16
20 :
21 : MODEL EQUATIONS
22 :
23 : DYNAMIC
24 :
25 :  $D=F/V$ 
26 :  $RX=U*X$ 
27 :  $U=UMAX*S/(KS+S)$ 
28 :  $X'=RX-D*X$ 
29 :  $RSX=U*X/YXS$ 
30 :  $RP=U*X*YPX+KP*X$ 
31 :  $S'=D*(SI-S)-RSX$ 
32 :  $P'=RP-D*P$ 
33 :
34 : SIMULATION RESULTS
35 :
36 : OUTPUT T,D,X,P,S,U
```

**Appendix B.2: Comparison of Results from Simulation
with actual Experimental Results**

