FERMENTATION METHODS FOR DEXTRANSUCRASE PRODUCTION

A thesis submitted by

PANAYIOTIS ZANGANAS B.Sc, M.Sc

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SUMMARY

Several fermentation methods for the production of the enzyme dextransucrase have been employed. The theoretical aspects of these fermentation techniques have been given in the early chapters of this thesis together with a brief overview of enzyme biotechnology. A literature survey on cell recycle fermentation has been carried out followed by a survey report on dextransucrase production, purification and the reaction mechanism of dextran biosynthesis.

The various experimental apparatus as employed in this research are described in detail. In particular, emphasis has been given to the development of continuous cell recycle fermenters.

On the laboratory scale, fed-batch fermentations under anaerobic low agitation conditions resulted in dextransucrase activities of about 450 DSU/cm^3 which are much higher than the yields reported in the literature and obtained under aerobic conditions. In conventional continuous culture the dilution rate was varied in the range between 0.375 h^{-1} to 0.55 h^{-1}. The general pattern observed from the data obtained was that the enzyme activity decreased with increase in dilution rate. In these experiments the maximum value of enzyme activity was ~ 74 DSU/cm^3. Sparging the fermentation broth with CO_2 in continuous culture appears to result in a decrease in enzyme activity.

In continuous total cell recycle fermentations high steady state biomass levels were achieved but the enzyme activity was low, in the range 4 - 27 DSU/cm^3 .This fermentation environment affected the physiology of the microorganism. The behaviour of the cell recycle system employed in this work together with its performance and the factors that affected it are discussed in the relevant chapters.

By retaining the whole broth leaving a continuous fermenter for between 1.5 - 4 h under controlled conditions, the enzyme activity was enhanced with a certain treatment from 86 DSU/cm^3 to 180 DSU/cm^3 which represents a 106% increase over the enzyme activity achieved by a steady-state conventional chemostat. A novel process for dextran production has been proposed based on the findings of this latter part of the experimental work.

Keywords: Fermentation, Dextransucrase, Dextran, Leuconostoc mesenteroides, Cell recycle.

Dedicated to my mother

VASSILIKI ZANGANA

for her love, support, patience and advice

throughout all my studies.

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CHAPTER 1:

INTRODUCTION

1.1 DEXTRANSUCRASE

Dextransucrase is an extracellular enzyme which is produced by a large number of bacteria, the most widely used being Leuconostoc Mesenteroides NRRL B512(F). This enzyme is inducible and sucrose is the only substrate that is known to induce it. Dextransucrase acts as a biocatalyst on sucrose and the products of this reaction are dextran and fructose. Dextran is a polymer and in particular a polysaccharide consisting of D - glucose monomers.

The action of dextransucrase on sucrose is given by the following stoichiometric equation:

$$n\ C_{12}H_{22}O_{11} \xrightarrow{\text{dextransucrase}} (C_6H_{10}O_5)n + n\ C_6H_{12}O_6$$

sucrose dextran fructose

In the variable length dextran chain there are also $\alpha(1\text{-}4)$ and $\alpha(1\text{-}3)$ linkages forming branch points along the chain.

Dextran produced by cultivation of Leuconostoc Mesenteroides and in particular the NRRL strain B512(F) produces dextran containing 95% 1,6 - and 5% 1,3 - aD - glucopyranosidic linkages and is the strain in

common use for commercial dextran production in the USA and Europe.

Dextran produced by different organisms differs in the type and degree of branching, which, along with molecular weight greatly affects its solubility and rheology. These molecules have molecular weights up to several million.

Probably the main uses of dextran is in the pharmaceutical market as a blood volume expander (substitute for blood plasma), for people who suffer a blood loss. For this purpose clinical dextran with a molecular weight of ~70,000 daltons is required.

Using dextran as a substitute for blood plasma has a number of advantages over the traditional method that involves an infusion of human blood plasma. These advantages are: i) dextran is heat sterilizable ii) eliminates the transfer of human infections and iii) there is also the possibility of long term storage.

Other clinical dextran products include blood flow improvers (MW $\approx$ 40,000) used to overcome vascular occlusion eg. in crush injuries and also complexes with iron which are used to treat iron deficiency anaemia.

Dextran has also many uses other than pharmaceutical such as a gelling agent in the food industry, for the manufacture of molecular sieves used for separation processes, deflocculants in paper products, metal plating processes and as chromatographic media, such as Sephadex.

The other product of the overall reaction of dextran synthesis, fructose, is also commercially important since it finds uses as a sugar substitute.

1.2 DEXTRANSUCRASE PRODUCTION

Dextransucrase is produced by growing cells of Leuconostoc Mesenteroides under well defined conditions of temperature and pH. Enzyme can be formed at temperatures between 20 and 30°C. High temperatures in this range induce higher cell growth but not necessarily higher enzyme elaboration rates. Optimum conditions for enzyme production with respect to temperature and pH have been found to be 23 - 24°C and 6.65 - 6.7 respectively. The enzyme is a growth related product and is produced mainly while the cells are growing or in other words during the exponential growth phase of the cells. When cell growth has ceased the fermentation is terminated, cell separation takes place and enzyme harvesting is carried out. Since the enzyme is growth related it is desirable to operate the fermentation in such a way as to extend the exponential growth phase of the cells thus increasing the enzyme activity of the fermentation broth and fermenter productivity. This is usually carried out by feeding extra substrate during a batch cultivation. This technique is known as fed batch fermentation. There are two ways of producing dextran. These are: the traditional whole cell fermentation process and the cell-free fermentation method.

In the whole cell fermentation process there is sucrose excess in the nutrient medium. The enzyme which is elaborated by the growing cells

is excreted to the culture supernatant and reacts with the excess sucrose to produce dextran. The whole process is carried out without pH control. The fermentation broth at the end of the process is viscous due to the presence of dextran which can be isolated by precipitation and other purification steps. Subsequently the crude dextran is fractionated in order to produce dextrans of desirable molecular weight distributions.

Another method for producing dextran is by a cell free method. In this method the overall reaction of the dextran synthesis is split into two stages. In the first stage the enzyme is produced under optimum conditions for its elaboration. This enzyme solution may or may not undergo purification or processing for cell removal and is then transferred to a sucrose solution to produce dextran under optimum conditions for its production.

Industrial production of dextran is currently carried out using the whole cell process while dextran production by the cell free method has only been employed on a laboratory scale.

Apart from published work on dextransucrase and dextran production using conventional fermentation techniques such as batch and fed batch cultivation, there is published work on enzyme purification and

concentration, on enzyme immobilization and recently on Leuconostoc Mesenteroides whole cell immobilization, for dextransucrase production.

1.3 AIMS OF THE PROJECT

The aims of the project were to investigate the effects of various fermentation methods on dextransucrase yields. The fermentation methods to be employed included conventional continuous fermentations, either anaerobic or CO_2 sparged, and fermentations under total and partial cell recycle conditions. In all these experiments various process parameters were to be changed and their effects on dextransucrase yields as well as on residual sucrose and fructose concentrations were to be studied.

The aim of residual fructose analyses in part of the experimental work was to investigate if fructose is consumed by the microorganism after sucrose has been depleted from the fermentation broth.

Experimental work was also to be undertaken on "the enzyme activity enhancement" of the whole broth from a continuous fermenter when collected and kept in a resevoir without pH control and batch cultivation of the microorganism.

Finally, a flow scheme for a plant to produce dextran using more advanced processing techniques than that currently carried out industrially was to be proposed.

1.4 OUTLINE OF THE THESIS

The work carried out during the course of this project is presented in a number of different chapters according to the fermentation technique employed. Prior to the presentation of the experimental work and discussion of the results obtained, the mathematical analyses of these fermentation methods are given. Some aspects of enzyme bio-technology are briefly covered as well as the literature on cell recycle fermentation, the applications and the behaviour of this type of system in various fermentation processes and under various operating conditions. A literature survey on the production of dextransucrase and on relevant aspects of this process has also been included.

In particular, chapter 2 presents the theory of fed-batch, continuous, total cell recycle and partial cell recycle continuous fermentations. In this chapter also, the principles of cross-flow filtration are given together with a brief presentation of the features of the various cross-flow filtration membranes available.

Chapter 3 contains some basic principles of enzyme technology.

Chapter 4 reviews briefly the literature on cell recycle fermentation. Only the highlights of the application of this technique on various

fermentations are presented.

Chapter 5 contains a review of the literature on dextransucrase production.

In chapter 6, a description of all the experimental apparatus used during the course of this project together with the experimental techniques employed is given.

Chapter 7 describes the analytical equipment, the materials and the analytical methods employed throughout this work.

Chapter 8 contains a presentation and discussion of the results of fed batch fermentations.

Chapter 9 gives the results of conventional either anaerobic or CO_2 sparged, continuous fermentations and their discussion.

Chapter 10 presents the results of total cell recycle continuous fermentation experiments and their discussion.

Chapter 11 contains the results of partial cell recycle fermentations and their discussion.

Chapter 12 gives the results of the enzyme activity enhancement experiments, their discussion and the proposal of an advantageous process for dextran production.

Finally, in Chapter 13 the conclusions drawn from this work are reported.

CHAPTER 2.

FERMENTATION THEORY

2.1 INTRODUCTION [2.1]

The term fermentation was first defined by Pasteur as "life in the absence of oxygen". Today the term fermentation is used both in the broad sense (incorrectly) for any biotransformation, and in the limited (correct) sense for a metabolic process in which organic compounds serve both as electron donors and acceptors. Fermentation involves the catabolism (biochemical breakdown) of an organic compound (usually a carbohydrate) to compounds whose average oxidation state is the same as that of the initial substrate (ie. the carbohydrate) but whose total energy content is lower. The consequence of this is that both reduced and oxidized products are formed.

The organisms which ferment are either facultative or obligate anaerobes. Facultative anerobes grow aerobically in the presence of oxygen or anerobically in its absence. These organisms, of which E.Coli is an example, use fermentation only under anaerobic conditions and carry out respiration in the presence of air. Obligate anaerobes, however, grow only in the absence of oxygen. Tolerance to oxygen varies from one organism to another. Strict anaerobes will tolerate virtually no oxygen while many strains are less fastidious. Strict anaerobes include such potentially useful organisms as C.thermoaceticum and A.Woodii. The former converts glucose to acetic

acid and the latter carbon dioxide and hydrogen into acetic acid.[2.1]

In this work "fermentation" will be used in its broader sense, i.e the application of microbial metabolism to transform simple raw materials into valuable products. Fermentation processes can produce a very wide range of useful substances, for example, chemicals such as citric acid, antibiotics, biopolymers and single cell proteins. The potential is as broad and varied as microorganisms themselves. What is needed is the knowledge of these microorganisms, the control of their metabolism and growth and the ability to handle them on a large scale. All these developments in molecular biology, genetic manipulation and in microbiology in general, have given the well established fermentation industry the opportunity to develop new processes and to improve existing ones. Here it must be stressed that the exploitation of all these major advances depends upon the techniques of large scale handling and large scale culture of these microorganisms. These techniques and skills have been built up by industry over many years.

Structure of a fermentation process

The central part of a fermentation process is the growth of the industrial organism in an environment which stimulates the synthesis of the desired commercial product. This is carried out in a fermenter which is, essentially a large vessel in which the organism may be maintained at the required temperature, pH, dissolved oxygen concentration and substrate concentration. However, the actual culturing of the organism in the fermenter is only one of a number of stages in a fermentation process, as illustrated in Fig. 2.1. The medium which the organism is to be grown has to be formulated from its raw materials and sterilized; the fermenter has to be sterilized and inoculated with a viable, metabolically active culture which is capable of producing the required product; after growth, the culture fluid has to be harvested, the cells separated from the supernatant and the product has to be extracted and purified. One must also visualize the research and development programme superimposed upon this process. For a fermentation to be possible in the first place, an acceptable, productive organism must be obtained and its productivity increased to economic levels by medium improvement, mutation, recombination, and process design. Thus a successful fermentation is based on the skills of people from many different disciplines such as chemical engineers, biochemists, biologists, microbiologists, chemists and others.

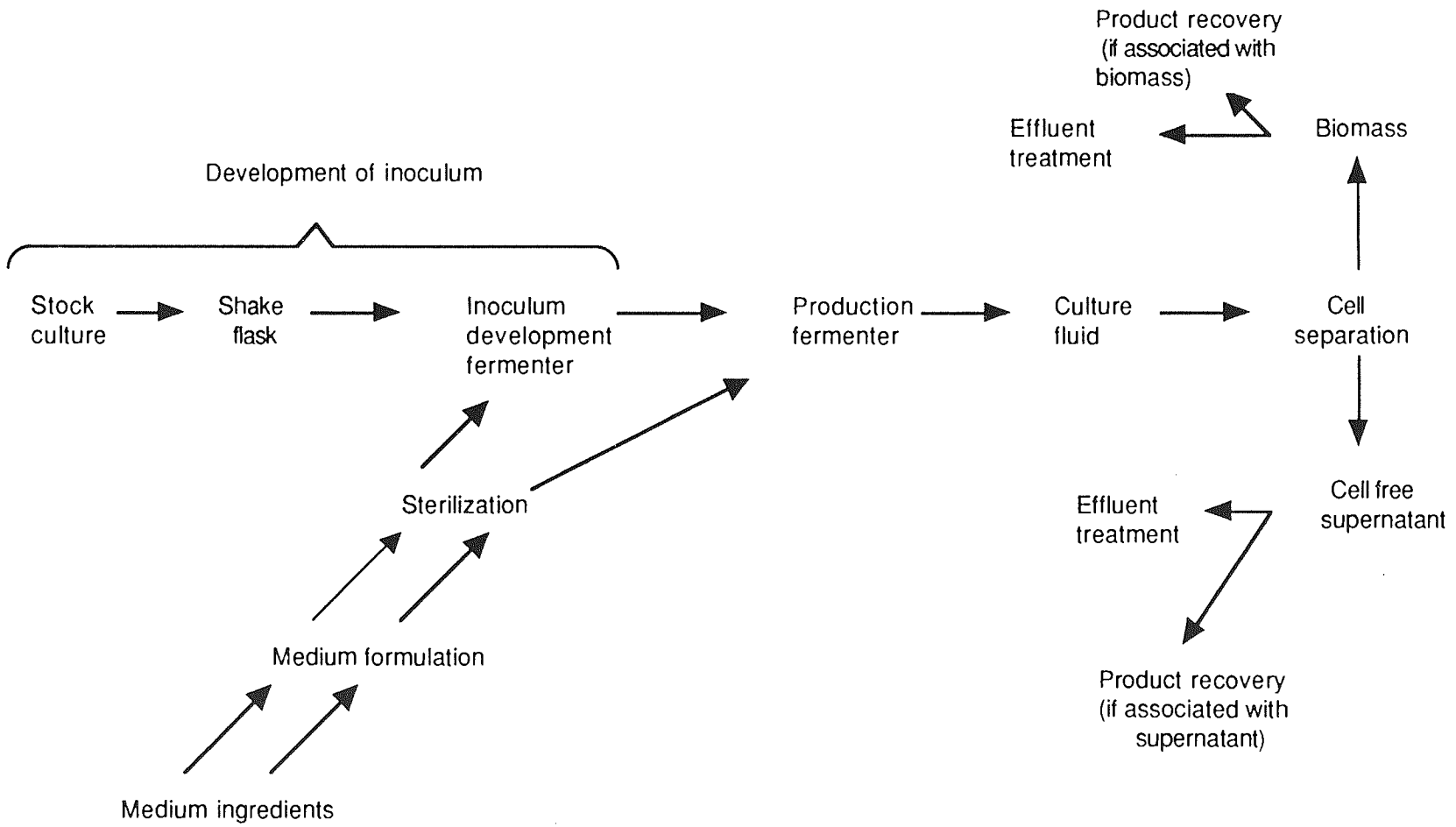


Fig. 2.1 A Generalized Schematic Representation of the stages of a typical fermentation process

2.2 DESIGN OF THE FERMENTATION MEDIUM (2.2), (2.3)

The fermentation medium which is used in a fermentation process must not only meet the requirements of the microorganism and product formation, but also those of the industrial process. Hence its cost, efficiency of utilization, rheology, and its consequent effects on downstream processing, are all important factors in formulating a suitable fermentation medium.

All microorganisms require for their growth and product formation water, carbon sources, nitrogen, mineral elements and in some cases other nutrients such as vitamins and amino acids. The vast majority of the commercially significant microorganisms are chemo-organotrophic and therefore the energy and carbon sources are one and the same.

The main sources of carbon are the carbohydrates. Simple sugars like glucose, sucrose and lactose are available in a variety of purities as powders or syrups. Available polysaccharides are starch, dextrins, hemicellulose and cellulose. Impure sources of carbohydrates are cane molasses, beet molasses which contain glucose, fructose and sucrose. Beet molasses also contain raffinose while both contain other organic and inorganic impurities.

In the vast majority of fermentations, cheap carbohydrate sources are sought in order to reduce the cost of the process. It is considered that an effective medium for use in a competitive enzyme production process must be both cheap and where possible, reproducible over a long production period. In many cases these last two requirements tend to be mutually exclusive, since cheap substrates are often illdefined and probably their composition may vary.

Nitrogen may be provided in a variety of forms such as ammonia or ammonium and nitrate salts or more complex mixtures such as yeast extract, peptone, cornsteep liquor, fish meal, gluten meal, urea, yeast hydrolysates. Essential cations are usually supplied as inorganic salts. Trace elements are sometimes added, but often they are contained in the other medium components. In some cases growth factors such as vitamins are necessary for growth of microorganisms.

In general, nitrogen is used in the anabolic cellular synthesis of amino acids, purines, pyrimidines, proteins, DNA and RNA.

The inorganic nitrogen salts added in the fermentation medium may serve the dual purpose of pH control and nitrogen source.

Phosphates may be incorporated in the medium as buffering agents although pH control is normally used.

TABLE 2.1 gives some examples of carbon and nitrogen sources commonly used in fermentation media.

TABLE 2.1

SOME EXAMPLES OF CARBON AND NITROGEN SOURCES COMMONLY USED IN FERMENTATION MEDIA

Carbon sources	*Nitrogen sources*
Starch	Ammonia
Glucose	Ammonium salts
Malt	Nitrates
Beet molasses	Corn-steep liquor
Cane molasses	Peanut granules
Vegetable oils	Soybean meal
Hydrocarbons	Dried blood
	Pharmamedia
	Soya meal
	Distillers' solubles.
	Yeast extract

The yield of biomass obtainable per unit of carbon incorporated into the medium is an important criterion in the choice of a carbon source. The cellular yield coefficient for a carbon source is defined as:

$$\frac{\text{The quantity of the dry matter produced}}{\text{The quantity of carbon substrate utilized}}$$

Sometimes hydrocarbons give better yield coefficients than carbohydrates. The reason for this is that the highly reduced hydrocarbons contain more carbon per gram of compound than do the carbohydrates. The improved yield results in high oxygen demand for cells growing in these highly reduced carbon compounds and consequently far more oxygen has to be provided to the hydrocarbon based fermentation than to the carbohydrate based one. The consumption of large amounts of oxygen will result in the generation of considerable heat which must be removed from the fermentation broth so to keep its temperature at an optimum value.

Hence, the choice of carbon source has an important effect on the fermentation vessel design which has to provide on the one hand the necessary amount of oxygen for complete or satisfactory utilization of the carbon source and on the other hand sufficient heat exchange area to remove the heat produced.

Inducers

Many enzymes are synthesized in quantity, only in the presence of a specific inducer. This occurs more often in the case of catabolic enzymes which are only produced in the presence of an inducer. These compounds are usually the substrate of the enzyme or structurally similar compounds.

Repressors

The production of catabolic enzymes as well as secondary metabolites is often repressed by the presence of certain compounds in the fermentation medium. A remedy would be to feed this particular compound to the culture at a low rate.

Apart from the repressive effects of the carbon source used it has been shown that nitrogen sources as well, may have a repressive effect on secondary metabolite production.

Precursors

The productivity of certain metabolites may be increased considerably if a precursor of the metabolite is included in the fermentation medium or

added during the process.

The processing cost in a fermentation process in relation to the fermentation medium employed is influenced in a variety of ways such as 1) pretreatment 2) microbial content affecting sterilization cost 3) physical properties, such as surface tension in aerobic fermentations influencing bubble size distribution and foaming tendency and 4) rheological properties affecting agitation, heat transfer, aeration and downstream processing.

2.3 INOCULUM PREPARATION [2.1), (2.4), (2.5)]

It is important that the culture used to inoculate a fermentation medium must meet the following specifications:

1. It must not be contaminated.

2. It must have a sufficient large volume so as to meet an optimum size for the fermentation.

3. It must be in an active condition to minimize the length of the lag phase and consequently the length of the fermentation process.

The culture medium used for the preparation of the inoculum may be different from that used in the main or actual fermentation process. The requirements for the medium used for inoculum development are to provide an environment for sufficient growth of the microorganism and at the same time to provide an environment where the microorganism would be in a very good active state. The nutrient medium employed in the main fermentation is designed on the one hand to meet the nutrient requirements of the microorganism and on the other hand to enable the microorganism to produce the desirable product at a maximum level.

However, in many cases it has been shown that it is advantageous for the medium for inoculum preparation to be the same as the medium which is used in the main fermentation. With this procedure the period of adaptation of the inoculum culture to the production medium is minimized, hence resulting in a reduction of the lag phase and total fermentation time.

The quantity of the inoculum used should be in the range of 3-10% v/v, so starting from the master culture the inoculum must be built-up in a number of stages to produce sufficient biomass in order to inoculate the production stage fermenter. Throughout the inoculum development stages there is a risk of contamination and strain degeneration, necessitating strict quality control procedures.

As regards inocula development for bacterial fermentation processes the objective is to produce an active inoculum which will give as short a lag phase as possible. In the main fermentation the length of the lag phase is affected by the size of the inoculum and its physiological condition. In developing inocula for this type of fermentation the inoculum should be transferred when the cells are in their exponential phase of growth.

2.4 FED BATCH CULTURE [2.8], [2.9], [2.4]

Recently, many batch operations have been transformed into fed-batch (semicontinuous) fermentations by introducing the nutrient gradually into the reactor instead of charging everything at the beginning of the operation. The main features and advantages of fed batch fermentation include flexibility in the introduction of regulating compounds at the desired point of the batch operation as the reaction process occurs, maintenance of low nutrient levels which is essential in substrate inhibited [2.10] cultures, as well as extension of the stationary or exponential phase [2.11], by nutrient addition, so to obtain additional product. Other advantages of fed batch operation include deliberate variation of the feed concentration and a variety of control actions. It is also a very important fermentation technique for high cell concentration batch fermentations which lead to substrate limitation. High cell concentrations require a high concentration of nutrients in the medium. If only batch operation is applied, the culture will soon become substrate limited. If fed batch operation is introduced the nutrients which are fed into the fermenter will keep pace with the nutrient requirements of the cells and thus additional product will be produced. The treatment of the fed batch culture in terms of theoretical analysis is given in the following section 2.4.1.

2.4.1 THEORY OF FED BATCH CULTURE [2.4), (2.6), (2.7)]

Suppose that we have a homogeneous batch culture with only one growth limiting substrate. Let s_r = initial concentration of growth-limiting substrate and x = biomass concentration at time, t. Then we have

$$x=x_o+Y(s_r - s) \qquad \text{Eqn. 2.1}$$

where x_o = inoculum concentration, s = concentration of residual growth-limiting substrate and Y is the growth yield. It is assumed that when the biomass concentration reaches its maximum value, x_m, the growth-limiting substrate is practically exhausted so that $s<<s_r$. Assuming that the inoculum is small compared with the final biomass, equation 2.1 reduces to $x_m = Ys_r$.

Now suppose that when $x_m \approx Ys_r$, a feed medium is started at a flow rate F with the growth-limiting substrate at concentration, s_r, in the feed medium. The total biomass in the culture is given by X = xV where V is the culture volume at time, t. Since x = X/V differentiation of this quotient gives for the growth rate

$$dx/dt=(VdX/dt - XdV/dt)/V^2 \qquad \text{Eqn. 2.2}$$

We can substitute $dX/dt = \mu X$, where μ is the specific growth rate, $dV/dt = F$ and $F/V = D$ where D is the dilution rate. Eqn. 2.2 then becomes:

$$dx/dt = (\mu - D)x \qquad \text{Eqn. 2.3}$$

This equation is generally true for a fed batch culture. It is assumed that the relation of the specific growth rate to the concentration of growth-limiting substrate is of the Monod type, that is

$$\mu = \mu_m s/(s+K_s) \qquad \text{Eqn. 2.4}$$

When $s_r >> K_s$ over most of the range of μ from zero upwards, the growth-limiting substrate will be almost completely utilized so that when $x = x_m \approx Ys_r$, $dx/dt \approx 0$. With this condition it follows from Eqn. 2.3 that $\mu \approx D$

Let S = total growth-limiting substrate in the culture, then for the substrate balance we have:

rate of increase = rate of input - rate of consumption for growth, that is

$$dS/dt = Fs_r - \mu X/Y \qquad \text{Eqn. 2.5}$$

When $X = Vx_m$ virtually all of the substrate is consumed as fast as it enters the culture so that $Fs_r \approx \mu X/Y$, hence dS/dt and ds/dt are approximately zero. This state, when $dx/dt \approx o$, $ds/dt \approx o$ and $\mu \approx D$ is called the quasi-steady state.

To obtain the concentration of growth-limiting substrate as a function of dilution rate in the quasi-steady state, we substitute $D \approx \mu$ in Eqn. 2.4 which gives

$$s \approx DK_s/(\mu_m - D) \qquad \text{Eqn. 2.6}$$

The rate of increase in the total biomass during the quasi-steady state is given by

$$dX/dt = FYs_r \qquad \text{Eqn. 2.7}$$

thus

$$X=X_o+FY_{s_r}t \qquad \text{Eqn. 2.8}$$

Comparing a fed batch culture in the quasi-steady state with a chemostat culture in the steady state, we have in both cases that $\mu = D$ but, whereas D is constant in a chemostat, in a fed batch culture D is decreasing and μ is assumed to decrease at the same rate. The unique feature of fed batch culture is that, in the quasi-steady state, the biomass is in a transient state with the growth rate under control.

2.5 CONTINUOUS CULTURE (2.4), (2.6), (2.12), (2.13) (2.14)

Chemostat culture has revealed new horizons in microbial physiology and the history of the method shows the prime importance of the development of theory before experiment. The method is applicable to all types of protist and animal or plant tissue cells which can be grown in a homogeneous submerged culture.

The chemostat consists of a culture into which fresh medium is continuously introduced at a constant rate and the culture volume is kept constant by continuous removal of fermentation broth. Among the various methods of maintaining constant volume in the fermenter is that of an overflow tube or weir set to a constant height within the vessel.

The design and analysis of continuous fermentation operation is based on the concept of the continuously stirred tank reactor or fermenter (CSTF).

The assumptions made for the mathematical analysis of the CSTF are:

a. Perfect mixing occurs so that the exit stream has the same composition as the rest of the vessel contents.

b. Mixing is such that the concentration of all components within the vessel is the same in all parts of the vessel.

c. If the process is aerobic, the concentration of dissolved oxygen is the same in all parts of the vessel.

d. The heat transfer characteristics of the system are constant (heat of fermentation is removed continuously).

2.5.1 SPECIFIC GROWTH RATE

The object of the quantitative theory is to predict the values of the growth rate and the concentrations of biomass and substrate under different conditions. Let μ = specific growth rate and F/V = D is the dilution rate which represents the flow rate per unit volume.

A material balance on biomass can be written as:

net increase in biomass = growth - output

For an infinitely small time interval dt this balance for the whole culture is

$$V.dx = V.\mu x.dt - Fx.dt \qquad \text{Eqn. 2.9}$$

Dividing throughout Eqn. 2.9 by V.dt we obtain

$$dx/dt = (\mu - D)x \qquad \text{Eqn. 2.10}$$

In the steady state we have dx/dt = 0 and from equation 2.10 we obtain

$$\mu = D \qquad \text{Eqn. 2.11}$$

2.5.2 STEADY-STATE BIOMASS AND GROWTH-LIMITING SUBSTRATE CONCENTRATIONS

The material balance for the growth limiting substrate is:
net increase = input - output - substrate consumed. For an infinitely small time interval, dt, this balance for the whole culture is

$$V.ds = F.s_r.dt - Fs - V.\mu x.dt/Y \qquad \text{Eqn. 2.12}$$

were Y is the growth yield defined as:

$$Y = \frac{\text{mass of cells formed}}{\text{nutrient consumed}}$$

If we divide equation 2.12 throughout by V.dt we obtain

$$ds/dt = D(s_r - s) - \mu x/Y \qquad \text{Eqn. 2.13}$$

In the steady state $dx/dt = ds/dt = 0$ which results in

$$(\mu - D)\,\tilde{x} = 0 \qquad \text{Eqn. 2.14}$$

and

$$D(s_r - \tilde{s}) - \mu\tilde{x}/Y = 0 \qquad \text{Eqn. 2.15}$$

where the tilde denotes a steady state value.

To obtain $\tilde{x}$ and $\tilde{s}$ we substitute for the specific growth rate

$$\mu = \mu_m s/(s + K_s) \qquad \text{Eqn. 2.16}$$

Substituting $\mu = D$ in Eqn. 2.16 we obtain for the steady state

$$\tilde{s}=K_s D/(\mu_m-D) \qquad \text{Eqn. 2.17}$$

and from Eqn. 2.15 if we put $\mu = D$, we obtain

$$\tilde{x}=Y(s_r-\tilde{s})=Y\{s_r-K_s D/(\mu_m-D)\} \qquad \text{Eqn. 2.18}$$

2.5.3 CRITICAL DILUTION RATE

The maximum growth rate is obtained when $\tilde{s} = s_r$.

If we insert this value in Eqn. 2.16 we obtain

$$\mu = D_c = \mu_m s_r/(s_r+K_s) \qquad \text{Eqn. 2.19}$$

where D_c is the critical dilution rate. At this value the steady state biomass concentration is zero. In the case that $s_r >> K_s$ it follows from Eqn. 2.19 that $D_c \approx \mu_m$.

2.5.4 DETERMINATION OF MAXIMUM GROWTH RATE

When $s >> K_s$ Eqn. 2.16 becomes $\mu = \mu_m$. If we insert this value in

Eqn. 2.10 and integrate we obtain

$$\ln x = (\mu_m - D)t + \ln x_o \qquad \text{Eqn. 2.20}$$

With respect to the dilution rate if we select a value of D such that $D > D_c$ the biomass concentration decreases or biomass washout occurs conforming to Eqn. 2.20. As can be seen from this equation (Eqn.2.20) the slope is (μ_m - D), enabling the value of μ_m to be calculated.

2.5.5 RATE OF OUTPUT OF BIOMASS IN A CHEMOSTAT

In a chemotat culture, the biomass productivity is given by R = Dx and in the steady state we have

$$R = DY \{s_r - K_s D/(\mu_m - D)\} \qquad \text{Eqn. 2.21}$$

The productivity with respect to biomass output reaches a maximum which is determined by differentiating R with respect to D and equating the derivative to zero. The value obtained is as follows:

$$D_m = \mu_m \left\{ 1 - \left(\frac{K_s}{s_r + K_s} \right)^{1/2} \right\} \qquad \text{Eqn. 2.22}$$

The steady state biomass concentration at the dilution rate D_m is

obtained by inserting this value of D_m into equation 2.18. This procedure yields the following expression as regards $\tilde{x}_m$ i.e the biomass concentration, at steady state, at dilution rate D_m

$$\tilde{x}_m = Y[s_r + K_s - \{K_s(s_r + K_s)\}^{1/2}] \qquad \text{Eqn. 2.23}$$

If $s_r >> K_s$ then the maximum productivity of the chemostat, with respect to biomass, is given by the following expression

$$D_m \tilde{x}_m \approx D_m Y s_r \qquad \text{Eqn. 2.24}$$

2.6 CELL RECYCLE FERMENTATION

2.6.1 THEORY (2.4), (2.6), (2,12), (2.15), (2.16)

To a large extent the feasibility of economic production of valuable chemicals, in biotechnological processes, depends on the final concentration of the product obtained and also on the volumetric productivity of the bioreactor. The low concentration of product obtained makes the separation process more expensive and low productivity increases the cost of the bioreactor for the desired output. Productivity is limited due to the low concentration of cells in the reactor but this limitation can be overcome by separating the cells from the product stream and returning them back to the fermenter. The increase in cell mass per unit volume in the fermenter means in most cases higher fermenter productivity. In this case it may be possible, depending on operating parameters, for dilution rates higher than μ_{max} to the employed.

In cell recycle fermentation and in particular in a chemostat with cell recycle a separating device is fitted to the continuous fermenter. The fermentation broth is passed through this device and the concentrated

cell suspension stream is recycled to the fermentation vessel. The result is, as mentioned earlier, an accumulation of biomass to a level higher that can be achieved in a conventional chemostat culture.

To concentrate the biomass in a cell recycle fermentation system usually membrane filters or centrifuges are used. The centrifugation method is not workable in high density systems such as concentrated fermentation broth and also the whole process is too complicated and expensive to be economical. Another disadvantage of the centrifugation method is that aseptic conditions are difficult to maintain.

With respect to the use of membrane filters for cell recycling purposes there are two configurations, as regards their positioning in the fermentation system, which are commonly used; external or internal.

A few cases have been described where the membrane was placed in the fermenter vessel[2.16] . Such a configuration has certain advantages but also certain disadvantages. The advantages include the fact that everything is kept within the fermenter allowing sterility problems to be dealt with as in a conventional continuous fermenter. A disadvantage of placing the membrane within the fermenter is that if a problem occurs during the fermentation the whole system must be shut down to enable access to the membrane.

The use of external membranes gives a higher degree of flexibility but it may suffer as regards sterility. However, recent developments in membranes using materials that allow autoclaving or treatment with chemicals, under arduous conditions, eliminate the risk of contamination.

In the present study the external recycle mode of operation was employed.

Cell recycle fermentation was first analysed by Herbert[2.12] in 1961, then by Pirt in 1969. Its theory was extended and experimentally tested for the first time by Pirt and Kurowski[2.16] in 1970.

Bull and Young[2.15] (1981) also presented a theoretical treatment of a cell recycle fermentation and it is their treatment which has been used in this work.

A schematic of a cell recycle fermentation is shown in Figure 2.2. The ratio of the cell free flow rate leaving the fermenter to the total outflow rate is a measure of the fraction of the cells in the combined outflow streams that is returned to the fermentation vessel. This ratio is termed recycle ratio and the range of its possible values is $0<R\leq1$.

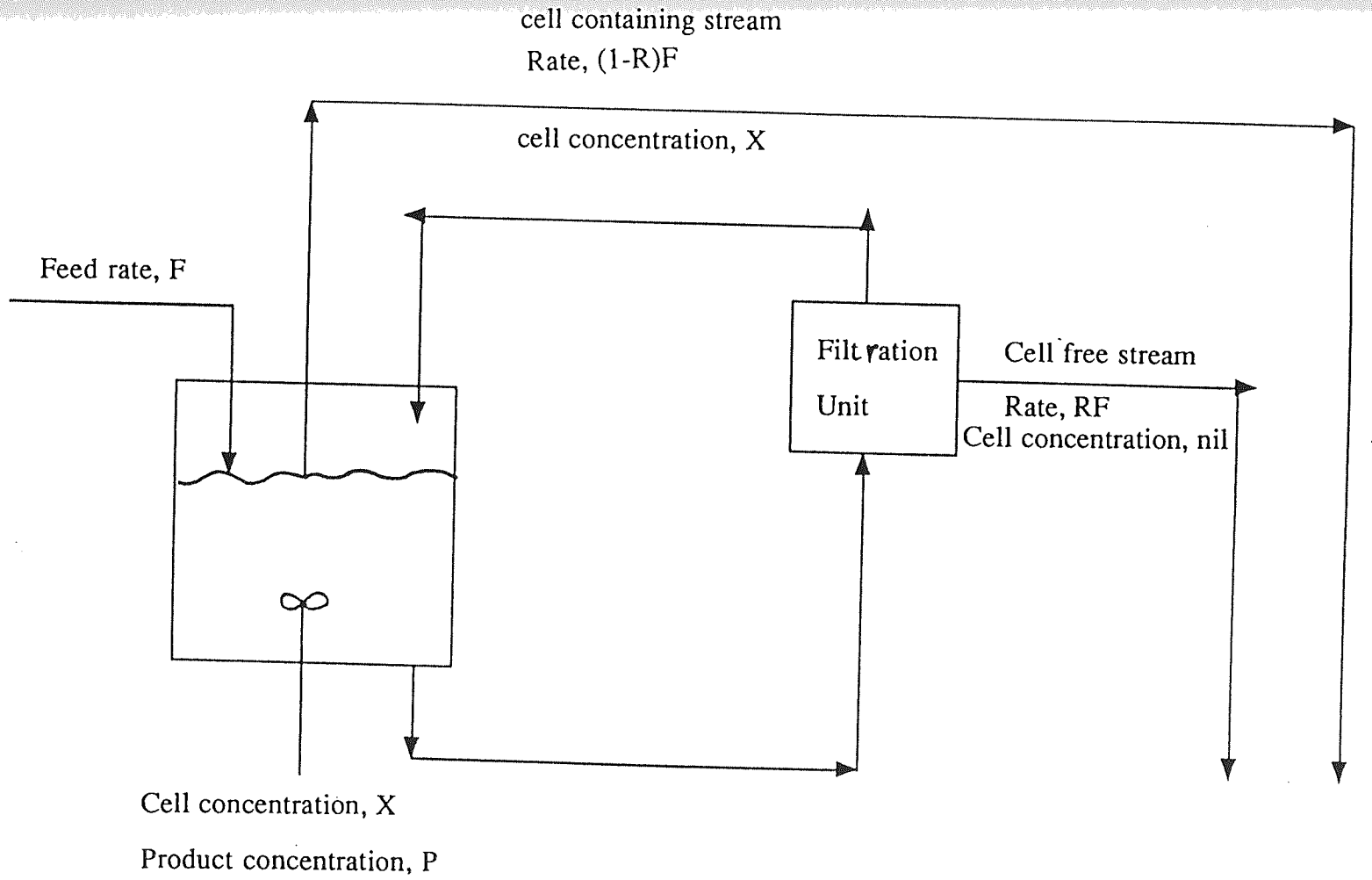


Figure 2.2 A Simplified diagram of a cell recycle fermentation system

When R = O the fermenter is a conventional chemostat while when R = 1 no cells leave the fermentation system and consequently total recycle of biomass is applied.

The theoretical treatment of this particular cell recycle fermentation is presented below together with the assumptions on which it is based.

It is assumed that: (i) the Monod equation is valid and applicable to this system; (ii) the retention time of culture in the recycle loop is negligible so the culture, in the fermentation system, is at all times under the set environmental conditions; (iii) there is no reduction in cell viability due to cell recycle operation; (iv) the maintenance energy requirements are negligible; (v) biomass production is not inhibited by product accumulation.

a. Specific growth rate (μ)

The biomass balance for the culture can be written as:

Net increase in biomass = Growth - Output

which for the whole culture will be

$$Vdx = V.\mu x.dt - (1-R)F.x.dt \qquad \text{Eqn. 2.25}$$

where V is the culture volume in the fermentation system

Dividing throughout by V.dt we obtain

$$dx/dt = \mu x - (1-R)(F/V)x \qquad \text{Eqn. 2.26}$$

F/V = D, the dilution rate.

By inserting this value in equation 2.26 we obtain

$$dx/dt = [\mu-(1-R)D]x \qquad \text{Eqn. 2.27}$$

At a steady-state we have dx/dt = 0 and consequently equation 2.27 becomes

$$\mu = (1-R)D \qquad \text{Eqn. 2.28}$$

When R=1, μ=0. In this case all the substrate which is fed into the fermentation system is only enough for the cells to meet their maintenance requirements.

b. Growth limiting substrate concentration (s)

The Monod equation is used to describe the relationship between the specific growth rate and limiting substrate concentration.

$$\mu = \mu_m s / (K_s+s) \Rightarrow s=K_s\mu\ (\mu_m-\mu) \qquad \text{Eqn. 2.29}$$

where K_s is a saturation constant and μ_m is the maximum specific growth rate. If in equation 2.29 we insert the value of μ from equation 2.28 we obtain the expression of the steady state concentration of the growth limiting substrate, i.e.

$$\tilde{s}=K_s(1-R)D/[\mu_m-(1-R)D] \qquad \text{Eqn. 2.30}$$

As can be seen from this equation for a given value of dilution rate, the steady state value of growth limiting substrate concentration will be lower in a CRF than in a conventional chemostat.

c. Biomass concentration (x)

The concentration of biomass in the fermenter is equal to the concentration in the combined outflow streams divided by (1-R).

Since $x = Y(s_o - s)$ the expression of x for the cell recycle fermenter at steady state is

$$\tilde{x}=Y(s_0-\tilde{s})/(1-R) \qquad \text{Eqn. 2.31}$$

where s_o is the concentration of the growth limiting substrate in the feed stream.

Equation 2.31 reduces to

$$\tilde{x}=Y s_0/(1-R) \qquad \text{Eqn. 2.32}$$

when $s_o >> \tilde{s}$ and can be used to predict biomass concentrations in the cell recycle fermentation system.

d. Volumetric cell growth rate ($\mu\tilde{x}$)

The volumetric cell growth rate is given by the product of the specific growth rate and steady state biomass concentration, thus:

$$\mu\tilde{x}=DY(s_0-s) \qquad \text{Eqn. 2.33}$$

Substituting for s in equation 2.33 by equation 2.30 we obtain

$$\mu\tilde{x}=D\,Y(s_0-[K_s(1-R)D/\mu_m-(1-R)D])$$ Eqn. 2.34

e. Critical dilution rate (D_{crit})

The critical dilution rate is defined as the dilution rate at which s becomes s_o and the microorganism is washed away from the fermentation system.

If in the Monod equation we substitute the value of μ from equation 2.28, we obtain:

$$D = (\mu_m s / K_s+s) / (1-R)$$ Eqn. 2.35

Substituting s for s_o in the above equation, (2.35), we obtain

$$D_{crit} = (\mu_m s_o / K_s+s_o) / (1-R)$$ Eqn. 2.36

which for values of $s_o >> K_s$ reduces to

$$D_{crit} = \mu_m / (1-R)$$ Eqn. 2.37

The physical interpretation of equation 2.37 is that for any value of R

(which is always in the region of $0<R\leqq 1$) $D_{crit.}$ will be higher than μ_m and consequently the fermentation can be run at dilution rates higher than μ_m. In the extreme case that $R = 1$ the microorganism can not be washed away (in this case $D_{crit.}$ equals infinity).

2.6.2 CROSS-FLOW FILTRATION [2.17), (2.18), (2.19), (2.20), (2.21)]

There are two modes of flowing fluids through membranes, dead-ended and tangential. In dead-ended flow, the fluid passes perpendicular to the plane of the filter, whereas in the tangential regime, fluid passes essentially parallel to the filter surface. Common sterile filtration procedures are operated in the dead-ended flow pattern but for processing cells, dead-ended filtration is somewhat limiting in terms of total throughput. In dead-ended filtration the material not passing through the filter remains close to the upstream surface of the filter and forms a layer that can severley limit subsequent flow rates and throughput. In tangential flow, this material is swept along with the fluid which acts as a cleaning force to keep the upstream surface of the membrane relatively clear of retained material. For the same cell suspension, tangential flow procedures can produce flow rates and throughputs 100 to 1,000 times higher than corresponding dead-ended procedures.

The layer of the retained material on the upstream side of the membrane is very important and it has been given the name concentration polarization or gel layer and it always serves to limit the flow of fluid through the membrane. This concentration polarization layer becomes actually a secondary membrane that has properties

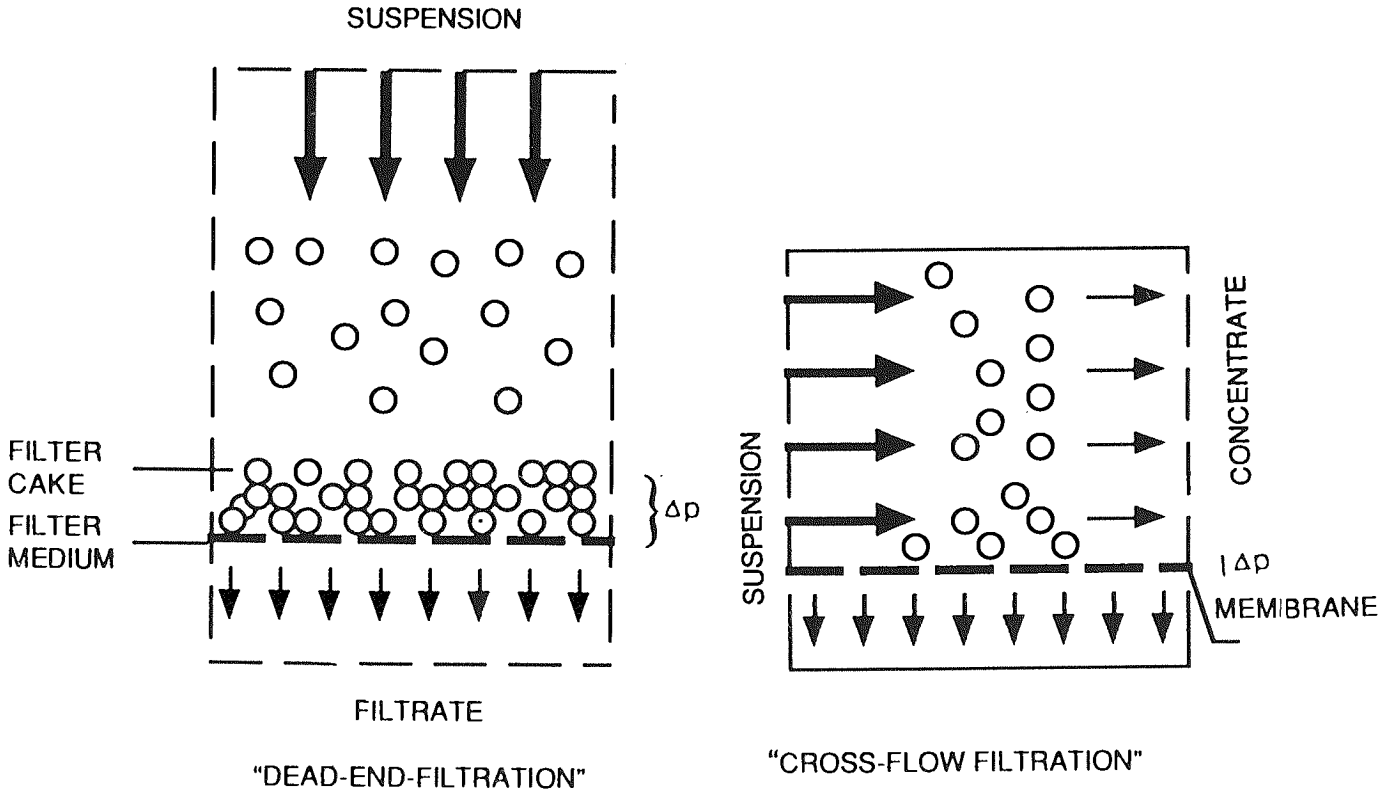


Fig 2.3 Comparison of filtration principles

different to those of the actual membrane.

Theory of cross-flow filtration

The theory of cross-flow filtration is not treated here in detail, only the highlights are presented. When fermentation broth or a cell suspension is passed tangentially across the upstream surface of a membrane filter a concentration polarization layer is formed that causes decrease in the flow rate of fluid that passes through the membrane. Under conditions of equilibrium the rate of solvent flow through a membrane is given as:

$$J=B\,(\ln C_g/C_B)\left(\dot{\gamma}\frac{D^2}{L}\right)^{1/\beta} \qquad \text{Eqn. 2.38}$$

where

J = solvent flux in volume per unit area of membrane.

B = a constant depending on wall boundary conditions.

C_g = concentration of macrosolute in the polarization layer.

C_B = concentration of bulk species retained upstream of the membrane.

$\dot{\gamma}$ = fluid shear rate at the membrane surface.

D = diffusion coefficient of macrosolute species in the polarization layer.

L = length of the flow channel.

For cell processing the pump that must be used to put the cell suspension through the cross-flow filtration unit should provide the necessary flow rates that minimize polarization effects on the membrane.

In cross flow filtration the main parameters one has to control are the inlet and outlet pressures. These parameters control the recirculation rate and transmembrane pressure that, in turn, controls the permeate flux. Filtrate pressure is usually kept to zero, although to minimize polarization effects non zero values must be used.

Recirculation rate is the flow rate of a fluid that passes in parallel across the upstream side of the membrane. The average transmembrane pressure (TMP) is given from the following equation.

$$\text{Avg. TMP} = \frac{P_{in} + P_{out}}{2} - P_{filtrate} \qquad \text{Eqn. 2.39}$$

and is the driving force that makes the fluid pass through the membrane. The value of (P_{in} - P_{out}) is proportional to the shear rate or the velocity of the fermentation broth or cell suspension that passes tangentially across the upstream side of the membrane.

Membranes[2.17],[2.18],[2.19],[2.20],[2.21]

There are basically three types or classes of membrane filters: microporous or microfiltration (MF), ultrafiltration (UF) and reverse

osmosis (RO).

With respect to ultrafiltration membranes, their active portion which actually rejects molecules or cells is extremely thin. The underlying structure of this type of membranes serves as a support matrix of the thin film. Resistance to fluid flow occurs mainly at the rejecting layer at the upper surface. Another feature of ultrafiltration membranes is that they are very asymmetric.

UF membranes are rated by their molecular weight retention characteristics, that is the molecular weight of a globular protein which will be rejected at the upstream surface of the filter.

Microporous or microfiltration membranes are usually more symmetrical than (UF) membranes and usually flow can proceed in either direction. With these types of membranes resistance to flow is encountered throughout the entire volume of the filter. Regenerating and cleaning of UF membranes is relatively easy compared to MF membranes because only the top surface needs to be cleaned, not the entire filter volume.

Microporous or microfiltration membranes are rated in terms of a mean pore size, usually measured in microns. A representative pore

size for a microfiltration membrane would be in the region of 0.2 to 0.45 μm. Recently, microfiltration membranes which have a highly asymmetric pore structure through their depth are commercially available. This asymmetric morphology minimizes the potential internal pore fouling usually associated with symmetric membranes. As a result membrane life, recovery and long term performance usually exceed those of isotropic membranes.

Membrane Fouling

Concentration polarization and fouling of membranes are two phenomena that manifest themselves in the same manner although they are two distinct phenomena. Fouling is caused when a chemical constituent of the fermentation broth or cell suspension adhers onto the membrane. This adsorbance causes a decrease in flux. Fouling is differentiated from concentration polarization by the fact that fouling is immune to variations in cross-flow rate, shear or tangential velocity of the fermentation fluid, across the upstream side of the membrane, whereas concentration polarization is not.

Microfiltration and ultrafiltration membranes are usually constructed in two configurations: plate and frame device and spiral configurations. The difference in shear between these two configurations is governed by

the spatial separation of the membranes and the geometry of the flow channel. Crossflows for spirals usually are lower than for cassette devices. Another advantage of the cassette configuration is that it can be operated at high pressures while spiral configurations cannot.

Membrane selection criteria

One criterion for choosing a membrane is certainly the particular application for which the membrane is required. If the removal of a protein from a fermentation broth is desired then probably a MF membrane is more suitable than an UF membrane because of the relatively large pore sizes peculiar to MF membranes. In general UF membranes give higher rejections of proteins in comparison with MF membranes.

Another criterion for choosing a particular type of membrane is its chemical compatibility with the process fluid as well as cleaning agents which are used to clean the membrane and recover, as much as possible, its initial performance.

If aseptic conditions are required, as for example in the case of cell

recycle fermentation, the membrane must be autoclavable or be chemically compatible with sterilization agents which are used for sanitation.

In selecting a suitable membrane the material of construction of the membrane must also be considered in relation to the possible interactions of macromolecules, such as proteins, with the membrane material. In most cases membranes manufactured with low protein binding properties are preferred.

Applications

The applications of cross flow filtration which are covered in this section involve cell harvesting, cell washing and cell recycle fermentation.

The first steps after a fermentation has been completed are to perform a solid/liquid separation, in order to reduce on the one hand the bulk volume of the phase where the product is found and on the other hand to enable other product isolation procedures to be carried out. If the desired product is intracellular then the phase of interest is the cell mass itself, the volume of which is much smaller than that of the original crude fermentation broth. If the product is extracellular, then it will be

found in the filtrate, the volume of which is close to that of the original solution.

Cell washing applications using tangential flow filtration are similar to cell harvesting in many respects. However while filtrate is being collected, new solvent is simultaneously being added to the cells. The net result is a changing of solvent conditions such as pH or ionic strength, or the removing of a cellular component such as a protein. Cell washing procedures can be used to remove an unwanted protein or to separate a valuable intracellular protein from lysed cells and cell debris.

In cell recycle fermentation, the fermentation broth is continuously put into a cross flow filtration device and the retentate is returned back to the reactor resulting in an increased cell concentration in the fermenter. This type of fermentation can change the dynamics of a conventional batch fermentation process when the desirable product is produced at a rate which is a positive function of biomass concentration or rate of biomass production or broth.

CHAPTER 3:

ASPECTS OF ENZYME BIOTECHNOLOGY

3.1 INTRODUCTION

Enzymes are biocatalysts which make possible the numerous biochemical reactions in every living organism. In a single biochemical pathway many enzymes are involved. Enzymes are very powerful catalysts in four respects[3.1]. Firstly they are very efficient, catalysing reactions often 10^8 - 10^{11} times more rapidly than the corresponding non enzymic catalysts. Such high rates are achieved even though the enzyme catalysed reactions require much less extreme conditions, such as temperature, pH and pressure, and most of these reactions take place in the cheapest, safest and most abundant solvent, water.

Secondly, the range of reactions catalysed is extremely broad such that a much greater variety of reactions can be catalysed than with chemical catalysts.

Thirdly, enzymes are usually very specific in terms of the type of reaction catalysed.

Fourthly, enzymes are naturally subject to a number of controls both in terms of the gross control of how fast they are synthesised and degraded, and in the fine control of their activity by the binding of small molecule modifiers which can either increase or decrease the activity of the enzymes.

Although much progress has been made, there are still practical difficulties in using purified single enzymes in a biochemical reactor[3.2]. The most important problems are related to their stability and their purification costs. Sometimes it is advantageous to use whole cells (microbial, plant or animal) containing an appropriate enzyme system as a catalyst, especially when complex reactions are involved. In large scale enzyme production the problems of residual biomass and waste water disposal can become unmanageable. The use of the whole cells instead of enzymes may also be preferred when the enzyme isolation and purification is too expensive. The lower specific activity of whole cells and the eventual decrease in selectivity is often balanced by lower price and simple use of such a catalyst.

Table 3.1 gives the amylase activities of various grain sources. From this Table[3.3] it can be seen that the enzyme activity varies considerably according to the source of the enzyme.

TABLE 3.1
AMYLASE ACTIVITIES OF VARIOUS GRAIN SOURCES

	Total relative activity			
	β-Amylase		α-Amylase	
Cereal	Ungerminated	Germinated	Ungerminated	Germinated
Barley	29.8	34.4	0.058	94.0
Wheat	25.1	23.7	0.063	214.7
Rye	17.8	17.6	0.111	119.8
Oats	2.4		0.297	60.3
Maize			0.249	35.6
Sorghum			0.127	75.6
Rice		0.2		2.3

Table 3.2 gives some turnover numbers for enzyme - and inorganic - catalysed reactions.(3.3)

TABLE 3.2
SOME TURNOVER NUMBERS FOR ENZYME - AND INORGANIC - CATALYSED REACTIONS

Catalyst	EC number	Reaction	Turnover number	Temperature (°C)
Enzymes				
Bromelain	3.4.22.4	Hydrolysis of peptides	4×10^{-3}–5×10^{-1}	0–37
Carbonic anhydrase	4.2.1.1	Hydration of carbonyl compounds	8×10^{-1}–6×10^{5}	0–37
Fumarase	4.2.1.2	L-Malate $\rightleftharpoons$ fumarate + H_2O	10^{3} (forward)	0–37
			3×10^{3} (backward)	0–37
Papain	3.4.22.2	Hydrolysis of peptides	8×10^{-2}–10	0–37
Ribonuclease	3.1.4.22	Transfer phosphate of polynucleotide	2–2×10^{3}	0–37
Trypsin	3.4.21.4	Hydrolysis of peptides	3×10^{-3}–10^{2}	0–37
Inorganic catalysts				
Aluminium trichloride		*n*-Hexane isomerization	10^{-2}	25
alumina			1.5×10^{-2}	60
Copper/silver (Cu_3Au)		Formic acid dehydrogenation	2×10^{-7}	25
			3×10^{10}	327
Silica-alumina		Cumene cracking	3×10^{-8}	25
			2×10^{4}	420
Vanadium trioxide		Cyclohexene dehydrogenation	7×10^{-11}	25
			10^{2}	350
Zeolite (decationized)		Cumene cracking	$\sim 10^{3}$	25
			$\sim 10^{8}$	325

3.2 ADVANTAGES OF PRODUCING ENZYMES BY FERMENTATION

Enzymes are components of all types of cells i.e. animal, plant and microbial. Many early applications utilized enzymes from higher organisms. For example, studies of the mashing process in beer-making revealed the importance of the malt enzymes in utilizing carbohydrate and protein reserves, and this led to the utilization of enzyme-rich malt extracts in other applications.

Other enzymes are made from unwanted or low-value organs from domestic farm animals, for example, trypsin and chymotrypsin from pig pancreas, alcohol dehydrogenase from horse liver and catalase from bovine liver. These sources seem to offer economic advantages over microorganisms produced by fermentation. An important proportion of current enzyme markets are satisfied by products from plants or higher organisms.

These sources for enzymes, however, have some major disadvantages. The tissues required for the extraction of enzymes from animals tend to be limited in supply and hence it is difficult to match supply to demand if the latter increases suddenly, and labour intensive methods are frequently needed for cultivation and collection.

There is often competition for material from which to extract

alternative valuable biological products. Supplies of agricultural materials used for extraction of enzymes are usually seasonal and supply may be subject to uncontrollable climatic conditions.

It was early recognized that microbial cells had great potential as sources of enzymes [3.1], [3.3], [3.9], [3.11] and they offer a number of advantages.

Production of microbial cells can be scaled up relatively easily to allow increased production if market demands so dictate.

Microbial cells have very diverse natures and the number of enzymes that can be produced is very large. Microbial cells, by contrast with higher organisms are relatively easy to culture in a controlled environment, are highly amenable to genetic alteration, and thus, because of their relatively short generation times can be rapidly 'improved' by strain development techniques.

A production cycle for a batch fermentation will last between a half day and ten days. In contrast, for higher organisms, a life cycle lasts typically from two months to several years [3.4], allowing little scope for investigation of improvement of yield and productivity within a reasonable time. Fermentation also allows the chosen strain to be cultivated under near-ideal and closely controlled conditions to give high and consistent productivities.

A fermentation plant can be designed to an appropriate scale for the level of demand.

Furthermore another advantage of the microbial production of enzymes by fermentation is the flexibility of the fermentation plant. This can be used for a variety of products or switched from one type of product to another in the event of changing demands. For example, in the early 1970s a sudden fall in demand for detergent proteinases coincided with an increase in utilization of enzymes for starch processing, and excess capacity built for proteinase production was utilized to make amyloglucosidase.

Enzymes from higher organisms can also be produced by transferring production into fermentation processes. Culture of animal and plant cells is now established on the laboratory scale (3.4), (3.5) and in some cases these sources have been specifically used for enzyme production. So far, however, it seems unlikely to be successful on a production or industrial scale. Plant cells in particular grow very much more slowly than those of microbes in culture giving throughputs which are one or more orders of magnitude lower. Animal cells in culture are extremely fragile and it has not yet been possible to design efficient large scale fermentation vessels in which they can survive. An alternative approach is to transfer the relevant genes into microorganisms together with the appropriate promoters to allow high levels of expression.

This has already been achieved in the laboratory for calf rennin. Precursors of the enzyme have been expressed from cloned genes in E.coli[3.6] and Saccharomyces cerevisiae[3.7],[3.8]. The precursors have been purified, activated, and shown to display the same milk-clotting properties as the enzyme purified from calf stomach.

This latter approach, i.e the transfer of relevant genes into micro-organisms together with the appropriate promoters, also illustrates the advantages of producing enzymes by microbial fermentation.

3.3 ENZYME CATALYSIS[3.1],[3.2],[3.3],[3.10]

Enzyme action depends on the formation of an enzyme-substrate complex (ES). The enzymes are subject to the same kinetic and thermodynamic constraints as are chemical catalysts.

Enzyme action resembles very much that of an ordinary heterogeneous catalyst. Enzymes change the rate of reaction but not the final position of the equilibrium between substrates and products.

A means other than catalysis to accelerate the reaction S (substrate) $\rightarrow$ P (product) is to increase the temperature until a significant number of S molecules attain the transition state. This is, however, not feasible for living cells.

Catalysis can be realized either by proceeding by an alternative reaction mechanism, which has an alternative transition state with a lower free energy, or by lowering the free energy of the conventional transition state intermediate. Another mode of action of a catalyst is to alter the environment such as to cause a decrease in the free energy of the product so that the net ΔG is decreased and consequently substrate conversion to product is attained.

In an enzyme catalysed reaction three phases are generally recognized. In the first phase the concentration of the free enzyme is decreased

drastically since most of it is combined with the substrate in a dynamic equilibrium which continue to occur as long as there are fresh substrate molecules available.

In the second phase there is a dynamic equilibrium between enzyme molecules and reactants. The enzyme reforms enzyme-substrate molecules after having transformed the previous substrate molecules.

In the third phase of the reaction, the substrate concentration has been diminished and hence the rate of reaction falls asymptotically. This part of the reaction is very important, especially in industrial processes where complete reaction is desired in order to maximize product yield and concentration. This phase of the reaction may occupy the majority of the reaction time and this is more pronounced in the case of product inhibition which often occurs when high substrate concentrations are employed.

Fig. 3.1 depicts the free energy change ΔG and activation energy E_a of enzymatic and non-enzymatic reactions. The energy of activation is determined experimentally by application of the Arrhenius equation which relates to the rate constant of an elementary chemical reaction, K, to temperature, T;

$K = K_0 \exp(- E_a / RT)$ or in a logarithmic form;

$\ln K = -E_a / RT + \ln K_0$. A plot of this equation yields a straight line with a slope ($- E_a / RT$) thus enabling the activation energy to be

calculated.

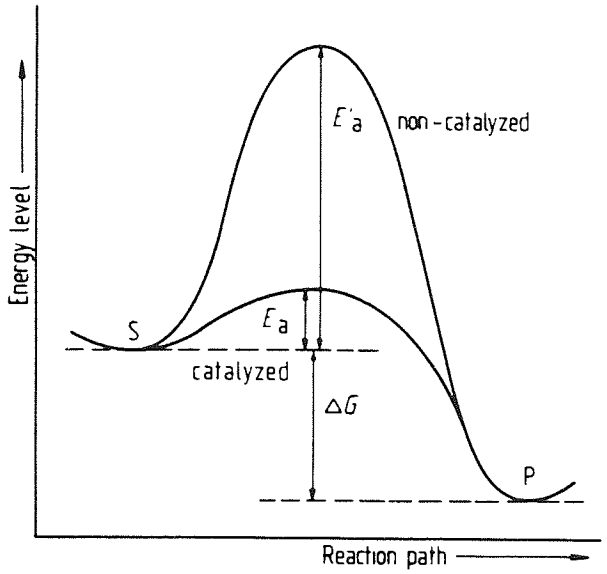


FIG 3.1 Free energy change ΔG and activation energy E_a of enzymatic and non-enzymatic reactions, (ref. 3.2).

3.4 REACTION KINETICS AND DETERMINATION OF KINETIC CONSTANTS[3.1), (3.2), (3.3), (3.10), (3.12)

The Michaelis-Menten Equation.

The first general rate equation for reactions involving enzymes was derived by Henri in 1903 and after 10 years was confirmed by Michaelis and Menten. This mathematical treatment of enzyme catalyzed reactions was based on the assumptions that:

i) a steady state level of the enzyme-substrate complex is sufficient for this mathematical treatment i.e. the rate of formation of the intermediate enzyme-substrate complex equals the rate of its decomposition. A further assumption that was made is that:

ii) the overall rate of the reaction is limited by the breakdown of the ES complex into free enzyme and product. This situation can be represented by the following reaction scheme:

$$\underset{[E]}{\text{Enzyme}} + \underset{[S]}{\text{Reactant (substrate)}} \underset{K_{-1}}{\overset{K_1}{\rightleftharpoons}} \underset{[ES]}{\text{Complex}} \xrightarrow{K_2} \underset{[E]}{\text{Enzyme}} + \underset{[P]}{\text{Product}}$$

The complex formation step is considered to be reversible since it has an energy state similar to that of the enzyme-substrate mixture. The subsequent breakdown of the complex to give the product and the enzyme is exothermic and it is this part of the overall reaction which is irreversible in many cases. The rate expression of this reaction is given below and it is assumed that the intermediate complex concentration remains constant;

$$[E] + [S] \underset{K_{-1}}{\overset{K_1}{\rightleftharpoons}} [ES] \xrightarrow{K_2} [E] + [P]$$

The steady state assumption as regards the intermediate complex concentration i.e. Formation = Breakdown is :

$$\frac{d[ES]}{dt} = K_1 [E] [S] - (K_{-1} + K_2) [ES] = 0$$

Formation Breakdown

Eqn. 3.1a

and therefore,

$$K_1 [E] [S] = (K_{-1} + K_2) [ES]$$

Eqn. 3.1b

In order for the above assumption to be accurate the rate of reaction muct be linear and so it is determined as close to time zero as possible, before the reactant concentration changes appreciably the initial rate.

Equation 3.1b can be rearranged to give:

$$\frac{[E] [S]}{[ES]} = \frac{K_{-1}+K_2}{K_1} = K_m$$

Eqn. 3.2

where K_m is the Michaelis-Menten constant.

A mass balance on the enzyme requires that the total enzyme $[E_O]$ does not change, i.e. $[E_O] = [ES] + [E]$.

Therefore $[E] = [E_0] - [ES]$

If we substitute for [E] in equation 3.1b and rearrange we obtain:

$$[ES]=\frac{[E_0][S]}{K_m+[S]} \qquad \text{Eqn. 3.3}$$

The rate constant K_2 is a first order rate constant having units of time^{-1}. Multiplication of both sides of equation 3.3 by K_2 will give the rate of product concentration with time as a function of the maximum possible rate, the Michaelis constant and the substrate concentration:

$$\frac{dP}{dt}=K_2[ES]=\frac{K_2[E_0][S]}{K_m+[S]}$$

This is usually written in the form of

$$\upsilon=\frac{\upsilon_{max}[S]}{K_m+[S]} \qquad \text{Eqn. 3.4}$$

which is the Michaelis-Menten equation.

When $K_2 << K_{-1}$ then equation 3.2 becomes $K_m = K_{-1} / K_1$ which demonstrates that K_m is, in this case, the dissociation constant of the enzyme-substrate complex. The constant K_2 is called the turnover number and represents the maximum reaction velocity per mole of

enzyme or per mole of active site, depending on the concentration units in which [E] is expressed. Usually $K_2 = \upsilon_{max} / [E]$ and its units are : moles of product per minute per mole of enzyme.

The Michaelis Menten equation describes a right rectangular hyperbola with limits υ_{max} and $-K_m$ as can be shown by rearrangement of equation 3.4 in the form:

$(\upsilon_{max} - \upsilon)(K_m + [S]) = K_m \ \upsilon_{max}$

Thus examining the υ vs [S] curve, three distinct kinetic regions can be found. At very low and very high substrate concentrations the υ vs [S] curve is essentially linear. The first region can be approximated by first order kinetics, for $[S] << K_m$ equation 3.4 becomes

$$\upsilon = \frac{\upsilon_{max}\,[S]}{K_m} \qquad \text{Eqn. 3.5}$$

while the kinetics in the second region approaches zero-order since for $[S] >> K_m$ eq. 3.4 yields $\upsilon = \upsilon_{max}$

Figure 3.2 shows the reaction rate, υ, versus substrate concentration, [S]. In this Figure $\upsilon \equiv r_S$ and $[S] \equiv C_S$.

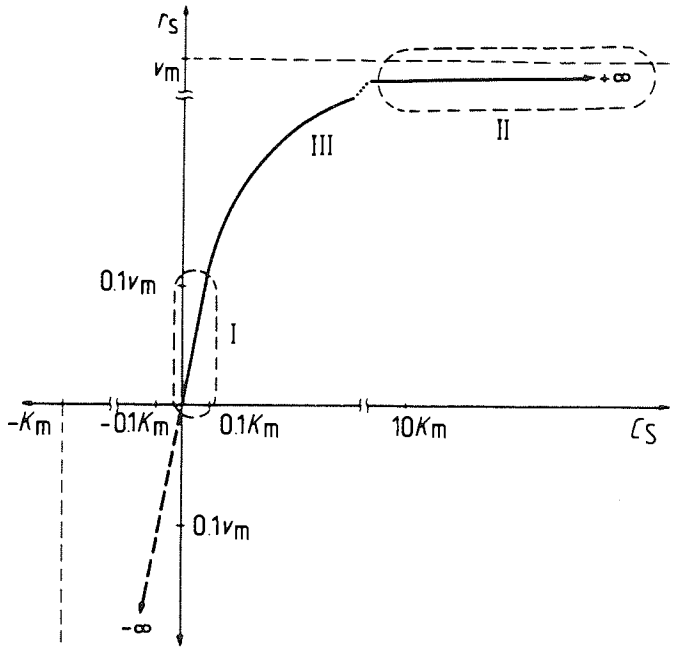


Fig. 3.2 Reaction rate, $\upsilon \equiv r_S$, versus substrate concentration, $[S] \equiv C_S$, (ref. 3.3).

Note the changes of scale.

I. First-order rate approximation.

II. Zero-order rate approximation.

III. Michaelis-Menten equation

Data given in Figure 3.3 supports the proportionality between the rate of reaction, υ, and enzyme concentration [E] at fixed substrate concentration.

Note that in this Figure $\upsilon \equiv V$ and $[E] \equiv [E_T]$.

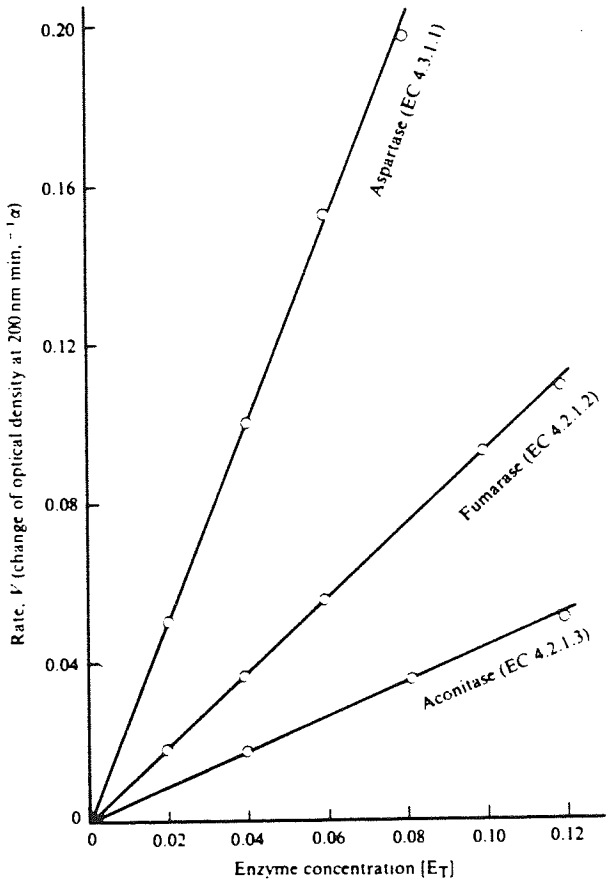


Fig. 3.3. Influence of enzyme concentration on the rate of enzyme-catalyzed reactions, (ref. 3.3).

Determination of Kinetic constants

The parameters of the Michaelis-Menten equation can be determined either graphically after its linearization or, more advantageously, numerically. The Michaelis-Menten equation can be rewritten in several ways to get linear plots of experimental data on reaction rates.

The most common method used to determine kinetic constants is the double reciprocal plot of Lineweaver - Burk. This is obtained by taking reciprocals on each side of the Michaelis-Menten equation yielding the expression:

$$\frac{1}{\upsilon} = \frac{K_m}{\upsilon_{max}} \cdot \frac{1}{[S]} + \frac{1}{\upsilon_{max}}$$

Plotting $\frac{1}{\upsilon}$ vs. $\frac{1}{[S]}$ gives a straight line extrapolation of which allows the determination of both constants. The disadvantages of this method are 1) that two reciprocal values are needed, 2) with this method most significance is focused on the rates obtained at low substrate concentrations which involve significant experimental error.

Another method for the determination of kinetic constants which has not the drawbacks of the double reciprocal plot is presented below. This method is based on treating υ_{max} and K_m as variables and [S] and υ as experimentally determined constants.

Eq. 3.4 can be modified to give:

$$\upsilon_{max}=\frac{\upsilon_1 . K_m}{[S_1]} + \upsilon_1 \quad \text{for any individual measurements } [S_1], \upsilon_1.$$

This expression will be valid for any pair of [S] and υ values. Thus:

$$\frac{\upsilon_1 K_m}{[S_1]} + \upsilon_1 = \frac{\upsilon_2 K_m}{[S_2]} + \upsilon_2$$

If we solve for K_m we obtain:

$$K_m=\frac{(\upsilon_2 - \upsilon_1)}{\left(\frac{\upsilon_1}{[S_1]} - \frac{\upsilon_2}{[S_2]}\right)}$$

Using the same methodology for υ_{max}, we obtain the expression

$$K_m=\upsilon_{max}\frac{[S_1]}{\upsilon_1} - [S_1] = \upsilon_{max}\frac{[S_2]}{\upsilon_2} - [S_2]$$

Solving for υ_{max} yields

$$\upsilon_{max}=\frac{([S_2] - [S_1])}{\left(\frac{[S_2]}{\upsilon_2} - \frac{[S_1]}{\upsilon_1}\right)}$$

With this method the values of υ_{max} and K_m are determined for every pair of experimental data. A line is drawn connecting - $[S_1]$ to υ_1 and then extended to the first quadrant. This procedure is then repeated for the rest of the data i.e. -$[S_2]$, υ_2; - $[S_3]$ υ_3.

If there is not any error in the data a unique intersection is obtained from which the values of υ_{max} and K_m are read directly. In practice more than one intersection point will be obtained and in this case the median value is taken as the best estimate of K_m and υ_{max}.

In conclusion, the main features and advantages of the direct linear plot are:

- Every line represents one pair of experimental data.
- Probably is the best method.
- No reciprocal values are needed, and finally, the method enables instantaneous recognition of deviating points.

Table 3.3 gives Michaelis constants and maximum rates for cellulase complexes from various sources and of varying degrees of purity with respect to cellobiose[a], (ref. 3.10).

TABLE 3.3

MICHAELIS CONSTANTS AND MAXIMUM RATES FOR CELLULASE COMPLEXES.

Source	Preparation[b]	K_m	V_{max}
Trichoderma lignorum	I	1.3	7
	II	1.3	12
	III	1.2	40
	IV	1.3	550
Geotrichum candidum	I	2.5	5
	II	2.0	45
	III	2.5	180
	IV	2.8	270
	V	2.4	550
T. koningii	I	1.0	6.5
	II	1.8	20
T. reesei	I	2.0	20
	II	1.8	70
Aspergillus niger	I	2.5	35
	II	2.7	1200
Asp. foetidus	I	2.0	75
	II	2.0	10000
	III	1.8	9000
T. viride		2.0	23
T. longibrachiatum		1.5	10

a. Measured at pH 4.5 and temperature of 40°C.

b. Preparations of varying degrees of purity and from different manufacturers.

Kinetics of reversible enzyme reactions - Extent of the reaction

In many cases enzyme catalyzed reactions have equilibria which greatly favour the formation of products. In such a case these reactions are considered irreversible. In some other cases equilibria are reached demonstrating that the reverse reaction must also be considered. This feature of such a type of reactions is illustrated by the following reaction mechanism:

$$[E] + [S] \underset{K_{-1}}{\overset{K_1}{\rightleftharpoons}} [ES] \underset{K_{-2}}{\overset{K_2}{\rightleftharpoons}} [E] + [P]$$

When the overall reaction reaches an equilibrium (the rates in both directions are equal) the following equations are set which describe it.

$$K_1 [E] [S] = K_{-1} [ES]$$

$$K_2 [ES] = K_{-2} [E] [P]$$

Rearranging, we obtain:

$$\frac{[ES]}{[E]} = \frac{K_1}{K_{-1}} [S] = \frac{K_{-2}}{K_2} [P]$$

it follows $\frac{[P]}{[S]} = \frac{K_1 K_2}{K_{-1} K_{-2}} = K_{eq} \equiv$ The equilibrium constant.

Since the rate constants are difficult to determine, the equilibrium

constant must be expressed in terms of υ_{max} and K_m for both forward and backward reactions.

The forward reaction, i.e. the reaction which leads to the formation of products can be expressed as $\upsilon_{max}(f) = K_2[E_o]$ while the backward reaction which releases the substrate can also be expressed similarly, i.e. $\upsilon_{max}(b) = K_{-1}[E_o]$. Taking the ratio;

$$\frac{\upsilon_{max}(f)}{\upsilon_{max}(b)} = \frac{K_2[E_0]}{K_{-1}[E_0]}$$

As has already been shown in section 3.3 $K_m(f)$ can take the form of Eq. 3.2,

$$\text{i.e.} \quad K_m(f) = \frac{K_{-1} + K_2}{K_1}$$

Similarly for $K_m(b)$ we obtain;

$$K_m(b) = \frac{K_{-1} + K_2}{K_{-2}}$$

Taking the ratio $K_m(b)$ over $K_m(f)$ leads to the expression;

$$\frac{K_m(b)}{K_m(f)} = \frac{(K_2 + K_{-1}) \cdot K_1}{K_{-2} \cdot (K_2 + K_{-1})}$$

and consequently the expression for Keq is;

$$K_{eq.} = \frac{\upsilon_{max}(f)\ K_m(b)}{\upsilon_{max}(b)\ K_m(f)}$$

which is known as the Haldane relationship.

With respect to the determination of kinetic constants it must be noted that if initial rate measurements are taken then the reaction can be considered irreversible since there is no product in appreciable amount.

3.5 ENZYME INHIBITION

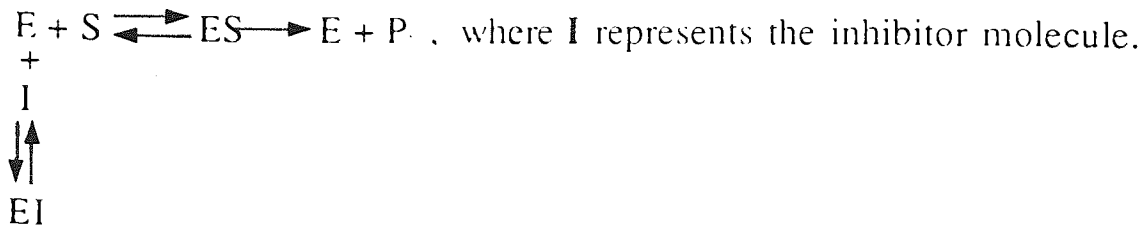
(References: (3.1),(3.2),(3.3),(3.4),(3.9),(3.10),(3.12),(3.13),(3.14))

Inhibition is caused by substances called inhibitors which reduce the rate of an enzyme-catalyzed reaction and they can have an irreversible or reversible effect on the enzyme activity. Basically three simple types of reversible inhibition systems are considered according to the inhibitor action.

Competitive inhibition

Competitive inhibitors are similar to the substrate in shape, size and charge distribution and hence are able to bind to the enzyme active-sites, preventing the formation of the enzyme-substrate complex. Inhibition of this type is reversible and is overcome in the presence of excess substrate.

A simple schematic of the mode of action of a competitive inhibitor is given below:



, where I represents the inhibitor molecule.

A typical plot of an enzyme-catalyzed reaction in the presence of a

competitive inhibitor is given in Fig. 3.4.

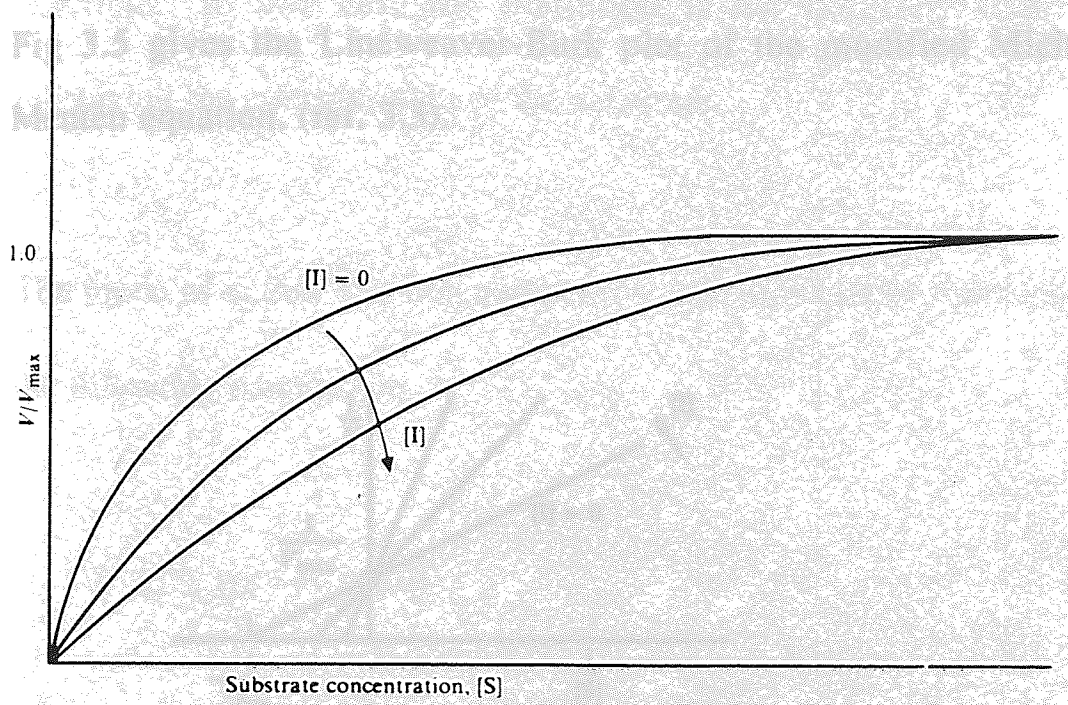


Fig 3.4 Michaelis-Menten plot for competitive inhibition, (ref. 3.2). Note that $V/V_{max} \equiv \upsilon/\upsilon_{max}$.

The modification to the Michaelis-Menten equation is shown in Eqn. 3.6.

$$\upsilon = \upsilon_{max} \frac{[S]}{K_m[1 + ([I]/K_i)] + [S]} \qquad \text{Eqn. 3.6}$$

where [I] is the concentration of the inhibitor and K_i is the inhibition constant, or dissociation constant of the enzyme-inhibitor complex,

which is defined as the concentration of inhibitor at which the K_m value is doubled.

Fig 3.5 gives the Lineweaver-Burk plot of the modified Michaelis-Menten equation, (ref. 3.3).

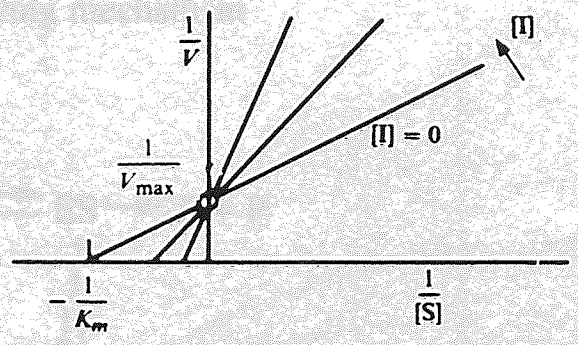


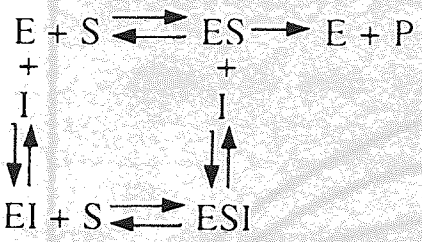
Fig 3.5. **Lineweaver-Burk plot for competitive inhibition. Slope = 1 + ([I] / K_i), ordinate = 1 / V_{max}, abscissa = $-\frac{[I]/K_m}{1+([I]/K_i)}$. Note that $1/V \equiv 1/\upsilon$ and $1/V_{max} \equiv 1/\upsilon_{max}$.**

Non competitive inhibition

Non competitive inhibition is a type of inhibition where the inhibitor also forms a reversible complex with the enzyme but these inhibitors do

not bind at the active site of the enzyme but at more remote position(s) resulting in a decrease in the maximum specific rate of the catalysed reaction. In this case the inhibition is not normally reduced by increasing the concentration of the substrate.

The mode of action of a non competitive inhibitor can be represented by the following mechanism



where I is the inhibitor molecule.

The effect of low concentrations of this type of inhibitor on the Michaelis-Menten equation is given by Eqn 3.7 which is derived using the steady-state procedure.

$$\upsilon = \left[\frac{\upsilon_{max}}{1 + ([I]/K_i)} \right] \frac{[S]}{K_m + [S]} \qquad \text{Eqn. 3.7}$$

where [I] is the inhibitor concentration, K_i is the inhibition constant,

which is defined as the concentration of the inhibitor at which the υ_{max} of the enzyme is halved. Plots for noncompetitive inhibition are given in Fig 3.6 and Fig 3.7

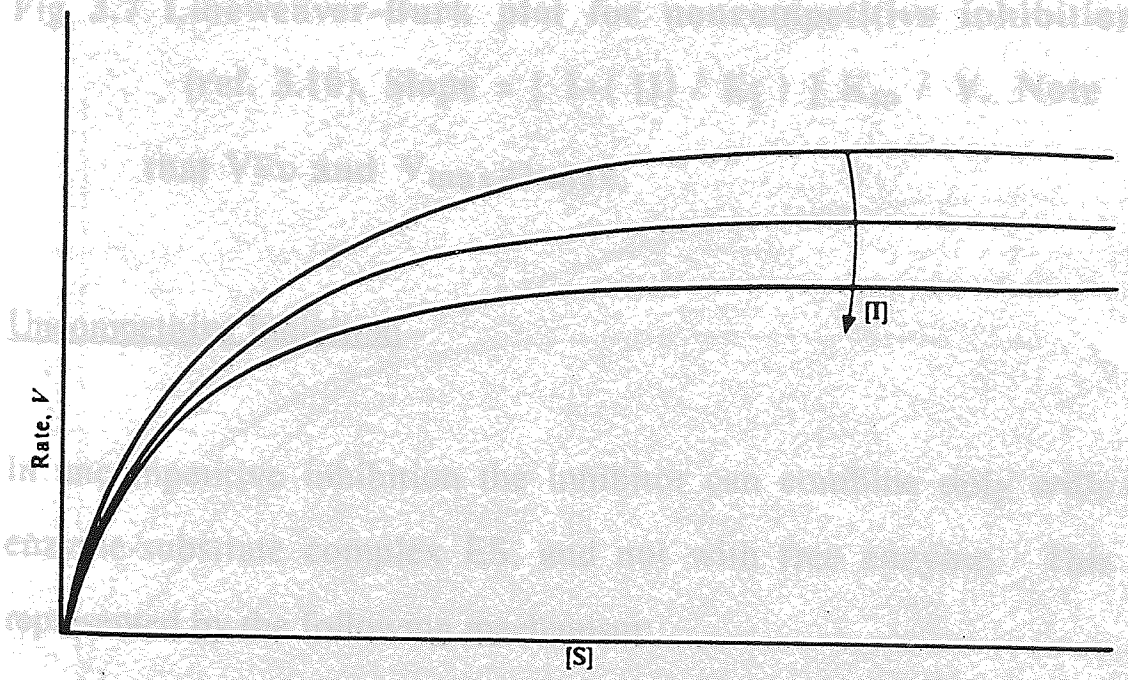


Fig 3.6 Michaelis-Menten plot for noncompetitive inhibition, (ref. 3.3). Note that V≡υ.

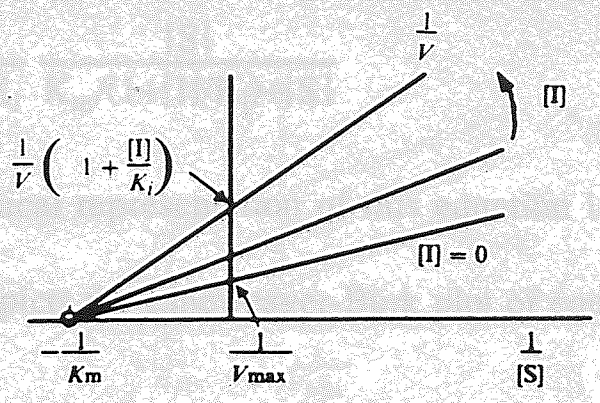


Fig 3.7 Lineweaver-Burk plot for noncompetitive inhibition, (ref. 3.10). Slope = [1+([I] / K_i)] K_m / V. Note that $V \equiv \upsilon$ and $V_{max} \equiv \upsilon_{max}$.

Uncompetitive Inhibition

In uncompetitive inhibition the inhibitor can combine only with an enzyme-substrate complex ES, and not with free enzyme. This is represented by the following mechanism:

$$E + S \rightleftharpoons ES \rightarrow E + P$$
$$+$$
$$I$$
$$\downarrow\uparrow$$
$$ESI$$

The modified Michaelis-Menten equation, obtained using the steady-state

procedure, for this type of inhibition is:

$$\upsilon = \frac{\upsilon_{max}}{1+[I]/K_i} \frac{[S]}{K_m/(1+[I]/K_i)+[S]} \qquad \text{Eqn. 3.8}$$

The graphical representation of this equation is given in Fig 3.8 while Fig 3.9 depicts the Lineweaver-Burk plot of equation 3.8.

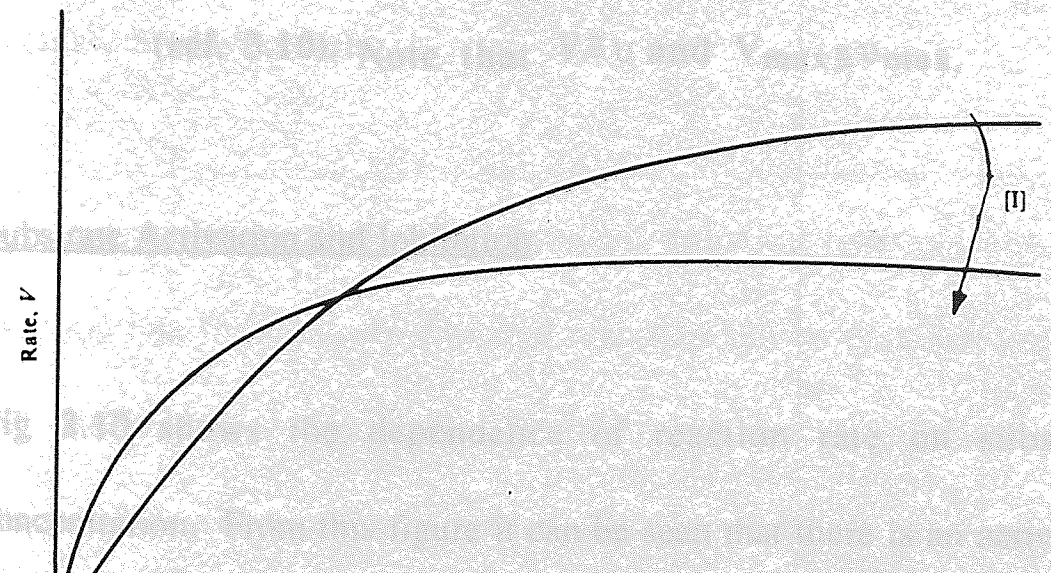


Fig 3.8 Michaelis-Menten plot for uncompetitive inhibition, (ref. 3.10). Note that V≡υ.

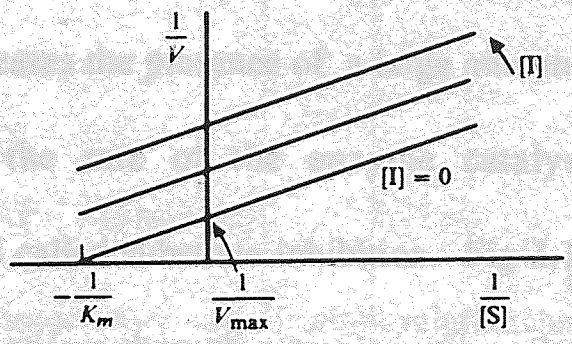


Fig 3.9 Lineweaver-Burk plot for uncompetitive inhibition, (ref. 3.10). Note that $V \equiv \upsilon$ and $V_{max} \equiv \upsilon_{max}$.

Substrate Activation and Inhibition

Fig 3.10 shows the dependence of reaction rate on substrate concentration. From this figure it can be seen that there is an activation effect of increasing substrate concentration, on the rate of an enzyme catalyzed reaction. This is valid for low or relatively low substrate concentrations. To explain this behaviour it is assumed that the enzyme has multiple binding sites for substrate and the binding of the first substrate molecule causes the remaining active sites of the enzyme to

have a stronger affinity for the substrate.

In some other cases the presence of a large amount of substrate causes a reduction of the rate of the enzyme catalysed reaction. This phenomenon is called substrate inhibition. Fig 3.11 shows that the rate of the reaction passes through a maximum as substrate concentration is increased. When the substrate concentration S is larger than $S(\upsilon=\upsilon_{max})$, a decrease in substrate concentrations will result in an increase in reaction rate.

The quantitative relationship between substrate concentration and reaction rate for substrate inhibited reactions can be obtained using the Michaelis-Menten approach. In the mathematical treatment which follows it is assumed that a second substrate molecule can bind to the enzyme. When S is incorporated into the ES complex a non reactive intermediate results:

Reaction steps at equilibrium:

$$E + S \underset{K_{-1}}{\overset{K_1}{\rightleftharpoons}} ES$$

$$ES + S \rightleftharpoons ES_2$$

$$ES \xrightarrow{K} E + P \qquad \text{(Slow step)}$$

If we use the dissociation - equilibrium relationships and the application of mass conservation on the enzyme, the modified Michaelis-Menten equation is obtained which accounts for substrate inhibition of an enzyme catalyzed reaction.

$$\upsilon = \frac{\upsilon_{max}[S]}{[S] + K_m + [S]^2/K_i} \qquad \text{Eqn. 3.9}$$

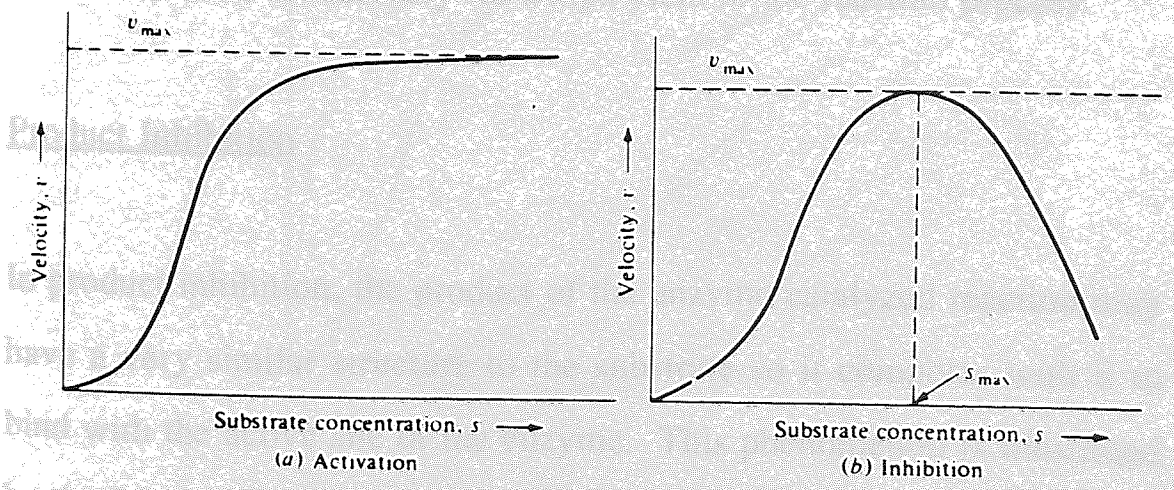


Fig 3.10 Substrate activation (a) and inhibition (b), (ref. 3.2).

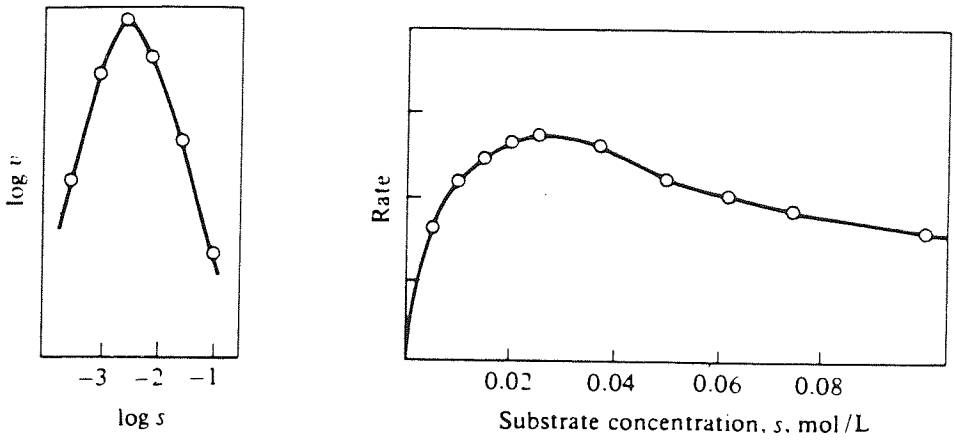


Fig 3.11 Substrate Inhibition, (ref. 3.2). Note that s≡S.

In some enzyme catalyzed reactions the substrates used may contain inhibitors. Their negative effects are more intensive when high throughputs of substrate are required. In such cases treatment of the feedstocks used is necessary for a high yield of the reaction process.

Product Inhibition

In product inhibition, the product of the enzyme catalyzed reaction may have a very similar structure to the substrate so it competes with it to bind with the active site of the enzyme. This phenomenon is illustrated by the following mechanism.

$$[E]+[S] \rightleftharpoons [ES] \longrightarrow [E]+[P]$$
$$\downarrow\uparrow \; [P]$$
$$[EP]$$

Using the same methodology reported earlier in this section the modified Michaelis-Menten equation is obtained which accounts for end-product inhibition.

$$\upsilon = \frac{\upsilon_{max}[S]}{[S]+K_m\left(\frac{1+[P]}{K_i}\right)} \qquad \text{Eqn. 3.10.}$$

Figure 3.12. shows the effect of the activator and inhibitor on the rate of the enzyme catalyzed reaction.

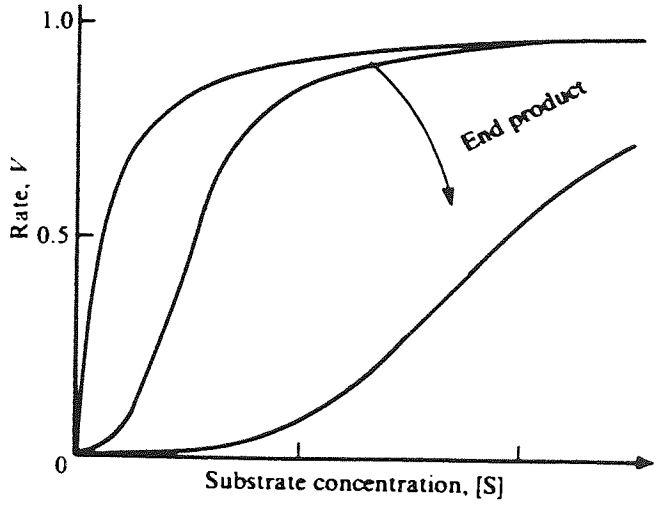


Fig 3.12 Michaelis-Menten plot showing end-product inhibition, (ref 3.10). Note that $V \equiv \upsilon$.

For product inhibition, the rate of the reaction must not be studied in its very early stages, because there is no product in an appreciable concentration and its inhibitory effects would not normally be observed.

3.6 EFFECTS OF pH AND TEMPERATURE ON ENZYME ACTIVITY AND STABILITY

(References:(3.2), (3.3), (3.10), (3.12), (3.13), (3.14), (3.15))

Effect of pH

Enzymes possess many ionizable groups and those involved in the active site(s) of the enzyme molecule must be in a proper ionic form for the enzyme to sustain its catalytic activity by maintaining a proper conformation of its active site(s). On the other hand the substrate itself may possess ionizable groups and only one ionic form of the substrate may be able to bind to the enzyme or react. Consequently pH affects the rate and the realization of an enzyme catalyzed reaction.

The effect of pH can be reversible or irreversible. In the former case the initial activity is restored after a complete pH shift cycle. In the later case the secondary or tertiary structure of the enzyme is altered irreversibly during the time course of the pH change.

The pH, apart from altering the rate of the enzyme catalyzed reaction, affects as well the stability of the enzyme. The optimum operational pH then must be a compromise of enzyme activity and enzyme stability.

In conclusion, three pH-effects can be distinguished which sometimes

occur simultaneously.

- change in the enzyme / substrate binding affinity.
- reversible change in the conformation of the active site(s) of the enzyme.
- irreversible change in the stability of the enzyme, denaturation, at extreme pH values on both sides of the pH optimum. This is a rate process and therefore time dependent.

Effect of Temperature

Enzyme catalyzed reactions, like any other chemical reactions, generally increase their rate with temperature. However, the highly ordered tertiary structure of an enzyme molecule is maintained primarily only by weak non-covalent bonds. These can be easily disrupted when the enzyme molecule absorbs too much energy, resulting in its denaturation.

Denaturation, or a loss of catalytic activity is a chemical reaction which competes with the enzyme catalyzed reaction. As the temperature increases the thermal denaturation rate is accelerated dominating eventually its catalytic activity.

As mentioned earlier, enzyme catalyzed reactions increase in rate with rises in temperature, within a moderate range of temperatures which occur in biological systems.

This effect is described by an Arrhenius relationship:

$K = Ae^{-E/RT}$, where K is the reaction rate constant, A the Arrhenius constant, E the energy of activation, R the gas law constant and T the absolute temperature.

In enzyme catalyzed reactions, the activation energy is usually in the range of 4-20 Kcal/mole while enzyme denaturation has activation energies in the range of 40-130 Kcal/mole. Consequently the optimum temperature for the enzyme catalyzed reaction process must be a compromise between these dual effects of temperature, i.e increase in the rate of the reaction and enzyme denaturation.

A plot of catalytic activity A versus temperature exhibits a maximum called misleadingly the "optimum temperature". The position of this maximum often depends on the time of the measurement of the reaction rate. A method used for demonstrating and quantifying the effect of temperature on enzyme activity and stability involves the pre-incubation of the enzyme for a certain period of time in a buffer at various temperatures and determining its activity at 'T_{opt}'. The resulting curve of activity versus temperature is termed 'short time stability' which however is of limited value since it does not give any data about the time dependence of enzyme activity. The best method is to continuously measure the decrease of activity (enzyme activity versus time) under the reaction conditions for various temperatures. This allows the estimation of the parameters of some suitable deactivation models which can be

used in order to predict the enzyme activity as a function of time at various temperatures.

On the contrary, a decrease in temperature may cause an enzyme to become less stable or there are activity losses when cooled, probably because of hydrophobic forces which play an important role in maintaining the active conformation and which decrease in strength with decreases in temperature.

3.7 STABILITY OF ENZYMES AND ITS ENHANCEMENT

(References: (3.1), (3.3), (3.4), (3.10), (3.12), (3.13), (3.14), (3.15))

The stability of biocatalysts is very important for the economy of their industrial applications. Since highly purified enzyme solutions are very expensive to prepare it is important to eliminate any activity losses of an enzyme under storage conditions (storage stability) and under reactor or process conditions (operational stability). Every increase in enzyme stability at both conditions usually results in significant cost savings.

From the engineering standpoint operational stability is more important than storage stability. In most cases it can be said that operational stability is more difficult to achieve mainly because operating or reaction conditions may be quite different from optimum storage conditions.

The various methods available for increasing enzyme stability fall into four categories.

a. Identification of microorganisms which produce enzymes that have better intrinsic stability.
b. Addition of stabilizing agents to the storage or reaction medium.
c. chemical modification of enzymes.
d. The immobilization of enzymes.

Chemical additives used for enzyme stabilization include substrates, salts and organic solvents.

Addition of substrates may stabilize enzymes by holding the enzyme structure in the form of an enzyme-substrate complex. This is especially so when the active site of the protein is the least stable region. This treatment for enzyme stabilization is not applicable to all enzymes. Examples of enzyme substrate addition to enzyme solutions causing destabilization of enzymes are also known.

With respect to salt cations such as Fe^{2+}, Mn^{2+}, Ca^{2+}, Cu^{2+} at low concentrations they interact with a group of enzymes called metalloenzymes. Some of these cations are cofactors and in their presence enzyme stability is increased. Ca^{2+} is important with respect to the stabilization of the tertiary structure of an enzyme. Its action is the formation of ionic bonds with two different amino acid residues.

The success or efficiency of a stabilization treatment of an enzyme by means of the addition of chemical agents depends on a successful combination of enzyme solution composition and its temperature.
For example, with respect to enzyme solution composition, the addition of some salts at low concentrations may stabilize the enzyme while the same salts at high concentrations usually cause denaturation.

Another method used to stabilise enzymes is their chemical

modification. This method has been used with some success and one class of this method involves addition to or modification of the R-groups of certain amino acid residues. In this class of chemical enzyme modification polyamino acid side chains may be added to amino groups of the native protein.

Another class of chemical modification of enzymes, with respect to increasing their stability and minimizing activity losses, involves the use of bifunctional reagents such as glutaraldehyde. The use of this chemical results in cross-linking of amino groups, thus preventing access of proteases to the enzyme molecule. Their effect is to somehow lock the enzyme in its active configuration and protect the enzyme molecule from the negative effects of the action of other co-existing enzymes and microbial activity.

A particular treatment or strategy, which is successful in stabilizing an enzyme, can not be extrapolated to other enzymes, i.e stabilization of an enzyme is designed according to its properties, structure and nature.

As regards the immobilization of enzymes, it is generally believed that in most cases it increases enzyme stability but on the other hand the risk of microbial contamination of the immobilized enzyme preparation is higher.

CHAPTER 4:

CELL RECYCLE FERMENTATION - LITERATURE SURVEY

To some extent the feasibility of economic production of microbial or fermentation products depends on the final concentration of the product obtained and also on the volumetric productivity of the bioreactor. The low concentration of product obtained in the effluent makes the separation process more expensive and low productivity increases the capital cost of the bioreactor for the desired output. Though batch and fed batch processes may give in some cases a fairly high concentration of the product, their productivity is low compared to continuous cultivation. In general, productivity is defined as the product of the dilution rate and the product concentration in the effluent stream. Increased productivity then is achieved by maximizing or establishing the optimum combination of these two parameters.

In the case of continuous fermentation the productivity is also limited due to the low concentration of cells in the reactor but this limitation can be overcome by separating the cells from the product stream and returning them back to the reactor. When the cells are recycled, it is possible to operate at dilution rates greater than μ_{max} (4.1). In conventional continuous fermentation a dilution rate employed must be less than the maximum specific growth rate otherwise washout of the microorganism from the fermentation system will occur. This limitation of the conventional continuous fermentation can be overcome

either by continuous fermentation with cell recycle or continuous fermentation with immobilized cells. These two systems are often termed as cell retention systems. An advantage of the former case over the latter is that the former eliminates diffusion limitation problems that are frequently found in systems of cells immobilized by entrapment in some carriers.

Batch and fed batch fermentations may give high product concentration at the end of the fermentation but the average productivity over the whole fermentation time is in general lower than that of continuous cultivation or cell recycle fermentation. Regarding these bioconversions, in general, the productivity will increase with the amount of viable biocatalyst in the fermentation zone.

An important application of cell recycle fermentation is in product inhibited cultures.

Some microorganisms when cultivated produce growth inhibitory substances which are excreted to the fermentation broth and in such cases it may be difficult to achieve the high cell density required. If however conditions are set to remove inhibitory products and thus increase cell productivity a system will be created with a high

production potential where high cell concentration is achieved and continuous removal of the growth inhibitory product, and other toxins which are produced by the biomass during growth[4.2] is also accomplished thus resulting in a possible decrease in product inhibition effects.

A typical example of the application of cell recycle fermentation in product inhibited cultures is that of ethanol production by fermentation.

M. Mota et al.[4.2] reported that in alcoholic fermentation there were toxins in the fermentation broth which were produced by the cells during their growth and which prevented the attainment of high levels of biomass in batch fermentation. The same authors in the same reference employed cell recycle fermentation and they concluded that the increased fermenter productivity was due to washing out the toxins by increasing the dilution rate in the fermentation system. Therefore, as they stated, optimal fermentation rates were achieved by maximizing the washing rate, i.e. the dilution rate.

It was also shown in their fermentation experiments that as soon as substrate was totally consumed cell viability decreased indicating substrate limitation. Also the performance of their system was limited

by viscosity constraints due to cell mass accumulation.

K.H. Khorakiwala et al.[4.3] worked on ethanol production in a membrane recycle bioreactor and they compared the performance of Saccharomyces cerevisiae and Zymomonas mobilis. These workers studied in particular the effect of cell concentration, dilution rate and inlet glucose concentration on the performance of their fermentation system. Cell concentration was maintained at required levels by bleeding cells intermittently as needed. In their work, the effect of cell concentration on the flux of their membrane system was illustrated. As expected, higher cell concentrations led to lower flux.

In other experiments, at high cell concentrations, it became difficult, as they reported, to control the system and maintain stable long-term operation due to foaming and viscosity of the fermentation broth. With respect to foaming, the addition of large doses of chemical antifoam was efficient but it was undesirable due to membrane fouling.

These workers finally concluded that:

1. The membrane recycle fermentation, MRF, is a continuous process which overcomes the inefficiencies associated with continual start-up and shutdown of batch processes.

2. High cell concentrations could be achieved, thus maximizing the rate of reaction. They succeeded in increasing cell concentrations 5 - 30 times over that normally achieved in batch fermentations, with correspondingly higher productivities.

3. High dilution rates, as much as 3-10 times higher than the maximum specific growth rates, could be used with complete substrate utilization, under the operating parameters employed, resulting in correspondingly higher productivities.

4. Inhibitory end-products were continuously removed, which is essential to long-term operation.

W.M Reed and M.E Bogdan [4.4] worked on continuous acetic acid production by cell recycle continuous fermentation.

These workers investigated the effects on volumetric productivity of varying the recycle ratio at constant dilution rate. They showed that increasing the recycle ratio resulted in increased fermenter volumetric productivity. There was a dramatic improvement over the volumetric productivities of a fermenter without recycle under similar conditions. However, the volumetric productivity increase, at high recycle ratios, was not proportional to the increase in cell concentration. They obtained a relative cell concentration increase of 500 % while the relative volumetric productivity only improved 76% as the recycle ratio was increased from 0.45 to 0.90. They stated that this disproportionate increase in volumetric productivity was due to a decline in specific productivity.

In general, a decline in specific productivity is an indication[4.5] of either medium limitations (i.e. exhaustion of one or more essential growth factors or mass transfer limitations caused by a physical change in the fermentation broth such as a dramatic increase in viscosity) or product inhibition. They showed that the former was not the case for the microorganism they cultivated and for the fermentation conditions employed and finally concluded that the decline in specific productivity

was due solely to free acetic acid inhibition of the cells as also demonstrated by pH decline as recycle ratio was increased.

The same authors investigated the effect on the volumetric productivity of increasing the dilution rate while maintaining a certain recycle ratio. As the dilution rate was increased the volumetric productivity increased. A further increase, as they demonstrated, was obtained by operating at a higher recycle ratio (R=1).

Another example of product inhibition fermentation[4.6] is that of lactic acid production from glucose and lactose. In this work it was demonstrated that a lactic acid concentration above 60 g/l resulted in complete inhibition of lactic acid production. The microorganism employed in this experimental work was the bacterium Lactobacillus delbreuckii ATCC 9649.

In many microbial processes the desired product is the cell mass itself. The cell mass often contains valuable compounds such as enzymes, lipids or cellbound membranes. One example is the intracellular enzyme superoxide dismutase[4.7], (SOD), which is of interest to medicine and food technology. SOD enzymes are known to scavenge the superoxide radical and are of vital importance for the oxygen tolerance of

organisms.

Since the desirable product is the cell mass itself, continuous culture under total cell recycle conditions is the most efficient fermentation system.

The authors of this publication, (4.7), investigated the effect of continuous culture with complete cell recycle at a constant dilution rate and they reported an increased cell concentration in the fermentation system. In their experiment, under the above mentioned conditions the substrate inflow to the fermenter was constant while the cell mass increased. The amount of substrate (galactose) available per cell decreased rapidly during cultivation. They also stated that the cell yield (g cell mass produced/g carbon source consumed) decreased with time since the substrate, (galactose), was also required for maintenance.

The same workers employed increasing dilution rates during cultivation under total cell recycle conditions and they stated that this resulted in even higher cell concentrations.

A comparison of the data of the former experiment with that of the latter one showed that about twice the cell mass was achieved at one third of the cultivation time when the cultivation was run at increasing

dilution rates.

Since this system was closed to solids (i.e. cells) and open to liquids, the continuous removal of the permeate from the fermentation system enables removal of the inhibitory lactic acid which was produced during the fermentation.

After the end of the fermentation the cellmass was washed, resuspended and its disintegration was performed in an ultrasonic disintegrator and/or in a bead-mill. From the cell-free extracts SOD was harvested.

Continuous culture, with complete cell recycle and constant feed rate was suggested as a method for determining the maintenance coefficient[4.8] and this will be discussed later in this chapter.

Melzoch et al.[4.9] studied the production of ethanol under total cell recycle conditions using a production strain of Saccharomyces cerevisiae. The cell suspension was retained in the fermentation zone while the cell-free permeate containing reaction products was continuously removed from the closed system.

These workers reported that in a system with total cell recycle it was

possible to reach , in a continuous process, a steady state characterized by the fact that the biomass concentration does not change and the fermentation activity of the cells also remained unchanged. Their experimental data showed that an increased cell concentration was reached and remained constant (steady-state) even after a comparatively long period. Responses of the whole system to changes in dilution rate (D) and to substrate feed concentration (S_o) were very sensitive. Increase in both parameters always resulted in a rise in biomass concentration until a new steady-state was achieved.

Even under these non-growth conditions S.cerevisiae cells retained their viability as data of the methylene-blue staining technique, used for yeast cell viability determination, demonstrated.

Melzoch et al. also demonstrated that increase in the feed substrate concentration at constant dilution rate resulted in higher biomass and ethanol concentrations.

Continuous culture, with complete cell recycle and constant feed rate, was suggested (4.8), (4.10) as a method for determining the maintenance coefficient of the microorganism under cultivation.
Traditionally the value of the maintenance coefficient has been

determined from chemostat data by extrapolation of the specific nutrient uptake rate (q_S) to zero dilution rate (4.11). As a result of the need for extrapolation this is an indirect method and may involve inaccuracies when the relationship between q_S and D is non-linear. Such behaviour has been reported for a number of fermentations.

Under total cell recycle conditions with constant dilution rate the biomass will accumulate in the bioreactor at a decreasing growth rate until finally no further growth occurs and a steady state biomass concentration is reached. Once this occurs the maintenance energy requirements of the total concentration of cells will just be met by the continuous rate of limiting nutrient addition. As mentioned earlier in this chapter higher dilution rates will result in higher cell concentrations until some other nutrient becomes limiting.

For the experimental determination of the maintenance coefficient, m, only direct measurements of dilution rate, D, growth limiting substrate concentration in the feed medium, S_O, residual growth limiting substrate concentration, S, and biomass concentration, X, are needed. The whole procedure is straight forward and by setting a number of values of D the value of m can be verified for different steady-state biomass concentrations

Questions that arise with respect to the validity and reliability of this technique are concerned with whether or not the cells suffer any viability loss or change in metabolic activity due to cell recycling and declining growth rate. A further reason why the result could be biased is the formation and enrichment of macromolecular inhibitors in the fermentation broth. If cell damage occurs underestimated values of m, the maintenance coefficient, would be obtained. Another possibility which needs to be considered when using this technique is that some nutrient other than the carbon source could become limiting at the higher steady state cell concentrations. However, once this occurs it would become evident as significant concentrations of the carbon source would be detected in the effluent permeate stream.

Apart from presenting briefly the main features and applications of cell recycle fermentation, the impact of the growth environment of such a system on cell physiology must also be discussed.

The growth environment within a cell recycle fermenter is very different(4.12) to that within a conventional batch or chemostat culture. This quite different growth environment leads to/or causes changes in the physiology(4.13) of the cultivated microorganism . Such phenotypic

changes in physiology are recognised as widespread phenomena within microbial cultures. Virtually every aspect of microbial structure and function has been shown to be variable in response to changes in such environmental parameters as nutrient status, temperature, pH and redox potential. Such variability may become a source of difficulty, resulting in the depletion or total loss of the desired activity due to the alteration of regulatory or secretory mechanisms.

N.Major and A.T Bull[4.13] characterized the physiology of lactate production in conventional chemostat cultures and they also investigated the physiological effect of defined degrees of cell recycle applied to chemostat cultures. The findings of these two authors are an example of physiological changes in a microorganism caused by its cultivation in such a different growth environment. When the cell recycle fermentation, CRF, was at steady state they observed, among others, a lower value of the growth yield, $Y_{X/S}$, than that of the steady state chemostat culture. This reduction in growth yield was described in terms of a specific rate of consumption of glucose, which was the carbon source of the nutrient medium in their fermentation, for non anabolic purposes.

Melzoch et al. [4.9] cultivated S.cerevisiae for ethanol production and

also reported on physiological state of cells cultivated under cell recycle conditions and in particular in a total cell recycle fermentation system. The behaviour of cells in their bioreactor arrangement showed some differences from cells cultivated in the ordinary way. The yeast cells retained their ability to bud (cell division) but the physical separation of cells was depressed. This phenomenon caused an increase in cell aggregates of different sizes and properties. These workers also stated that an appearance of diversity in cell size and a great number of budding cells under stationary conditions are characteristic of this type of fermentation. Since the biomass concentration virtually did not change they assumed that cell growth had stopped.

The technique of recycling cells by various means is often discussed in connection with cultivation of product inhibited microorganisms such as lactic acid bacteria, yeasts for production of ethanol and Clostridia for solvent production. A.C Berg et al. reported(4.14) on the use of membrane recycling for cultivation of microorganisms that are negatively affected by high concentration of substrates. When substrate inhibited microorganisms are to be cultivated the concentration of the deleterious substance in the reactor must be kept low. This is normally done in fed-batch fermentations where the feed pump is controlled by some measured signal. If no such signal is available or there is a risk of

high local concentrations of the toxic substrate at the point of addition (so called hot-spots), another approach can be used. The microorganisms can be cultivated in a system which is fed with a dilute substrate solution and the cells recycled to the fermenter e.g. by means of a membrane unit.

Several reasons for cultivating microorganisms on toxic substrates can be envisaged. Microorganisms can be used for detoxification of hazardous wastes. Furthermore, intracellular enzymes can be of interest in analytical applications and for bioorganic synthesis. Organisms of the genus Pseudomonas are capable of growing on a wide range of more or less toxic substances, such as aromatic compounds, as carbon and energy sources. A.C Berg[4.14] cultivated Pseudomonas cepacia ATCC 29351 on salicylate, which is a toxic substance, as sole carbon source in a system with complete cell recycling by means of a hollow fiber microfilter. The product of interest in their fermentation was the intracellular enzyme salicylate hydroxylase. These workers made comparisons between cell densities, yield coefficients, specific enzyme activities and volumetric productivities in batch, continuous and continuous with total cell recycle cultures. They found that in batch cultures the toxicity of the salicylate limited cell densities to below 1g/l. In a recycling cultivation a cell concentration of 15.1 g/l was achieved

and the volumetric productivity of the cell mass was increased by a factor of 3. With their work the advantageous features of this type of fermentation over conventional culturing techniques, regarding substrate inhibited microorganisms, were demonstrated.

Applications of cell recycle fermentation for the production of extracellular enzymes have also been reported.

A. Nipkow et al.(4.15) carried out research on the production of the extracellular enzyme exo-ß-amylase by culturing Clostridium thermosulfurogenes under cell recycle conditions in a pilot scale system. In their work they also studied, apart from fermentation kinetics, the effect of various parameters on filter performance.

These workers developed a sophisticated microfiltration cell-recycle fermentation system which comprised a conventional continuous-flow fermenter connected to an 'in situ' steam sterilizable cross-flow ceramic 0.2 µm pore size microfilter with a backflushing device. A microcomputer was used to control filtration pressure, tangential flow velocity and backflushing.

With respect to filter performance it was reported that when

maltodextrin, which is a polysaccharide, was used as the carbon source in their fermentation medium, filtration rates were impaired and filter fouling was more intense than when a simple saccharide, such as maltose, was used as the carbon source. To prove that dextrins of high molecular weight caused membrane fouling a small amount of a thermostable dextrin-degrading pullulanase was added to the system after some time from start up. This addition resulted in an increase in permeate flux demonstrating that this polysaccharide (maltodextrin) which had been degraded had clogged the filter pores.

The same workers also investigated the effect of filtration pressure and backflushing on filter performance. With respect to filtration pressure they noted that an increase in its value restored the permeate flux of the filter for a short time but it induced rapid fouling that was not reversible when the pressure was reduced again to its initial value. In addition, cell destruction was also noticed after the pressure increase.

With respect to backflushing, its effect on permeate flux of fouled filters was investigated at various backflushing pressures, blackflushing durations, and time intervals. The results of these experiments showed an initial increase in permeate flux but it was never possible to restore the permeate flux of a fouled filter, even at high backflushing pressures and durations. None of these treatments, such as an increase in

filtration pressure and backflushing, prevented long term fouling of the membrane.

Filter selectivity with respect to ß-amylase transmission showed it to be affected by the presence of a polysaccharide in the fermentation system. When maltodexrin was used as the carbon source in the fermentation, ß- amylase transmission was lower than the enzyme transmission when a simple saccharide was used as the carbon source. In both cases enzyme transmission through the membrane was low.

The same workers also reported that cell disruption in the recycle loop, due to operating conditions, was significant. The susceptibility of cells to disruption seemed to be a function of the culture age and the formation of cell debris accelerated filter clogging.

Studies on fermentation kinetics for exo-ß-amylase production under cell recycle conditions showed that productivity of ß-amylase and concentration of biomass were increased 11- and 12- fold respectively, if compared to values obtained in a chemostat. The concentration of the enzyme accumulated in the reactor to a value which was 5.5- fold more than under comparable conditions in a chemostat. The amount of enzyme that could be produced from a continuously operating cell recycle reactor within a month would be 25- fold higher than from

batch production (1 per 24h) using the same medium and reactor size.

As mentioned earlier an 11-fold increase of the biomass concentration was obtained under CRF conditions compared to chemostat conditions. This value was lower than the theoretical value, if Monod kinetics are assumed. They should have observed a 20- fold increase. The reasons for this discrepancy were not evident.

A common characteristic in all literature concerning cell recycle fermentation, both total and partial, is that relatively low values of dilution rate, D, have been employed in comparison with values of D in corresponding chemostat cultures. The selection and application of a particular value of dilution rate is limited by the permeate flow rates the membrane module can provide.

CHAPTER 5:

DEXTRANSUCRASE PRODUCTION LITERATURE SURVEY

5.1 INTRODUCTION

Dextransucrase (sucrose: 1,6-a-D glucan 6-a-glucosyltransferase, EC 2.4.1.5) is the name of the extracellular enzyme responsible for the conversion of sucrose to fructose and dextran according to the following equation:

$$\underset{\text{sucrose}}{n\ C_{12}H_{22}O_{11}} \xrightarrow{\text{dextransucrase}} \underset{\text{dextran}}{(C_6H_{10}O_5)n} + \underset{\text{fructose}}{n\ C_6H_{12}O_6} \qquad \text{Eqn 5.1}$$

Bacteria of the family Streptococceae produce dextransucrase. The genera belonging to this family that have generally been used for dextransucrase production are Streptococcus and Leuconostoc. Different species of these bacteria have been used. However, the enzyme and hence the dextran produced has been found to be species dependent. Dextransucrase is an inducible enzyme that requires only sucrose for its induction.

Numerous studies have been undertaken of the production and purification of dextransucrase, its reaction mechanisms, its immobilization and use, i.e. for dextran production. Differences between the enzyme produced by different strains of bacteria have also been explored and specific strains selected for different production purposes. An example is Leuconostoc mesenteroides (L.m) NRRL strain B512(F). The enzyme produced by these organisms synthesizes

dextran which is highly soluble in water. This dextran is used in the pharmaceutical industry as clinical dextran.

Dextran is the name given to a class of extracellular polysaccharides synthesized from sucrose by bacterial enzymes. The polymer chain consists of D-glucose units which are predominantly linear α - 1,6 glucosidic linkages in the main chain, and varying amounts of α-1,2, α-1,3 and α-1,5 branch linkages. These molecules have molecular weights up to several million. The degree and type of branching differs with the species of bacteria used, and affects along with the molecular weight the solubility and other rheological properties of the dextran. The main properties of dextrans are their viscous behaviour and the fact that they do not contain any charged groups.

The main uses of dextran have already been given in section 1.1.

5.2 DEXTRANSUCRASE PRODUCTION.

Many workers have reported on the fermentation medium components and their effects for maximal dextransucrase yields.

Tsuchiya et al.[5.1] reported on the carbon source which must be used for a high yield dextransucrase fermentation. In their work they employed various concentrations of sucrose in the fermentation medium and studied their effects on the results of the fermentation. They also considered physical properties of the fermentation broth after the fermentation was complete. These workers illustrated that a sucrose concentration of 10% w/v, used conventionally in the medium in whole-cell production of dextran, resulted in a very viscous fermentation broth from which cell removal was difficult. They also stated that the amount of dextransucrase produced increased with increasing initial sucrose concentration in the nutrient medium but the problem with such high sucrose concentration fermentations was, as in the previous case, that the cultures contained so much dextran as to render difficult the removal of cells. Even at levels of 3, 4 and 5 per cent the same problem was still pronounced. They finally concluded that 2 per cent sucrose was the optimum level for production of dextransucrase with Leuconostoc mesenteroides NRRL B-512.

The same workers cultivated also the same strain of Leuconostoc mesenteroides in a fermentation medium containing one of the following

carbohydrates: fructose, glucose and maltose, but no dextransucrase was produced. When in the fermentation medium a mixture of sucrose with one of the saccharides mentioned earlier was used, the dextransucrase yield obtained was proportional to the sucrose concentration in the fermentation medium.

Whiteside-Carlson and C.L.Rosano (5.2) employed a variety of media, containing glucose or fructose or sucrose for cultivating Leuconostoc cells. They showed that Leuconostoc grows preferably on sucrose containing media. Moreover they stated, that for dextran synthesis it was essential to cultivate these bacterial cells in sucrose containing media. In the fermentation media they employed in some cases they incorporated some amino acids and in some other cases they varied the concentration of these nutrients. They finally concluded that in no case was a nutrient found to be more essential for dextran synthesis than for growth in sucrose media.

Neely and Nott (5.3) cultivated Leuconostoc mesenteroides in a variety of media with respect to the carbon source.

Each of the media employed contained only one sugar. They reported that cell growth was more pronounced in the sucrose containing medium although some substantial cell growth was observed in some other non-sucrose containing media. Moreover, they reported that dextransucrase was only detected in the fermentation broth where sucrose had been

incorporated in the nutrient medium of the fermentation.

In an economic cell free process it could be postulated that cell growth could take place on a cheap carbohydrate source and after sufficient growth had been obtained sucrose could be added as the enzyme inducer. This concept was experimentally tested [5.4] using glucose / sucrose, fructose / sucrose and glycerol / sucrose mixtures, but it was unsuccessful.

It is known, in the case of other inducible enzymes that substrate analogs can be better enzyme inducers than the natural substrate. This idea was implemented employing sucrose palmitate as a substrate but very little growth and enzyme formation were detected. Alsop [5.4] finally concluded that no better enzyme inducer than sucrose, for dextransucrase production, has been found.

Nitrogen and other essential growth factors for growth of Leuconostoc mesenteroides are supplied by adding in the fermentation medium certain amounts of "corn steep liquor" or "yeast extract". These nitrogen sources supply the micro organism with the amino acids, valine and glutamic acid, as well as the vitamins, pantothenic acid, nicotinic acid, thiamine and biotin for which Leuconostoc mesenteroides has strict requirements.

Nitrogen supply in the form of inorganic salts must not be carried out

since in the case of nitrates, the bacteria are non proteolytic and consequently do not reduce them [5.5]. In addition nitrogen in the form on ammonium ions appears to have a negative effect on the production of the enzyme [5.1].

Ajongwen-Numfor [5.6] undertook research work in order to investigate the effects of yeast extract types on the outcome of the fermentation. He found that the type of yeast extract used in the fermentation medium had significant effects on enzyme yield. Of the different types studied, the Gistex Standard was found to be the type that favoured the highest enzyme production.

Phosphate salts (KH_2PO_4 or K_2HPO_4, depending on the fermentation technique employed) are used as an ingredient of the medium for inoculum preparation and enzyme and dextran production. Apart from their providing buffering capacity and thus enabling pH control of the culture, they are associated with Leuconostoc mesenteroides metabolism. For example, as Tsuchiya et al.[5.1] reported, increasing the initial concentration of organic nitrogen had little effect on the enzyme production, until the initial level of the phosphate buffer salt was simultaneously increased, thus demonstrating that phosphate plays a role in the microorganism metabolism. Furthermore, Rorem [5.7] showed that active cells associated with polysaccharide take up phosphate at a much higher rate than inactive cells although phosphate uptake in

fermentations for the production of dextransucrase needs to be calculated.

The use of the corn steep liquor or yeast extract for the preparation of the fermentation medium is expected to supply some necessary trace elements. In most of the fermentation media reported in the literature a supplement of trace elements had been included. The mixture of Magnesium, Sodium, Iron and Manganese known as R* salts was first introduced by Koepsell (5.8).

Tsuchiya et al.(5.1) also studied the effect of pH on dextransucrase production. They carried out a number of fermentation experiments in which the culture pH was controlled at 6.1, 6.3, 6.7, 7.0, ± 0.1 pH units. The maximum yield of the enzyme occurred in the culture in which the pH was controlled at 6.7. Towards the end or at the end of the fermentations they carried out, there was a decrease in enzymatic activity. This phenomenon was attributed to the instability of dextransucrase with time at the process temperature and pH they employed. The instability of dextransucrase at the particular pH value of 6.7 was also illustrated by the fact that the enzymatic activity of culture filtrates was retained at pH values of 5.0 to 5.3 for at least 24 hours at 25°C, while about 35% activity losses occurred at pH 6.7 in 1 hour.

In conclusion, the optimum pH for dextransucrase production is 6.7, but

at this pH value the enzyme is inactivated rapidly. However, peak enzyme potency can be retained for many hours at room temperature by adjusting the culture to pH ~ 5.2 which is the optimum pH for enzyme stability and activity at the time of maximum yield [5.8].

Schneider [5.9] also carried out experimental work to elucidate the pH dependence of enzyme production and he confirmed that pH 6.7 is the optimum for the production of the enzyme. For pH control purposes either NaOH or KOH may be used. Since ammonium ions have a detrimental effect on the microorganism, as discussed earlier, ammonium hydroxide must not be used to control the pH.

Tsuchiya et al.[5.1] also undertook research work to investigate the effect of temperature on enzyme production. Its effect was found to be interdependent with the pH at which the fermentation broth was controlled. These workers pointed out, as previously discussed that the enzyme is rapidly inactivated at pH 6.7 even at the temperature of 25°C. Moreover higher temperatures enhanced markedly the deleterious effect of pH on the enzyme. From a number of fermentation experiments they performed at different temperatures, in the range of 20 - 29°C, in which the pH of the fermentation broth was controlled at 6.7, they found that the optimum temperature for dextransucrase production was 23°C. Schneider [5.9] also investigated the effect of temperature and he

carried out fermentation experiments at 19, 23 and 27°C. He found that higher temperatures resulted in higher growth rates but lower yields of dextransucrase were obtained at a temperature of 27°C. He argued, using also the findings of Tsuchiya et al.[5.1], that this was due to the decomposition of the enzyme with time under these conditions of temperature and pH.

Schneider [5.9] also studied to effect of aeration of the fermentation broth on dextransucrase yields. He found that maximum enzyme activities were obtained when the dissolved oxygen concentration in the fermentation broth was maintained at 40-50% of the saturation level.

Ajongwen-Numfor [5.6] carried out experiments under conditions of no aeration and low agitation of the culture fluid. He found that the dextransucrase yields obtained were much higher than those achieved under the aerated conditions employed by Schneider [5.9].

The research work presented so far in this section has dealt with the optimum composition of the fermentation medium and optimum environmental factors, such as pH, temperature and aeration, for high yield dextransucrase production. The basic batch process remained unchanged until 1980 when Monsan and Lopez [5.10] and Schneider et al.[5.11] made further advances in terms of the maximum dextransucrase activity obtained. The work of both groups relied

heavily on the findings of Tsuchiya et al.(5.1) but higher enzyme activities were achieved by the use of a fed-batch technique to supply sucrose to the cells. The first of these two groups achieved 180 DSU/cm^3 while the second boosted the enzyme activity to 300 DSU/cm^3. Lopez and Monsan (5.10) used 2 percent sucrose and a yeast extract in the fermentation medium and in addition during the logarithmic phase of the fermentation, they fed continuously into the fermenter a sucrose solution of 1g/cm^3. Doubling in biomass production and a high increase in enzyme elaboration was experienced as a response of the fermentation system to this sucrose solution addition which was discontinued when the cells reached the stationary phase.

Schneider et al.(5.11) made a further advancement as regards this fed-batch fermentation technique. In the experimental work they undertook the amount of yeast extract and phosphate in the fermentation medium was taken to much higher levels than those used by previous workers. Their medium contained 4% w/v yeast extract and 2% w/v phosphate. These workers (5.11) stipulated that the background sucrose concentration in the fermentation broth should be maintained between 0.5 - 1.0% w/v. Frequent sampling of the culture fluid and carrying out of HPLC analyses for carbohydrates, and in particular for sucrose enabled the calculation of the required feed rate of the concentrated sucrose solution to be made.

This procedure of feeding the batch reactor with two liquid streams, i.e. the concentrated sucrose solution and sodium hydroxide solution added for pH control, and at the same time performing HPLC analyses for determining the required rate of the sucrose solution addition, would be time consuming and would cause technical difficulties if it had to be implemented at industrial scale.

Since there was a constant ratio between the amount of sucrose consumed and sodium hydroxide added to the fermentation broth to control the pH, these workers considered that it would be advantageous to feed a mixture of sucrose and sodium hydroxide to maintain the controlled pH. The concept was tested at laboratory and pilot plant scale and led, as stated earlier, to maximum dextransucrase yields of 300 DSU/cm^3.

Further research work on the development of the fed-batch fermentation technique for dextransucrase production was undertaken by Brown and McAvoy (5.12). The fermentation medium of Schneider et al.(5.11) was employed in their experiments, and the aeration rate was controlled at 40 - 50% of the saturation level by manual adjustments of impeller speed and/or air flow-rate. The feed mixture used to control the pH and at the same time to supply sucrose to the cells was formulated on the basis of an NaOH - to - sucrose molar ratio of 1.4.

There has been limited published work on the continuous production of

dextransucrase. Paul et al.[5.14] stated that in continuous culture enzyme productivity depends directly on the dilution rate employed. The other factors affecting enzyme productivity are: temperature, pH and sucrose concentration, as they have been illustrated earlier in this section.

This group of workers achieved a maximum enzyme activity of 70 DSU/cm^3 at a 0.4 h^{-1} dilution rate. This value of enzyme activity was the highest so far achieved in the literature. They also reported that the productivity of the continuous culture was 3 times greater than the productivity of the fed batch system. Both fermentation experiments carried out, were run under identical initial culture conditions, i.e: fermentation medium and environmental factors such as temperature and pH.

5.3 DEXTRANSUCRASE PURIFICATION.

In dextransucrase fermentation, the fermenter broths contain apart from dextransucrase, dead cells and cell debris, proteins, inorganic salts either from pH buffers or salts incorporated in the nutrient medium as well as other by products such as glucose, fructose, mannitol, dextran, leucrose, oligo-saccharides and other products of cell metabolism.

The first step in the purification of dextransucrase is the cell removal from the culture supernatant. This is accomplished by centrifugation or cross-flow filtration. Cell removal by centrifugation is still the most common method employed in industry for cell separation but it has the disadvantage that it is accompanied by enzyme activity losses due to very high shear rates generated during processing. This problem, however, can be reduced by employing cross-flow filtration to permit cell and cell debris removal.

Cell removal from the crude dextransucrase fermentation broth enables further purification steps or procedures to be performed.

In the early studies on Leuconostoc mesenteroides B512F dextransucrase production relatively crude enzymes were produced by precipitation of the enzyme from the culture medium using ethanol. As the pH of the culture media was usually allowed to fall below 6.7 large quantities of dextran accompanied the enzyme precipitation.

Robyt and Walseth [5.15] employed various techniques for dextransucrase purification. Among them are ammonium sulphate precipitation, and hydroxylapatite and DEAE-cellulose chromatography. They found that ammonium sulphate was completely ineffective in precipitation B-512(F) dextransucrase from a culture supernatant concentrate; 90% of the dextransucrase activity remained in solution when the concentration of ammonium sulphate was 80% w/v.

When culture supernatant concentrate was applied to Bio-Gel hydroxylapatite and eluted with a stepwise gradient of potassium phosphate, five fractions containing dextransucrase were obtained. All of these fractions were contaminated with glucosidase, sucrose phosphorylase, levansucrase, invertase and dextranase. Furthermore the total yield of dextransucrase was very low (<10%).

When the culture supernatant concentrate was chromatographed on DEAE-cellulose, dextransucrase activity could not be eluted from the column.

Finally they proposed a relatively simple, three-step purification method. According to this method the culture supernatant was dialyzed and concentrated with a Bio-Fiber 80 miniplant. This first purification step gave a 43-fold purification with a yield of nearly 100% dextransucrase. In the second step of this purification method the

dialyzed concentrate was treated with dextranase in a dialysis tube. Finally the dextranase treated enzyme was chromatographed on Bio-Gel A-5m. This last purification step of the dextranase-treated, culture supernatant concentrate gave a 240-fold purification of dextransucrase having a specific activity of 53 U/mg. One unit of enzyme (dextransucrase or levansucrase) was defined, in this work, as the amount of enzyme which will incorporate 1 μmol of D-glucose or D-fructose into polysaccharide in 1 min.

The purpose of the dextranase treatment of the dialyzed concentrate resulting from the first step of this purification method was to completely dissociate the dextransucrase - dextran complex. Consequently the molecular size of the dextransucrase was decreased. This treatment resulted in the separation of dextransucrase from levansucrase. Furthermore the added dextranase was retarded to a greater extent by Bio-Gel A-5m than dextransucrase, thus enabling the separation of dextransucrase from dextranase. Another consequence of removing the dextran, which is bound with the enzyme, with dextranase treatment was an increase in the instability of the purified dextransucrase.

In conclusion, with this purification method, purified dextransucrase was obtained in 33% yield and 99% of the protein and 84% of the carbohydrate had been removed from the starting material.

Paul et al.[5.14] purified dextransucrase using a process that consisted of phase partitions between dextran and polyethylenglycol. These workers showed that repeated phase partitions resulted in obtaining very highly purified dextransucrase preparations which had specific activities above 3,500 DSU/mg protein. These results are much higher than those obtained when using other purification techniques such as ultrafiltration and gel filtration. Moreover, as the same workers stated, the purification conditions inherent in phase partitions between dextran and polyethylenglycol allow dextransucrase concentration and stabilization. Another advantageous feature of this technique is that it can be very easily scaled up and coupled with the continuous culture of L. mesenteroides to achieve the continuous production of large amounts of purified dextransucrase. Finally with this purification method the highly purified dextransucrase preparations obtained were free from any contaminating enzyme activities.

Lopez and Monsan [5.10] also reported on dextransucrase purification. They initially tried to recuperate dextransucrase from the fermentation broth by ethanol precipitation; although a concentrated enzyme solution could be obtained, it formed insoluble active agglomerates that prevented any further purification. The contact of this solution with solid supports accelerated the agglomeration process. They therefore concentrated the enzyme by ultrafiltration followed by purification by GPC on Ultrogel AcA-34. 0.01M citrate buffer (pH 5) was used as elution agent. In the experiments they carried out 0.005% w/v $CaCl_2$

had been added to the fermentation broth as well as 0.05% w/v $CaCl_2$ had been added to the purified enzyme preparation.

The maximum specific activity they obtained was 2,400 DSU/mg and the purification yield was 82%.

Kobayashi and Matsuda [5.16] used a lengthy process to purify dextransucrase. In this process dextransucrase activity from cell-free culture supernatant of L.M. NRRL B1299 was purified by ammonium sulphate fractionation, adsorption on hydroxyapatite, chromatography on DEAE-cellulose and gel filtration on Sephadex G-75. The extracellular enzyme was separated into two principal forms, enzyme I and N, and the latter was shown to be an aggregated form of the protomer, enzyme I. The relative activities of enzymes I and N reached 820 and 647 times that of the culture supernatant, respectively. The overall recovery of purified enzyme activity was 0.9% for enzyme I and 2.0% for enzyme N.

M. McCabe and E. Smith [5.17] reported a specific method for the purification of Streptococcus mutans dextransucrase. The purification procedure they suggested involved employment of affinity as well as hydrophobic chromatography.

Dextransucrase activity was obtained from the cell-free culture liquor by $(NH_4)_2\ SO_4$ fractionation. Subsequently the crude enzyme was

pumped onto a Bio-Gel P2-insoluble dextran affinity chromatography column. The eluent was a solution of clinical dextran 0.5 mg/cm^3 containing 0.02% w/v sodium azide. Subsequently the dextransucrase pool obtained by affinity chromatography was applied to the butyl-agarose, hydrophobic, chromatography column, the eluent being NaCl (0 to 0.3M in 0.02% sodium azide).

The first purification step, i.e. that of affinity chromatography gave 300-fold purification, with 76% recovery of enzyme. Subsequently hydrophobic chromatography on butyl-agarose increased the overall enzyme purification to more than 1,000 fold with a 65% recovery of activity. In addition to removing inert protein and, hence, increasing the purity of the enzyme, the hydrophobic chromatography step served to separate soluble dextran (which passed through the butyl-agarose column) from the enzyme (which was retained by the column).

Dextransucrase purified by this method had no detectable invertase, levansucrase or dextranase activity, and the recovered activity presented two-thirds of the initial (crude) enzyme activity.

Fukui et al.(5.18) used a combination of ammonium sulphate fractionation, Sepharose 6B gel filtration, DEAE-cellulose, and hydroxyapatite column chromatography of culture fluids of streptococcus mutans HS-6. This purification process yielded three enzymes, invertase and water-soluble and water-insoluble glucan-

synthesizing glucosyltransferases. Chemical analysis of water-soluble glucan synthesized by the enzyme indicated the glucan consisted of over 94% a-(1 → 6) linkage. It was therefore concluded that the enzyme was the dextransucrase. This was the first case of dextransucrase preparation detected in cariogenic streptococci.

These workers also stated that the purified dextransucrase they obtained possessed invertase-like activity.

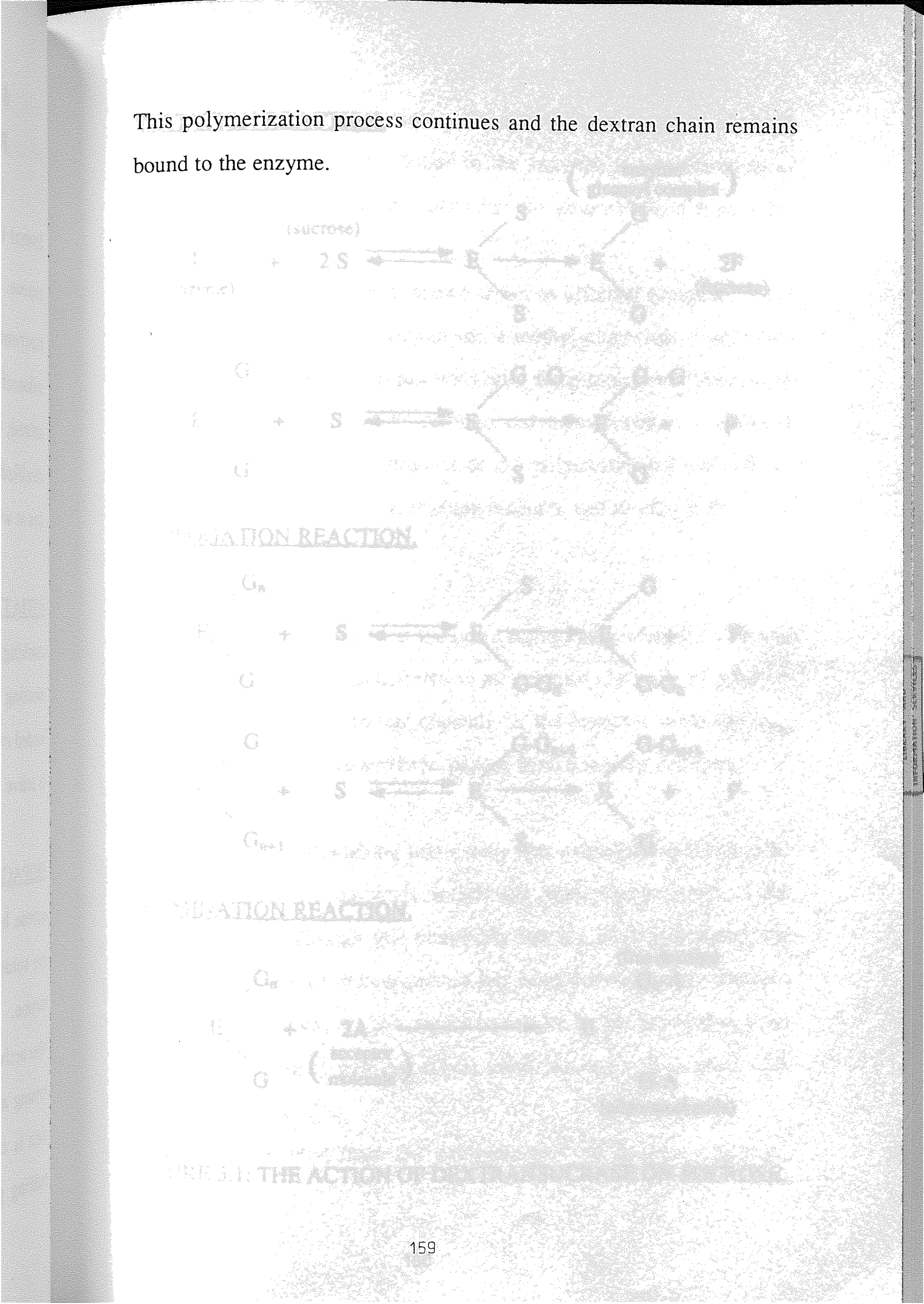
5.4 ACTION OF DEXTRANSUCRASE ON SUCROSE - REACTION MECHANISM OF DEXTRAN SYNTHESIS

Several theories have been proposed for the action of dextransucrase on sucrose. That proposed by Robyt and Ekland [5.19] has the most experimental support. By immobilizing the enzyme and using radioactively labelled substrates, intermediate reaction products are isolated, enabling their source, and hence individual reaction steps, to be identified. It has been shown that the enzyme has two identical reaction sites at which dextran synthesis occurs.

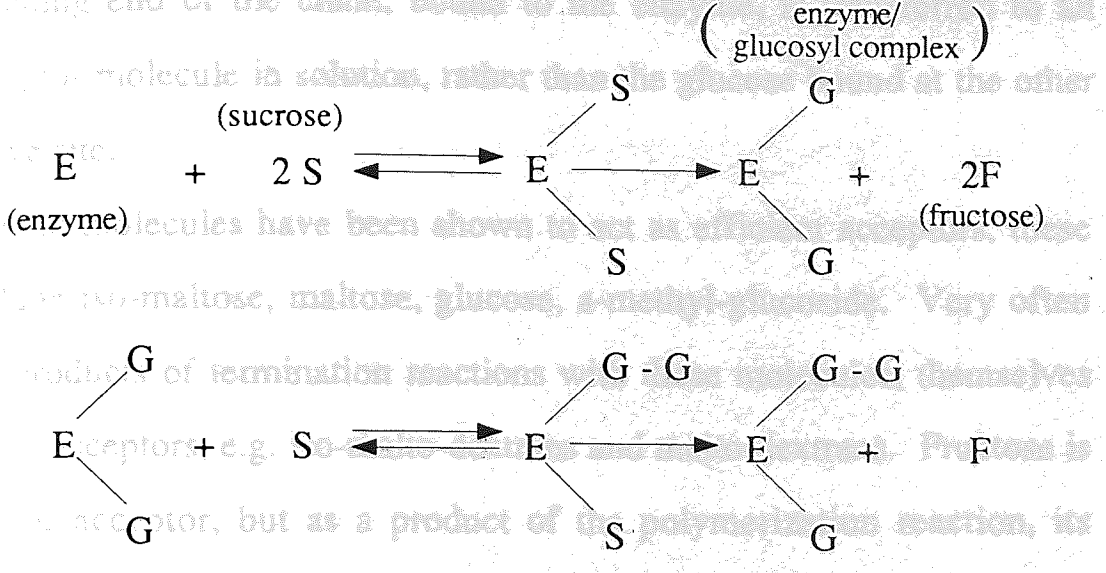
INITIATION: Polymerization is initiated by sucrose molecules attaching to each active site on the dextransucrase. Both are cleaved releasing fructose into solution. The glucose residues remain covalently bonded to the active sites. One of these glucose residues is drawn, from its active site, on to the other, forming an a(1-6) bond between them.

PROPAGATION: Further additions are made in a similar manner, i.e. sucrose binds at the free active site and is cleaved, releasing fructose into solution. The glucose residue remains covalently bound to the enzyme. The reducing end of the dextran chain (already bound at the other active site) is drawn on to the glucose at the "opposite" active site, forming an a(1-6) bond between them. In effect the growing dextran chain is extended by transferring it between the enzyme active sites, enabling glucose residues to be inserted between it and the enzyme.

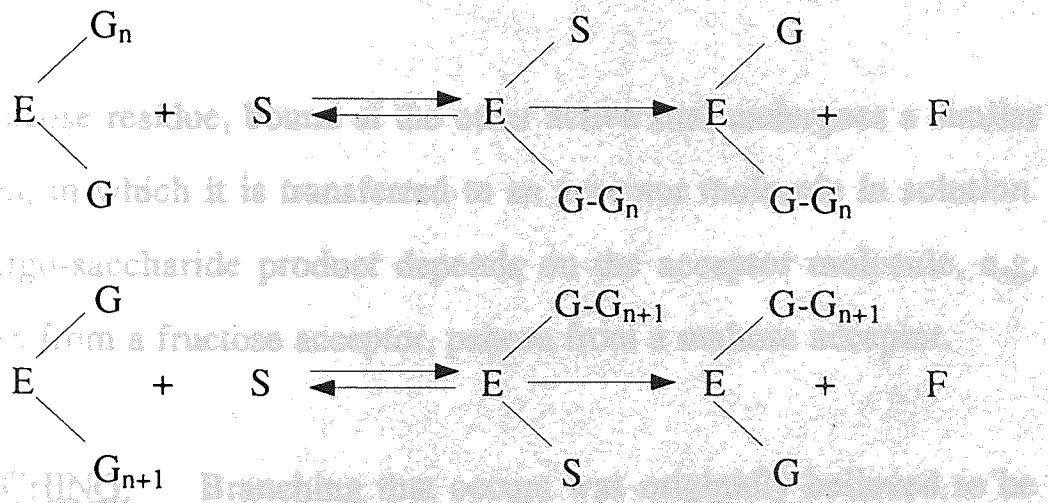
This polymerization process continues and the dextran chain remains bound to the enzyme.



INITIATION REACTION.



PROPOGATION REACTION.



TERMINATION REACTION.

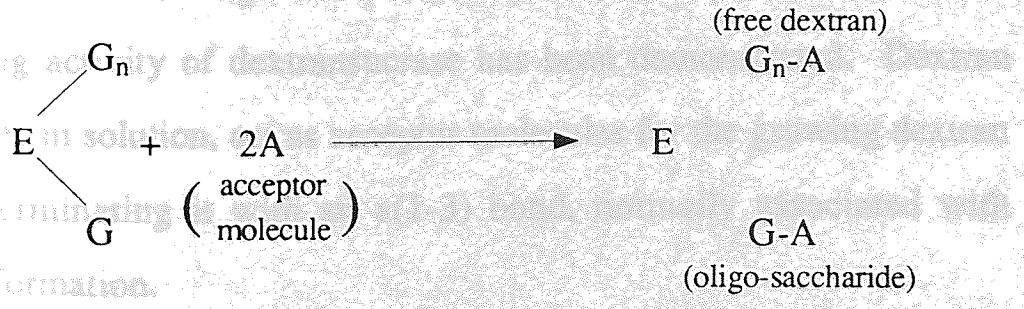


FIGURE 5.1: THE ACTION OF DEXTRANSUCRASE ON SUCROSE.

TERMINATION: Polymerization of a dextran chain is halted when the reducing end of the chain, bound to the enzyme, is transferred to an acceptor molecule in solution, rather than the glucose bound at the other active site.

Several molecules have been shown to act as efficient acceptors, these include iso-maltose, maltose, glucose, a-methyl-glucoside. Very often the products of termination reactions with these molecules, themselves act as acceptors, e.g. iso-malto-dextrans and malto-dextrans. Fructose is a poor acceptor, but as a product of the polymerization reaction, its concentration builds up in the reaction medium, and its effects cannot be ignored.

The glucose residue, bound at the other active site, undergoes a similar reaction, in which it is transferred to an acceptor molecule in solution. The oligo-saccharide product depends on the acceptor molecule, e.g. leucrose from a fructose acceptor, panose from a maltose acceptor.

BRANCHING: Branching that occurs was originally believed to be the result of other enzymes interfering with the activity of the dextransucrase. Though this possibility has not been eliminated, the branching activity of dextransucrase has been demonstrated. Dextran molecules in solution, act as acceptor molecules for the growing dextran chain, terminating it with an a(1-3) bond, normally associated with branch formation.

In dextran produced from the dextransucrase of L. mesenteroides

B512(F) only 5% of the links between glucose residues are formed by a(1-3) bonds.

5.5 DEXTRAN PRODUCTION

Dextran can be produced in two ways. The first method is known as the whole cell fermentation method and is the one which is used currently in industry. The second technique involves the separate production of the enzyme by fermentation which subsequently is transferred to another vessel, the dextran synthesis fermenter, to react with a sterile sucrose solution to produce dextran and fructose.

5.5.1 CONVENTIONAL FERMENTATION OF DEXTRAN.

Conventional production of dextran is carried out by fermenting dextransucrase-producing microorganisms under non-aerated conditions in sucrose rich media containing salts, vitamins and minerals in limited quantities. This medium is inoculated with cells of Leuconostoc mesenteroides NRRL B-512(F) at a neutral pH and the fermentation takes place without any subsequent control, usually with the exception of temperature, until its termination.

The bacterial strain, Leuconostoc mesenteroides NRRL B-512(F), is preferred for dextran production because it produces large amounts of dextransucrase with few contaminating enzymes.

In such a fermentation three stages take place: the growth phase of the

microorganism, the enzyme producing phase and the enzyme/sucrose reaction phase. The bacteria elaborate the dextransucrase into the fermentation medium during the early stages of the process when conditions (e.g. pH of 6.0 - 7.0) favour the enzyme production. As the fermentation proceeds, the pH of the medium slowly decreases to values below pH 6.0 at which point the enzyme converts the excess sucrose to dextran and fructose. Dextransucrase is inactivated at pH values less than 4.5 and when the fermentation reaches this point, the dextran produced is then isolated, purified and characterized to the final desired product through a series of precipitation, acid hydrolysis and spray drying processes. The enzyme activities in the fermenters during the whole cell fermentation processes are usually of the order of 0.10 to 0.24 U cm^{-3} and total fermentation times can be up to 72 h. One U (unit of activity) is the amount of enzyme that will produce one micromole of reducing sugar per minute at 25°C and pH 5.2.

5.5.2 ENZYMATIC SYNTHESIS OF DEXTRAN

An alternative process of producing the dextran involves the separate production of the enzyme followed by the dextran biosynthesis stage.

In the first stage of this process the enzyme is produced under conditions optimum for its elaboration. In the second stage, the cell-free enzyme is transferred to the dextran synthesis vessel to react with a

sterile sucrose solution to produce dextran under conditions optimum for its biosynthesis, or can undergo treatment with ethanol to precipitate a dextransucrase-dextran complex which can be removed and stored for subsequent use.

The intention of splitting the conventional whole dextran fermentation process into these two stages has been to optimize each one separately and consequently maximize dextran yields.

Schneider (5.9) produced dextran via the enzymatic route using cell-free enzyme. He added 15 cm^3 of purified enzyme with an activity of 554 DSU/cm^3, i.e. a total of 8,310 DSU of enzyme, to 2,100 cm^3 of a 10% sucrose solution in 0.05M acetate buffer, at a pH of 5.2 and temperature of 25°C. The yield of this reaction in terms of dextran produced was 88%. The same worker after comparing this result with the dextran yield obtained from a conventional process (whole cell fermentation process) concluded that the net increase in dextran production by the enzymatic route was 28%.

Schneider also found (5.20) that the dextran produced via the enzymatic route was identical in terms of structure to the dextran produced using the conventional whole cell fermentation process. He also demonstrated that the enzymatic route is a much more efficient means of obtaining dextran in terms of sucrose conversion.

CHAPTER 6:

EXPERIMENTAL EQUIPMENT AND TECHNIQUES

6.1 BACTERIAL STRAINS USED

The microorganism used throughout this study was the bacterium Leuconostoc mesenteroides NRRL B512(F). This was supplied in the form of freeze-dried vials by Fisons Pharmaceuticals plc., (C31 cultures, Fisons), Holmes Chapel, Cheshire, England. The vials were stored in a freezer at - 10°C until required.

These freeze-dried vials were reconstituted using 5 cm^3 of sterile MRS broth and the resulting suspension was used to inoculate sterile MRS agar slopes. The whole procedure was carried out using sterile sets of hypodermic needles and syringes and performed in the rear part of a laminar flow cabinet which had been previously cleaned several times with ethanol.

The inoculated MRS agar slants were subsequently incubated at 25°C for at least 48 hrs until sufficient growth on the agar slants was observed.

The inoculated and incubated agar slants were then stored in a refrigerator at 4°C until required for inoculum preparation for a fermentation run.

6.2 FED-BATCH FERMENTATIONS

6.2.1 DESCRIPTION OF THE BATCH FERMENTER

The fermenter used for fed batch culture was a 16-litre stainless steel SF116 "Microgen" fermenter, manufactured by the New Brunswick Scientific (NBS) Co. Inc., New Jersey, U.S.A., and supplied by N.B.S (UK) Ltd., Watford. 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.

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 dm^3, with a working volume of approximately 3 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 100 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.

The pH of the fermentation broth is controlled and monitored by a pH 19 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 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.

6.2.2 INOCULUM PREPARATION

The inoculum was prepared by aseptically transferring L.M. cells from one agar slope into 10-15 cm^3 of sterile MRS broth. This was incubated at 23-25°C for 10-15 hrs. At the end of this period, or when the medium became turbid, i.e. OD_{590} value of about 0.1, the contents of the broth bottle were transferred into a bottle containing 300-350 cm^3 sterile MRS broth. The latter was also incubated for the same period of time at the same temperature. At the end of this second stage, the inoculum was ready to be used in a 6 litre scale fermentation. This inoculum preparation procedure provided an inoculum size of about 5% v/v which was quite satisfactory.

6.2.3 FERMENTATION AND ADDITIONAL MEDIA

The composition of the medium used was identical to that of Schneider et al. [6.2] and is given below.

TABLE 6.1

	Sucrose	10.0 g/l
	Yeast extract	40.0 g/l
	K_2HPO_4	20.0 g/l
	R*salts	0.5% v/v
the R* salts contained:	$MgSO_4 . 7H_2O$	4.0 g
	NaCl	0.2 g
	$FeSO_4 . 7H_2O$	0.2 g
	$MnSO_4 . H_2O$	0.2 g
	Water	to 100 cm^3

The medium components were dissolved in tap water, made up to the required volume, pH adjusted to 7.0-7.1, sterilized at 116°C for 20 minutes and cooled to 23°C.

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 and alkali mixture used is shown below.

TABLE 6.2

Sucrose	900 g
NaOH	200 g
water	to 1,500 cm^3

The preparation of this solution was 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 cm^3, 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 mixing is highly exothermic and causes a high temperature rise. The mixture should be allowed to cool to around 25°C before more caustic is added.

The sucrose/alkali mixture had a golden yellow colour. In some cases the solution turned dark red when there was a large increase in temperature.

It was not necessary to sterilize this mixture as it was sufficiently strongly alkaline to prevent the growth of any microorganisms.

6.2.4 STERILIZATION AND ASEPTIC TECHNIQUES

The fermenter vessel, together with the pH probe was 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 ± 0.5°C and maintained at this 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 ± 1°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 ethanol. All fermenter addition ports, and the ends of all sterile tubings were also swabbed with ethanol before use.

6.3 CONTINUOUS FERMENTATIONS

6.3.1 DESCRIPTION OF THE CONTINUOUS FERMENTER

The continuous fermenter used throughout this study was an MBR 'mini" bioreactor (6.1) with total capacity of 2.5 litres. The fermentation vessel was a glass cylinder mounted on a stirrer motor. The stirrer shaft passed through a mechanical seal at the centre of the bottom end plate and it carried two disc turbine impellers for agitation of the fermentation broth. When the fermenter was not in use, water had to be put in the fermentation vessel to prevent the mechanical seal from drying out. The glass cylinder was sealed with bottom and top end stainless steel plates by PTFE sealing for the plates and rubber 0-rings at each end of the vessel.

The top plate of the fermenter had 8 ports of which seven were of 12mm diameter and the remaining one of 19mm diameter. The ports in the top plate of the bioreactor were used for the pH probe, the vent of the fermenter, addition of the inoculum, addition of a 5M NaOH solution for pH control, the reflux cooler of the fermenter as well as for the addition of nutrient feed. A stainless steel pressure safety valve was also mounted on the top plate of the fermenter during continuous operation. The dissolved oxygen probe was mounted in the fermentation vessel through the 19mm diameter port.

The bottom end plate of the fermenter had eight ports which were used

for the insertion of two heating elements, a temperature probe, a drain valve, a level immersion tube, a cooling coil, a sampling port and a safety thermostat.

The fermenter was equipped with an overflow weir of adjustable height which enabled continuous fertmentations to be carried out in a range of working volumes. At a given height of the level immersion tube, the volume of the culture in the fermenter depended on the agitation rate and the relative position of the weir in the fermentation vessel, i.e its relative position with respect to baffles, cooling coil, pH probe and with the other equipments within the fermentation tank.

For safety purposes a liquid level sensor was incorporated in the top plate of the fermenter. This consisted of two electrodes. The lower tip of the one electrode was immersed in the fermentation broth, while the lower tip of the other one was positioned approximately 10 cm higher than the level of the culture. The principle of operation of this liquid level sensor was based on the conductivity the fermentation broth exhibited. This was due to the fact that the fermentation medium contained inorganic salts (see section 6.3.3) and also was made up from tap water. If during a cell recycle fermentation run there was a tubing burst or other leakage, the level of the culture in the fermenter would drop down to a height lower than the tip of the low level sensor electrode. This would result in the shut down of the whole fermentation system by the liquid level sensor unit, thus preventing

damage to the mechanical seal and other fermenter components inserted in the vessel through its bottom end plate.

On the contrary a blockage of the outflow lines of the fermenter would result in accumulation of fermentation broth within the bioreactor vessel.

Contact of the culture with the tip of the upper level sensor electrode would result in shut down of the fermenter and associated equipment.

Temperature control of the fermentation broth was achieved by means of the two heaters inserted in the fermentation vessel through the bottom end plate and tap water circulating in the cooling coil in the fermenter. The output of the cooling coil was connected to a drain. The cooling water inlet was connected to a solenoid valve mounted on the bottom plate, outside the fermenter. The temperature was controlled by the on/off mode of operation of the heating elements and solenoid valve.

The pH probe of the fermenter was a glass Ingold type 465 electrode. It was positioned in a stainless steel housing and pressurized up to 1.5-2 bar. The callibration of the pH probe was carried out against pH buffers of pH 4 and pH 7. This pH probe is steam sterilizable, and was mounted on the fermenter through its top plate and was sterilized during the "in situ" sterilization of the bioreactor. After a fermentation

experiment the tip of the probe was cleaned with distilled water and stored in a 3M KCl solution until required for a subsequent run.

In experiments where a gas was used to sparge the fermentation broth, a cylinder containing the desirable gas was connected, via reinforced tubing, to a serto connector on the rear of the cabinet on which the fermenter was mounted. This gas passed through a rotameter and then through a silicone rubber tubing having two 0.2 μm fiber glass filters incorporated. The resulting sterile gas then entered the fermentation vessel through the top plate where the sparger inlet is found. All lines were sterile and connected to the bioreactor by needle connectors which pierced the rubber septa held in ports in the end plates and screwed into the septum holding cups.

The fermentation vessel was also equipped with a stainless steel condenser mounted on the central part of the top plate of the vessel. During sterilization of the fermenter ("in situ"), cooling water was circulated in the reflux cooler which was topped by a stainless steel exhaust air filter assembly on which a diaphragm valve was mounted. During conventional continuous fermentation experiments this diaphragm valve was kept fully closed. During conventional chemostat experiments in which the fermentation broth was sparged with CO_2 this valve was kept open allowing the gas to leave the bioreactor without causing pressure accumulation in the tank.

Other ancillary equipment and parts included the culture outflow line as well as the nutrient medium feed line. The culture outflow line consisted of an hypodermic, 5mm bore, needle connector and silicone rubber tubing. A 5 cm^3 pipette had been properly incorporated in this feed line enabling measurements of the inlet medium flow rates and consequently the setting of a desirable value of dilution rate.

Caustic addition (5M NaOH) for pH control of the fermentation broth was accomplished by a six-roller peristaltic pump (Eyela; MBR Bioreactor Ltd., High Wycombe, Bucks, U.K) activated by the pH control unit (MSC1) of the bioreactor. An Eyela pump was also used for the continuous addition of nutrient feed during a continuous fermentation.

6.3.2 INOCULUM PREPARATION

The preparation of the inoculum was carried out as follows:

10-15 cm^3 of previously sterilized MRS broth were used to harvest L.M B 512 (F) cells from an MRS agar slant. The resulting cell suspension was transferred aseptically to an inoculum bottle containing 100 cm^3 of sterile MRS broth of pH 7, adjusted before its sterilization . The MRS broth of the inoculum bottle was prepared according to the manufacturer's instructions and sterilized at 121°C, at a pressure of 104KPa (15 psig) for 15 minutes. The whole procedure for inoculum preparation was carried out in a laminar flow cabinet which had been previously cleaned several times with ethanol.

The inoculum then was incubated at 25°C for around 14-16 hrs. Usually this length of incubation time was satisfactory yielding an inoculum of an OD_{590} value of about 0.1.

It was noticed that if the agar slant used for inoculum preparation had been prepared recently the microorganism was growing much faster and an incubation time of 14-16 hrs was sufficient for the microorganism to attain a biomass level of $OD_{590} \approx 0.1$. If a Leuconostoc mesenteroides NRRL B512(F) slope was used which had been prepared a long time ago, the growth of microorganism was very slow necessitating prolonged incubation time for the biomass level to

build-up to an OD_{590} value, of the inoculum, of 0.1. In the latter case, an incubation time of about 24 hrs or more was required for sufficient growth of the microorganism.

It is generally known, that the inoculum size in bacterial fermentations should be in the region of 3-10% v/v of the working volume of the bioreactor. The fermentation medium that was put in the fermenter for the ""in situ"" sterilization of the fermenter was usually 1.2-1.3 dm^3 while the size of the inoculum was 110-115 cm^3. Consequently the inoculum development with respect to its size was satisfactory.

Inocula prepared with recently prepared slopes tend to give a shorter lag phase of the fermentation while inocula prepared with old slopes caused a longer lag phase of the fermentation.

6.3.3 FERMENTATION AND ADDITIONAL MEDIA

The fermentation medium composition used in continuous fermentations or in fermentation experiments which consisted partly of conventional continuous cultures was identical to that of Schneider et al. (6.2) and is given below.

TABLE 6.3

Sucrose	4.5 - 8% w/v.
Gistex Yeast extract	4% w/v.
KH_2PO_4	2% w/v.
R* salts	0.5% w/v.

The R* salts contained:		
	$MgSO_4 . 7H_2O$	4.0 g.
	NaCl	0.2 g.
	$FeSO_4 . 7H_2O$	0.2 g.
	$MnSO_4 . 7H_2O$	0.2 g.
	Water	to 100 cm^3.

The above medium components were dissolved in town's water and made up to the required volume.

Variations in sucrose concentration in the nutrient medium and other individual fermentation conditions are quoted in the relevant

experiments.

For pH control of the fermentation broth, a 5M NaOH solution was used. This caustic solution did not need sterilization for contaminant elimination.

6.3.4 STERILIZATION AND ASEPTIC TECHNIQUES

The "in situ" sterilization of the continuous fermenter was carried out as follows(6.1).

The level immersion tube was placed in the fermentation vessel and all available ports in the bottom plate of the fermenter were sealed by silicone rubber septa held in cups which were then sealed with blind plugs.

1.2-1.3 dm^3 of the fermentation medium was put in the bioreactor and the calibrated pH probe was mounted in the fermenter through a 12 mm diameter port in the top plate. The pH of this medium was adjusted to a pH value of 7 using 5M NaOH solution. All the remaining ports of the top plate were sealed with silicone rubber septa and blind plugs. The sterilization temperature was set at 116°C and the temperature was allowed to rise. Tap water was circulated in the stainless steel condenser and initially the diaphragm valve mounted on the top of the exhaust air filter assembly was open. During the whole sterilization process the stirrer speed had been adjusted to 1000 rpm. When the temperature was in the region of 100-105°C steam was leaving the fermentation vessel through the exhaust air filter assembly. At that moment the diaphragm valve was partially closed allowing only some steam to escape from the fermenter. When the temperature reached the set value of 116°C, it was controlled at this value for 20min. After the

sterilization time expired, the temperature was dropped down and when its value was about 90°C the previously sterilized vent line, bearing a Hepa-cap Whatman filter having mildly hydrophobic glass microfibre media, was connected aseptically to the fermenter at the top plate thus breaking the vacuum created in the vessel due to the sterilization process. This filter can retain 99.97% of all particles greater than 0.2 μm (6.3).

Other ancillary parts included the fermentation medium feed line for continuous feeding of nutrients, the broth outflow line connected aseptically to the level immersion tube via an hypodermic neddle connector, the double hypodermic needle used for inoculation of the fermenter as well as for caustic addition for pH control purposes. Another part was the line which supplied sterile CO_2 to the fermenter sparger in the experiments where CO_2 was put through the culture.

All these parts and other accessories were sterilized by autoclaving in a bench top Adelphi autoclave at 121°C for 20 min and at a pressure of ~ 104 KPa (15 psig).

The nutrient medium used to feed the continuous fermenter was sterilized at 116 ± 0.5°C for 20 min in the 16 dm^3 batch fermenter (New Brunswik Scientific, microgen fermenter, model SF 116). After the sterilization cycle had been completed the nutrient feed was kept in

the fermenter until required, under a positive back pressure of about 0.4 bar, to eliminate any risk of contamination.

The sterile nutrient feed was then transferred aseptically to 5 dm^3 aspirator bottles by means of sterile silicone rubber tubing. The aspirator bottles were then connected to the fermentation system.

The aspirator bottles were previously sterilized in the Adelphi autoclave at 121°C for 20-30 minutes at ~ 104 KPa (15 psig).

All connections to the fermentation system while an experiment was performed, including connection of the feed medium bottles, were carried out by swabbing any connectors or connection points liberally with ethanol.

6.4 CONTINUOUS FERMENTATION WITH TOTAL RECYCLE OF BIOMASS

6.4.1 THE PROCESS MICROFILTRATION MEMBRANE

The membrane used for total or partial cell recycle experiments was a Filtron (Northborough, Massachussetts, U.S.A) sigma series microfiltration membrane of 0.3 μm pore size supplied by Flowgen Instruments Ltd (Sittingbourne, Kent, U.K.). This pore size was considered to be suitable for this application since it was large enough for the enzyme molecules (~ MW ≈ 160,000 Dalton) to permeate through the membrane and, on the other hand, small enough, in comparision with cell size, for the cells to be kept in the retentate which is returned to the fermentation vessel. The membrane was manufactured by modifying polyethersulfone and it was especially synthesized for applications that require in-line sterilization or autoclaving (6.4).

This membrane also, according to the manufacturer, had low protein binding properties and stable performance after 20 total hours of sterilization cycles (cycles of 30 min each, at 121°C). With respect to its morphology, it had a highly asymmetric pore structure through its depth. This asymmetric morphology minimizes the potential internal pore fouling normally associated with symmetric pore membranes. In addition the membrane surface is smooth, allowing the free tangential

flow of cells across its surface. Rough surfaces may cause cells to become entangled and trapped causing a higher degree of polarization of the membrane surface.

This membrane was available both as a woven screen separator cassette configuration or as a linear open channel separator cassette configuration.

A woven screen separator creates gentle turbulence in the cross flow of the fluid minimizing concentration polarization but on the other hand might affect the cells in terms of viability and/or metabolic activity. Since in this project cell integrity and metabolic activity were of crucial importance, the open channel configuration of the membrane was chosen, although polarization effects in this configuration could be slightly more pronounced.

The area of this membrane was 0.75 ft^2 (0.07 m^2), the maximum inlet pressure was ~ 690 KPa (100 psig), and the recommended operating pressure was in the region of 34.5-517 KPa (5-75 psig).

After a fermentation run had been completed, cleaning of the membrane was carried out (6.5).

Both filtrate lines of the cross-flow filtration cell were kept closed. Initially a saline solution (0.9% w/v) was put through the membrane for

cleaning and to prevent protein precipitation. Then the membrane was flushed with 0.5 N sodium hydroxide solution at temperatures up to 40°C for 30-60 minutes and finally the cell was cleaned thoroughly with deionized water to remove the cleaning agent. Then the cassette was stored in a 0.1 N NaOH solution in a freezer until required for a subsequent experiement.

The features of the membrane presented in this section are those given by the manufacturer.

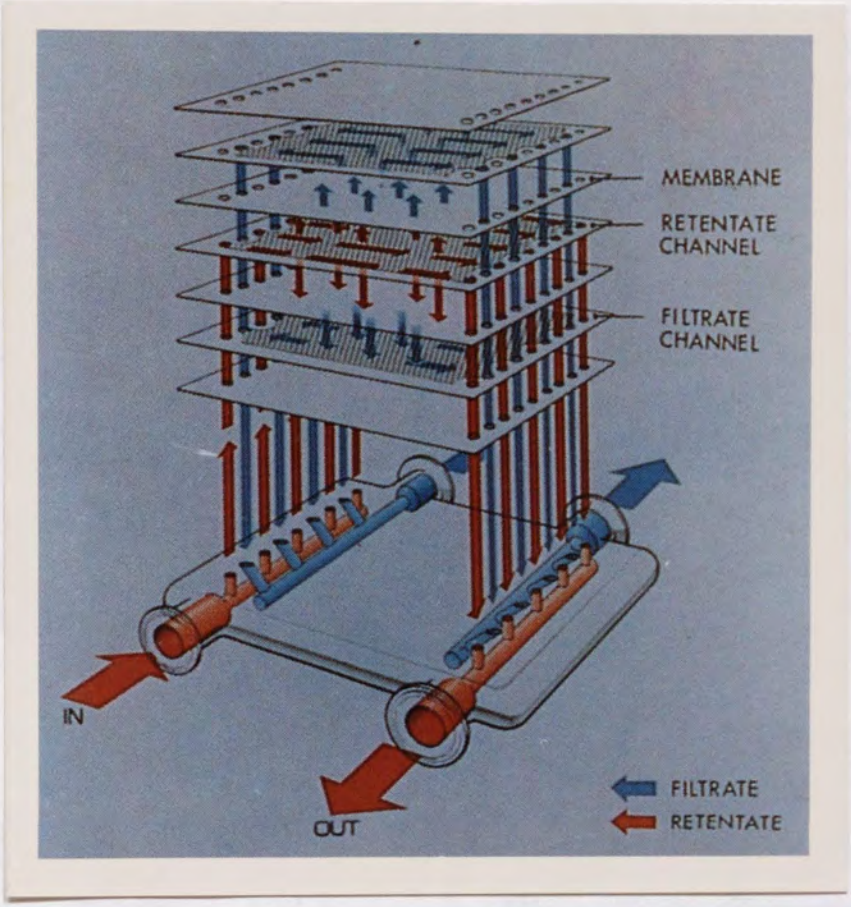


PLATE 1

Fluid flow pattern through a Centrasette cassette system. Similar fluid flow patterns exist in the Minisette cross-flow filtration cassette system.

Plates (1-4) have been reproduced with permission from Flowgen Instruments Ltd, Sittingbourne, Kent ME9 8AQ, U.K.

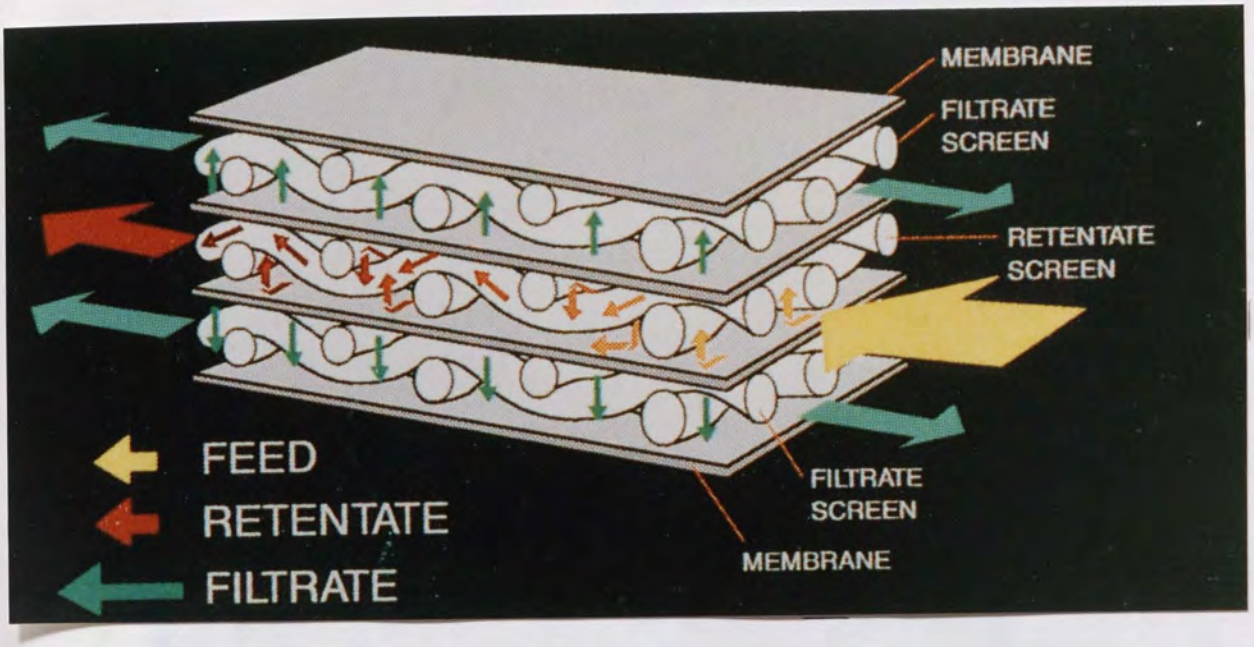


PLATE 2

Flow channel configuration-
retentate channel with woven screen separator.

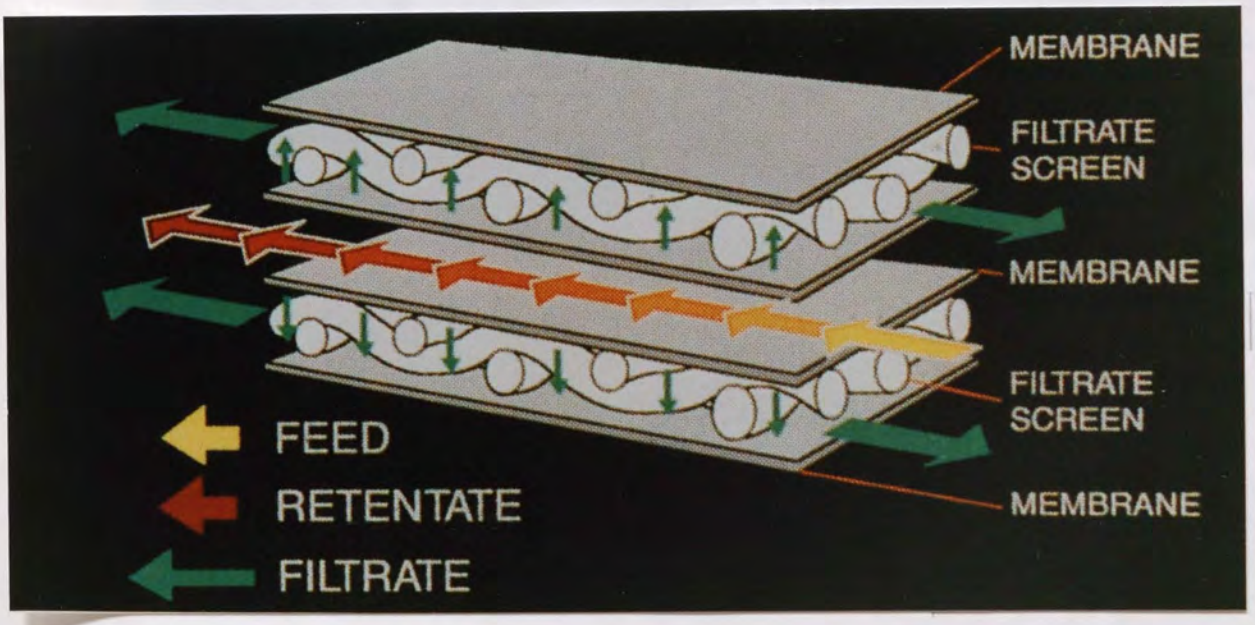


PLATE 3

Flow channel configuration-
retentate channel with open channel separator.

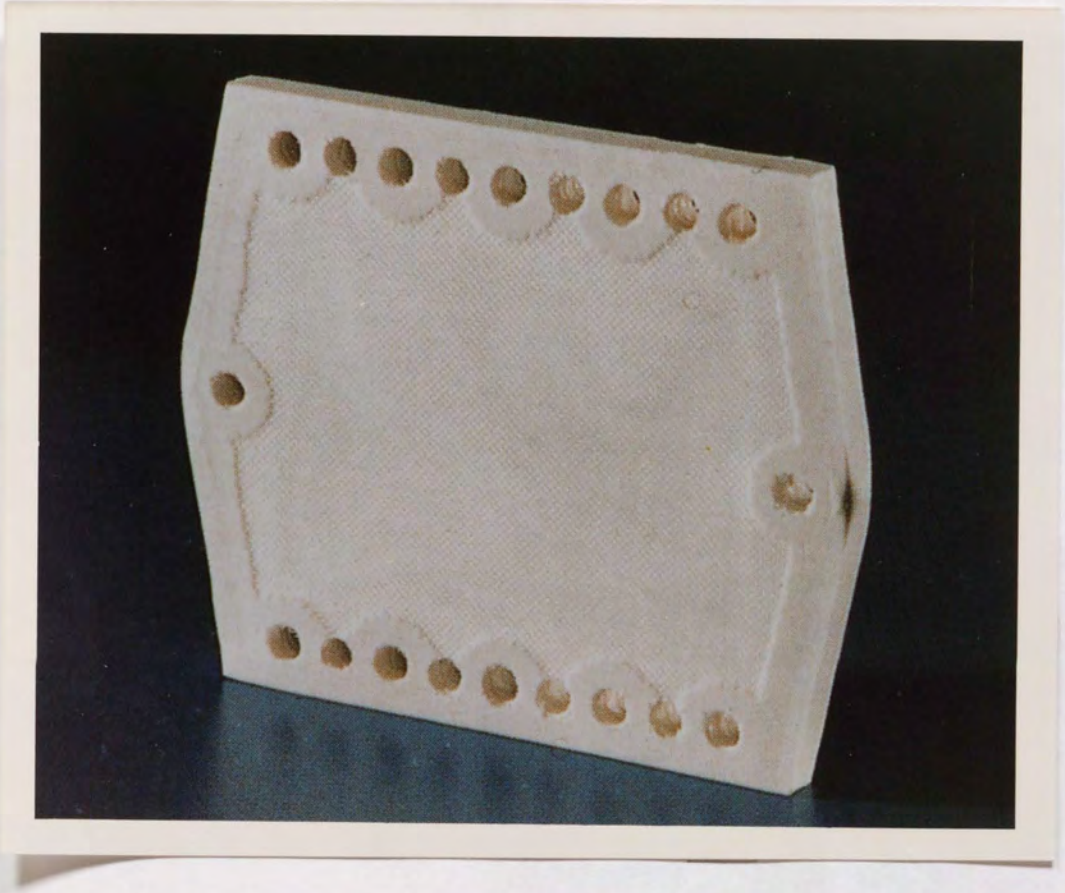


PLATE 4

The Filtron, 0.3 μm pore size, Sigma series Minisette cassette membrane.

6.4.2 EXPERIMENTAL APPARATUS AND MATERIALS FOR TOTAL CELL RECYCLE EXPERIMENTS

The cross flow filtration unit used in this study was a Filtron minisette, SS Cell NPT cell, supplied by Flowgen Instruments Ltd (Sittingbourne, Kent, U.K). At the front of this stainless steel filtration unit there were two ports, the inlet port and the retentate exit port while at the rear of the unit there were two filtrate exit ports. During operation, one filtrate line was closed and permeate was withdrawn or allowed to flow from the remaining filtrate line.

The minisette was connected to the fermenter using marphene II tubing, of 9.6 mm bore and 3.2 mm wall thickness supplied by Watson & Marlow (Falmouth, Cornwall, England), and an hypodermic needle connector put through a silicone rubber septa held in a port at the bottom end plate and screwed in the perforation septum holding cup.

The fermentation broth was withdrawn from the fermenter via a Watson & Marlow 701 S/R peristaltic pump and then passed through the filtration unit. The concentrated cell suspension exiting the cross-flow filtration module from the retentate port was returned to the culture vessel via a submerged inlet in the fermentation broth in order to reduce foaming within the vessel. The retentate line was silicone rubber tubing having a 4.8 mm bore and a 2.4 mm wall thickness and supplied by Watson & Marlow.

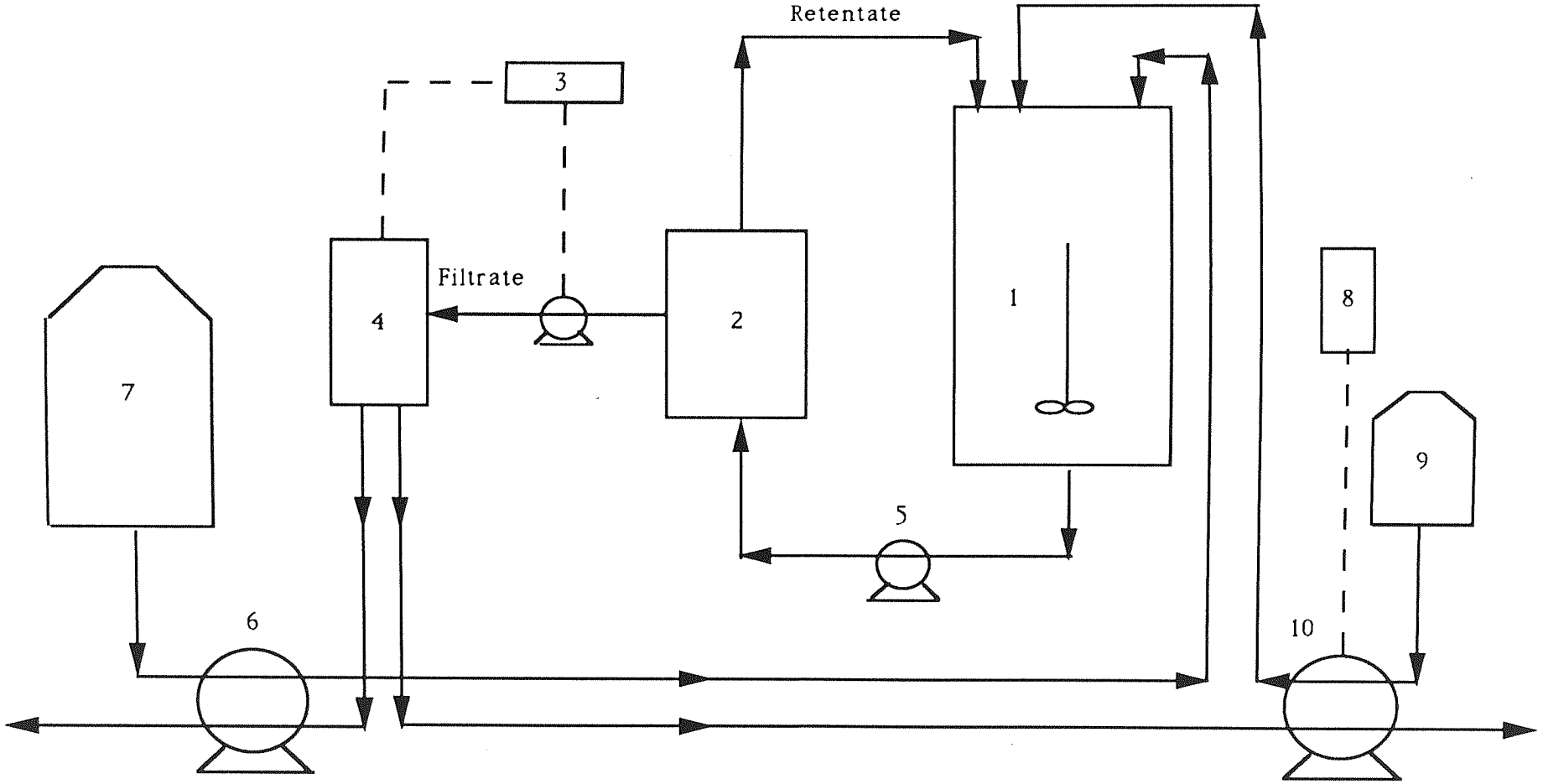


FIGURE 6.1 EXPERIMENTAL APPARATUS FOR TOTAL CELL RECYCLE EXPERIMENTS.
1: Fermenter, 2: Filtration Unit, 3: Level Controller, 4: Level Controller Bottle 5: Recirculation Pump, 6: Feed Pump, 7: Medium Feed Tank, 8: pH Controller, 9: Alkali Tank, 10: Caustic Addition Pump.

To keep a constant culture volume in the fermentation system a balance between all the liquid streams that are fed to the fermentation system and all the liquid streams that are removed from the bioreactor zone must be accurately made. The inlet streams, in total cell recycle experiments, were the nutrient feed and the 5M NaOH solution used for pH control. The liquid stream which was removed from the fermenter was the permeate from the cross-flow filtration cell.

The permeate from the filtration unit was fed, via a marprene II line of 4.8 mm bore and 1.6 mm wall thickness to a level controller bottle where the liquid level was controlled at a fixed height. This bottle was vented, thus preventing pressure build up in this small vessel caused by the pumping of permeate into it. In these experiments the pump used to feed the fermenter with nutrient feed was a duplex peristaltic Cole-Parmer masterflex pump, (CP Instrument Company Ltd, Hertfordshire, England). Two pumpheads of the same specification were connected to the pump drive. Tubing of the same specification was put through both pumpheads. This tubing was also of the same specification required for the pumpheads. During operation, the one pumphead was feeding the fermenter with nutrients and at the same time the other pumphead was withdrawing permeate from the level controller bottle. Both flow rates were the same for the reasons described above. With this arrangement the medium inlet flow rate was exactly balanced by the permeate flow rate which was withdrawn from

the fermentation system.

During the fermentation period caustic (5M NaOH) was added to the fermentation broth for pH control purposes. The pump used for pH control purposes was a duplex Cole-Parmer masterflex peristaltic pump which when switched on pumped caustic into the fermenter and at the same time withdrew permeate from the level controller bottle. Consequently an accurate balance of the caustic solution fed into the fermentation system and permeate removed from it, was achieved.

The level controller unit which was used to keep constant culture volume in the fermentation system consisted of i) the level controller bottle through the top plate of which an electrode had been inserted, ii) a peristaltic pump which was withdrawing permeate from the filtration unit and finally iii) the on/off control unit. The electrode of the level controller bottle was also connected to a variable sensitivity module for adjusting the sensitivity of the whole level controller system with respect to the conductivity of the liquid in the level controller bottle.

The operation of the level controller system was as follows:
When the level of the liquid in the level controller bottle was dropping to a level lower than the tip of the electrode (no conductivity) the on/off control subunit of the system activated the permeate withdrawal pump which then pumped permeate into the level controller bottle. When the

liquid in this bottle came into contact with the tip of the electrode this pump was shut down. Because of the withdrawal of permeate by the two duplex masterflex peristaltic pumps, for caustic dossage and for fermentation medium addition, the liquid level in the level controller bottle decreased to a level lower than the tip of the electrode. This resulted in activation of the permeate withdrawal pump and the system was automatically operated in this mode throughout the fermentation time.

It must be noted that the permeate withdrawal flow rate from the cross-flow filtration cell must be higher than the total permeate withdrawal flow rate from the level controller bottle. When the liquid level in this bottle drops to a level lower than the tip of the electrode the pump which withdraws permeate from the cross-flow filtration cell must have the capacity (which depends on the pump speed setting) to increase back the liquid level to the tip of the electrode of the level controller bottle.

At this point it must be noted that the speed of the pump that withdrew permeate from the filtration cell had to be high enough to meet the above requirement but at the same time its speed had to be not too high because it would result in blockage of the filter. The only remedy in this case was to employ high cross flow rates to minimize polarization effects and consequently enable high permeate flow rates to be achieved. The principle of operation of the level controller system, employed to

keep constant culture volume, was the conductivity of the product stream (in this case, the permeate) in the level controller bottle. The fermentation medium and the initial fermentation broth was made up with tap water and contained potassium salts as well as other inorganic salts present in the "R* salts" medium component and also inorganic salts present in the Gistex yeast extract.

The fermenter sampling procedure was as follows:

A small quantity (~5 cm^3) of fermentation broth was collected to clean the sampling line and then the actual sample was taken for analysis. The total sample volume was accurately measured and exactly the same volume of water was added to the level controller bottle. This resulted in an increase in the liquid height in the bottle, equivalent to the total sample volume, causing contact of the liquid with the tip of the electrode resulting in shutting down of the permeate withdrawal pump of the filtration cell until an identical volume of nutrient medium was fed into the fermenter. For this time period, there was nothing removed from the fermentation system and only feed of the same volume of the total sample, was pumped into the fermenter until the liquid level drops down to the tip of the electrode. With this procedure constant culture volume was maintained which otherwise would decrease with fermentation time, due to sampling.

As has been described in this section a peristaltic pump had been incorporated to one of the filtration lines of the minisette which was

withdrawing permeate and feeding it into the level controller bottle.

In developing this system for total cell recycle experiments it was considered that it would be advantageous to replace the peristaltic pump with a solenoid valve, located in the filtrate line of the minisette. The withdrawal of permeate by means of a peristaltic pump has the disadvantage that the pump may cause blockage of the filter when the permeate flow rates the membrane can provide are relatively low. This became more pronounced with fermentation time and unavoidable polarization effects which resulted in decreased permeate flow rates.

A solenoid valve operating on the on/off mode allowed, when in the on position, free flow of the filtrate into the level controller bottle without exerting suction or having any other interference with the filtration membrane.

The use of the solenoid valve had been tested in fermentation experiments and affected the membrane in a way that had not been predicted.

During the fermentation process and especially during the early stages, there was a high pressure accumulation in the filtrate line when the solenoid valve was closed. When the level controller unit opened the solenoid valve there was a large and instantaneous drop in the filtrate pressure that damaged the membrane.

This instant large pressure release can, however, be avoided by incorporating a peristaltic pump in the filtrate line. In such a case there is a smooth and gradual drop in pressure and not, as with a solenoid valve, a pressure shock that damages the membrane. It was decided therefore to use in subsequent experiments a peristaltic pump for permeate withdrawal.

In constructing the experimental apparatus for both total and partial cell recycle experiments the external or recycling loop was made as short as possible to minimize the retention time in the loop. The values of the retention time of the culture in the recycling line are quoted in the relevant experiments.

6.4.3 FERMENTATION MEDIUM AND ENVIRONMENTAL CONDITIONS

The fermentation medium composition was:

TABLE 6.4

Sucrose		4.5% w/v, unless otherwise stated.
Gistex yeast extract		4% w/v.
KH_2PO_4		2% w/v.
R* salts		0.5% v/v.
The R* salts contained:	$MgSO_4 . 7H_2O$	4.0 g.
	NaCl	0.2 g.
	$FeSO_4 . 7H_2O$	0.2 g.
	$MnSO_4 . 7H_2O$	0.2 g.
	Water	to 100 cm^3.

The fermentation temperature was controlled at 23.4°C, the pH was controlled at 6.65 by the addition of 5M NaOH solution, and the agitation was controlled at 700 rpm unless otherwise stated. Tap water was used for the preparation of all media used.

6.4.4 INOCULUM PREPARATION

The procedure for inoculum preparation was carried out as described in section 6.3.2.

6.4.5 STERILIZATION AND ASEPTIC TECHNIQUES

The continuous MBR 'mini' bioreactor was sterilized "in situ" as described in section 6.3.4.

Steam sterilization of the cross-flow filtration unit and associated tubing could be carried out in two ways. On-line sterilization and sterilization by autoclaving.

The procedure for on-line sterilization of the minisette involved initially removal of all the condensate from the steam line which provided steam to the laboratory. Subsequently, the feed line of the minisette was connected to the steam supply line. The sterilization time used was 30 minutes. The steam produced by the boiler of the department was not of a pharmaceutical grade and it was suspected that it could affect the performance of the membrane and thus this sterilization method was abandoned.

The procedure of sterilizing the minisette and associated tubing in an autoclave was carried out according to manufacturer's instructions and was as follows:

First the membrane was flushed with deionized water to remove the storage solution in which the membrane had been kept when not in use. The cell was finger tightened and placed in an autoclave and subsequently sterilized for 20 minutes at 121°C at 104 KPa (15 psig). After the sterilization cycle was complete the autoclave was allowed to reach room temperature, the minisette was taken out and the cell was tightened with a torque-wrench set to the membrane maufacturer's recommended value which was 65 inch lbs. Subsequently the filtration unit was connected to the fermenter. All connections were carried out by swabbing any connectors or connection points liberally with ethanol.

6.5 CONTINUOUS FERMENTATIONS WITH PARTIAL RECYCLE OF BIOMASS

The minisette was connected to the fermenter as described in section 6.4.2. The same type of tubing as used in the apparatus for total cell recycle was used.

The fermentation broth was withdrawn from the fermenter via a Watson & Marlow 603 S/R peristaltic pump set at 70 rpm and was put through the filtration unit. The concentrated culture stream, the retentate, was returned to the fermentation vessel via a submerged inlet in order to reduce foaming within the vessel.

Nutrient medium was added continuously to the fermenter using a six-roller peristaltic pump (Eyela, MBR Bioractor Ltd, High Wycombe, Bucks, U.K).

A Cole-Parmer masterflex peristaltic pump (CP Instrument Company Ltd, Hertfordshire, England) with a speed range of 1-100rpm was used to withdraw permeate from the cross-flow filtration unit at a controlled rate.

A constant culture volume in the fermentation system was achieved, as in conventional continuous fermentations, by using an overflow weir inserted in the vessel through its bottom end plate.

The controlling parameters for the fermentation, D, dilution rate, and R, recycle ratio, were set by manipulation of the speed of the growth medium addition pump and the cell-free filtrate peristaltic pump, as presented in the theoretical analysis of cell recycle fermentation, section 2.5.1.

Cross-flow rates in the filtration cell were determined initially with H_2O before an experiment began and with whole broth at the end of a fermentation experiment. The results are quoted in the relevant experiments.

Each experiment consisted of two phases. The first phase was a conventional continuous fermentation and the second phase was a partial cell recycle fermentation. The latter phase was initiated by applying cell recycle to the sucrose excess steady state chemostat of the first phase at $D = 0.29\ h^{-1}$.

The fermentation medium was the same as that employed for total cell recycle experiments apart from the sucrose concentration in the feed which was varied from 4.5% w/v to 8% w/v depending on the particular experiment.

Agitation rate was also varied and its values as well as the values of sucrose concentration in the feed and in the initial nutrient medium in

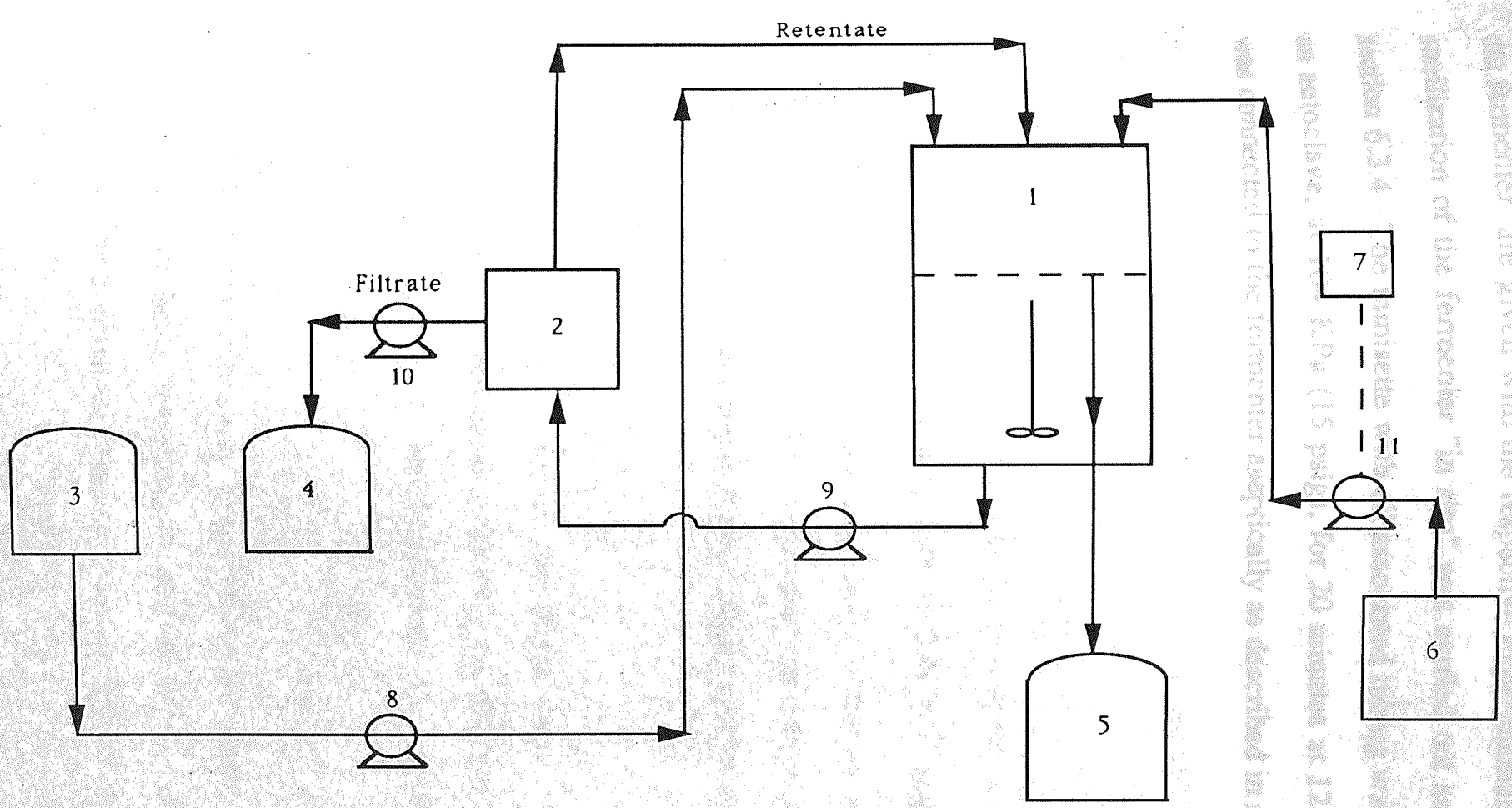


FIGURE 6.2 EXPERIMENTAL APPARATUS FOR PARTIAL CELL RECYCLE EXPERIMENTS
1: Fermenter, 2: Filtration Unit, 3: Nutrient Medium Tank, 4: Cell-Free Product Container
5: Effluent Reservoir, 6: Alkali Tank, 7: pH Controller, 8: Medium Feed Pump,
9: Broth Recirculation Pump, 10: Permeate Withdrawl Pump, 11: Caustic Addition Pump.

the fermenter are given with the respective experimental details. The sterilization of the fermenter "in situ" was carried out as described in section 6.3.4. The minisette with all associated tubing was sterilized in an autoclave, at 104 KPa (15 psig), for 20 minutes at 121°C. Then it was connected to the fermenter aseptically as described in section 6.4.5.

6.6 EXPERIMENTS ON "ENZYME ACTIVITY ENHANCEMENT".

Each of these experiments consisted of two phases. The first phase was a conventional chemostat experiment, using pH control while in the second phase the chemostat culture of the first phase was kept in the same vessel but with batch cultivation of the microorganism without pH control.

The conventional chemostat, comprising the first phase of each of these experiments was run at a dilution rate of 0.29 h^{-1} and the sucrose concentration in the initial medium in the fermenter as well as in the nutrient feed was 6% w/v. All the other medium components were the same as in the case of continuous fermentation experiments reported in section 6.3.3.

The environmental parameters for the first phase were:
The termperature was controlled at 23.4°C, the pH was controlled at 6.65 by the addition of a 5M NaOH solution. The agitation rate was controlled at 700 rpm.

The procedure for inoculum preparation for these continuous fermentations has been described in section 6.3.2. The "in situ" sterilization of the continuous fermenter was carried out as described in section 6.3.4.

After the steady-state data of the chemostat had been obtained the system was switched to the second phase. Immediately before switching to the second phase (batch cultivation, no pH control) 100 cm^3 of a suitable sterile sucrose solution was added aseptically to the fermentation broth to give a desirable initial sucrose concentration for the second phase. This addition was carried out through the inoculum port in the top plate of the fermenter.

The sucrose solution was sterilized at 116°C for 20 minutes in an Adelphi bench top autoclave. By bleeding this autoclave it was feasible to control the sterilization temperature at a desirable value.

In the second phase only temperature control and agitation were provided to the culture which were: 23.4°C and 700 rpm respectively.

CHAPTER 7:

ANALYTICAL EQUIPMENT AND TECHNIQUES

7.1 CELL GROWTH DETERMINATION

During the various fermentation experiments carried out it was necessary to follow the course of the growth of the microorganism in the fermentation system. A simple method was used for such determinations, which measured the turbidity or optical density of the culture. The procedure was as follows.

A sample was taken from the fermenter and diluted by a factor of 25 with saline (0.9% w/v NaCl water solution). Its optical density was measured at 590 nm with a Pye Unicam SP 1800 UV/Visible spectrophotometer, using quartz cuvettes. The spectrophotometer was initially set to zero using saline.

OD_{590} values greater than 0.6 were found to be outside the linear region of the spectrophotometer. The 25 fold diluted sample was further diluted, the OD_{590} value read and the result multiplied by the dilution factor.

During the lag phase of the fermentations the OD_{590} value was usually less than 0.1, but after further growth of the microorganism the OD_{590} value had increased to a level depending on the fermentation technique and conditions employed.

Figure 7.1 depicts the calibration curve [7.1] drawn for OD_{590} against cell dry weight (g/l).

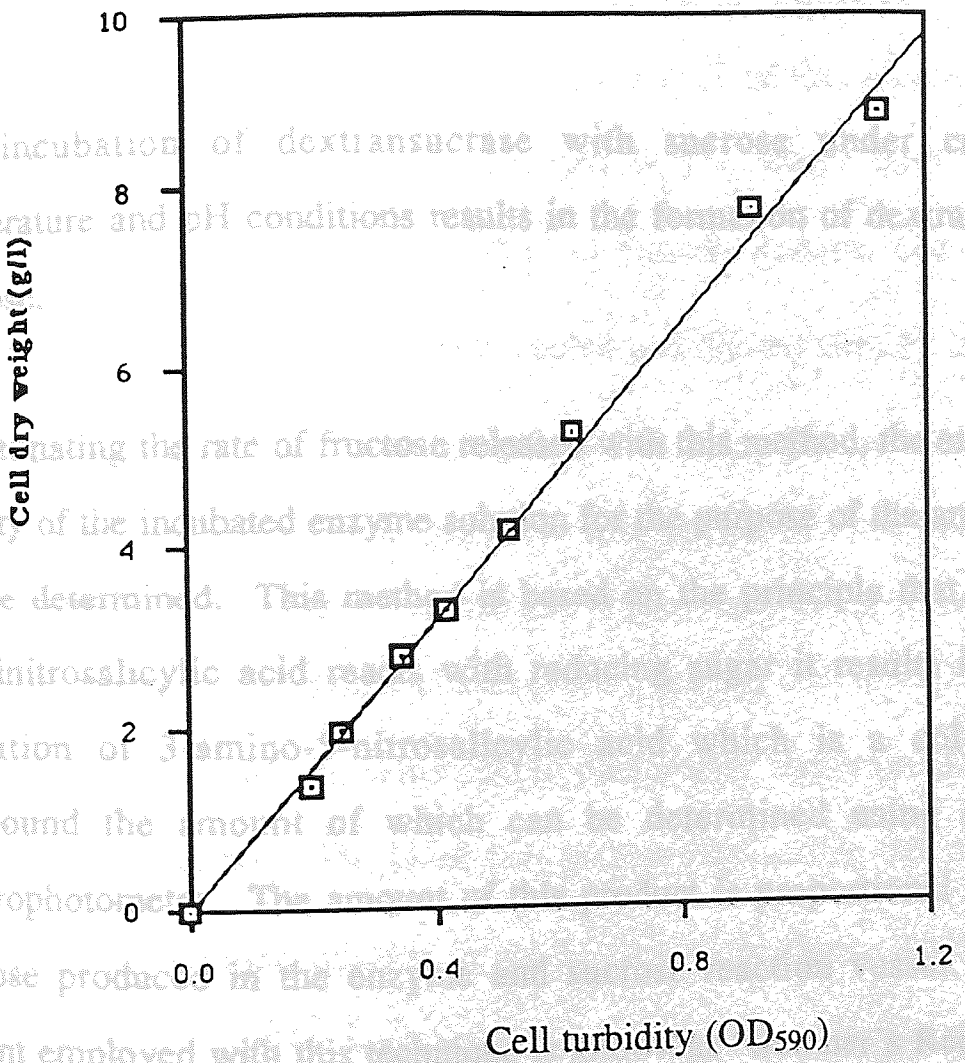


Figure 7.1: Cell dry weight calibration curve.

7.2 ENZYME ACTIVITY DETERMINATION

The activity of dextransucrase was determined using Hostettler's method. (7.2)

The incubation of dextransucrase with sucrose under certain temperature and pH conditions results in the formation of dextran and fructose.

By estimating the rate of fructose released with this method, the enzyme activity of the incubated enzyme solution for the purpose of the analysis can be determined. This method is based on the principle that when 3,5-dinitrosalicylic acid reacts with reducing sugar it results in the formation of 3-amino-5-nitrosalicylic acid which is a coloured compound the amount of which can be determined using a UV spectrophotometer. The amount of this product is proportional to the fructose produced in the enzyme and sucrose reaction vessel. The reagent employed with this technique is known as "Sumner's Reagent" which is prepared by dissolving 10 g of 3,5-dinitrosalicylic acid and 300g potassium-sodium-tartarate with 0.4 M NaOH water solution to give a total volume of 1 dm^3.

The analysis procedure, according to this method was as follows:

4 cm^3 of 6.25% w/v sucrose in 0.1 M sodium acetate buffer of pH 5.2 were incubated in a water bath at 25°C for some minutes, usually 7-10, to enable the sucrose solution to reach the set temperature of 25°C. Then 1 cm^3 of enzyme containing 30-50 DSU/cm^3 was added to the sucrose solution, which had previously been placed in the water bath, and gently mixed. Immediately, at t=0, 0.5 cm^3 of this mixture was taken out and the remainder incubated in the water bath. The withdrawn sample was added to 1 cm^3 of Sumner Reagent and boiled for 5 minutes. The sample was then cooled and diluted with 11 cm^3 of distilled water providing the unincubated sample. Further 0.5 cm^3 samples were withdrawn from the water bath at five minutes intervals, up to a period of 20 minutes with the above procedure being carried out for each sample. The optical densities were read at 530 nm (OD_{530}) against a blank, using the same spectrophotometer employed for cell growth determinations. The blank was prepared by adding 0.5 cm^3 of the 6.25% w/v sucrose solution in pH 5.2 acetate buffer to 1 cm^3 of Sumner Reagent, boiling for 5 minutes, cooling and adjusting to 12.5 cm^3 with distilled water. This was checked with a water blank (0.5 cm^3 of water) prepared in the same way. If the optical density, measured at 530 nm, of these two samples was the same it demonstrated that the sucrose solution was not contaminated and could be used in subsequent analyses.

The enzyme activity was calculated using the following formula:

$$DSU/cm^3 = \frac{(OD_{530}t - OD_{530}u) \times d \times 60 \times 2}{0.2 \times 0.52 \times t \times OD_{530}f} \qquad \text{Eqn. 7.1}$$

where $OD_{530}t$ = optical density at 530 nm of incubated sample at time t.

$OD_{530}u$ = optical density at 530 nm of unincubated sample.

d = dilution factor of enzyme solution.

$OD_{530}f$ = Optical density at 530 nm of a 2 g/l fructose solution,prepared using 0.5 cm^3 fructose solution, 1.0 cm^3 Sumner Reagent and treated as all other samples.

t = incubation time, in minutes.

One dextransucrase unit, DSU, is defined as the amount of the enzyme which will convert 1 mg of sucrose to dextran in 1h at 25°C and at a pH value of 5.2 (optimum pH for dextran production).

Another unit for dextransucrase activity which has been established recently and is in international use is the "U". This is defined as the amount of enzyme which will release of 1 μ mole of reducing sugar from sucrose in one minute. In this case the reducing sugar is fructose.

Equation 7.2 gives the correlation of dextransucrase unit DSU, and the standard, international unit for dextransucrase activity, U:

$$1U = 20.77 \times DSU \qquad \text{Eqn. 7.2}$$

7.3 FERMENTATION BROTH HPLC ANALYSIS FOR CARBOHYDRATES

Samples were taken from the fermentation system and analysed for sucrose. In some of the experimental work HPLC analyses for fructose were also carried out.

The HPLC system with which these analyses were performed consisted of an eluent reservoir, a dual piston Bio-rad model 1330 pump, a guard column, a Bio-rad Aminex carbohydrate HPX 87C column, a column heater for operating the column at the required analysis temperature, a Bio-rad refractometer and a Hewlett-Packard model 3390A integrator.

The eluent used was deionised distilled water containing 0.02% w/v calcium acetate.

Before the samples were injected into the HPLC unit either manually or via a Talbot ASI-4 automatic injector, they were filtered with 0.45 μm pore size disposable acrodisc filters supplied by Gelman Sciences, Northampton, UK.

Prior to the injection of the filtered media samples a standard sample containing known concentrations of carbohydrates was injected into the preconditioned analytical column of the system. A standard sample was also injected after every 3 - 4 media samples had been processed in the

HPLC, for the analysis and subsequent calculations to be as accurate as possible.

Table 7.1 gives the operating conditions, employed for carbohydrate analyses, of the HPLC system.

TABLE 7.1

HPLC OPERATING CONDITIONS

Eluent flow rate	0.5 cm^3 / min
Column temperature	85°C
Operating pressure	up to 100 bar

RETENTION TIMES (mins.)

Dextran	~ 8
Sucrose	~ 10
Glucose	~ 12
Fructose	~ 16

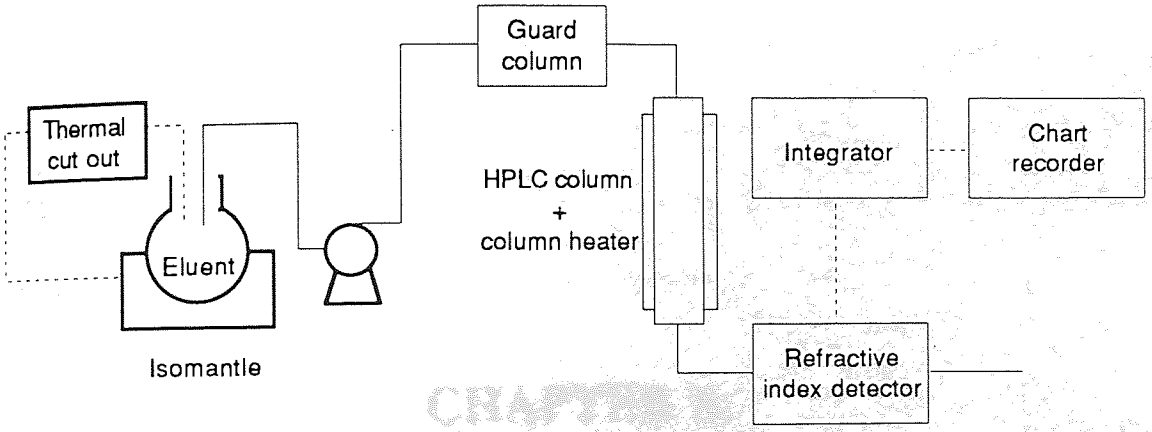


Figure 7.2: HPLC Analytical System

CHAPTER 8:

FED BATCH CULTURE
RESULTS AND DISCUSSION

8.1 UNAERATED LABORATORY SCALE FED BATCH CULTURE

A number of anaerobic fed batch fermentations were carried out on a 6 dm^3 scale using the SF116 "Microgen" N.B.S. fermenter and a typical fed batch unaerated fermentation is presented in this chapter. The apparatus has been described in section 6.2.1. The inoculum development procedure was given in section 6.2.2 and the fermentation and additional media used have been reported in section 6.2.3.

The operating parameters used were a temperature of 23 ± 0.5°C, a pH of 6.65 and an agitation rate of 100 r.p.m.

Figure 8.1 is a graphical representation of a typical anaerobic fed batch fermentation. It shows the enzyme activity, cell growth, total sucrose consumption values over the course of the fermentation. The results of this experiment are given in Table 8.1.

TABLE 8.1

RESULTS: FED BATCH FERMENTATION ON A 6 dm^3 SCALE

OD_{590} maximum value.	Enzyme activity. (DSU/cm^3)	Sucrose added. (g)	Growth rate, μ. (h^{-1})	Duration of fermentation. (h)
1.08	451	746	0.3	18.5

The lag phase for the anaerobic fermentations that were carried out was 6 - 9 h. As can be seen from Fig. 8.1, where the results of a typical fed batch fermentation are presented, the culture in this experiment exhibited a lag phase of approximately 7 h.

By the time the OD_{590} value was 0.3 - 0.4 the enzyme activity in the fermentation broth was about 50 DSU/cm^3. At the end of the lag phase as the cells started growing the pH dropped to the controlled value of 6.65 and the sucrose/alkali addition commenced. The enzyme was mainly elaborated when the cells were in the exponential phase of growth as evident from a rapid increase of enzyme activity in the culture supernatant, demonstrating that dextransucrase is a growth associated product.

The enzyme activity in these experiments reached maximum values in the range of 400 - 500 DSU/cm^3 in 6 - 8 h after the lag phase had expired.

The sucrose addition rate was constant during the exponential phase as indicated by the linear relationship of the sucrose consumption curve. Cell growth tended to level off at OD_{590} values of about 1.1 - 1.2.

In the fed-batch fermentations carried out after the cells had reached the stationary phase a decline in OD_{590} values and enzyme activity was observed. The decline in OD_{590} values was due to autolysis of the biomass while the drop in enzyme activity was due to the instability of dextransucrase at this temperature and pH. Consequently, the enzyme must be harvested as soon as the cells reach the stationary phase and maximum enzyme activity has been attained and before the decline or death phase of the culture occurs.

Using the data given in Table 8.1, approximate calculations for the determination of yield coefficients were carried out. These gave $Y_{x/s}$ = 0.10g cell dry weight/g sucrose consumed, $Y_{p/s}$ = 4840 DSU/g sucrose consumed, and $Y_{p/x}$ = 49400 DSU/g cell dry weight.

The calculations based on the data reported by Pennell[8.1], a previous worker in the same laboratory, gave for the yield coefficients the following values:

$Y_{x/s}$ = 0.087g cell dry weight/g sucrose consumed,

$Y_{p/s}$ = 4250 DSU/g sucrose consumed and $Y_{p/x}$ = 48900 DSU/g cell dry weight.

The results of Pennell from fed-batch fermentations were of the same order of magnitude as those given in Table 8.1 with respect to maximum OD_{590} value and maximum enzyme activity but were 10% higher for the total sucrose consumed. From the two sets of the yield coefficients given above, the values of $Y_{p/x}$ are seem to be similar but the values of $Y_{x/s}$ and $Y_{p/s}$ calculated from the work reported in this section are higher, 13% and 12.2% respectively, than those reported by Pennell. Obviously higher values of $Y_{x/s}$ and $Y_{p/s}$ indicate that more biomass and dextransucrase respectively, are formed per gram of sucrose consumed. This implies a more efficient fermentation in terms of substrate utilization. The fact that the value of $Y_{p/x}$ is approximately the same in both cases indicates that the amount of enzyme produced per unit of biomass is approximately the same implying that in both cases the productivity of biomass, in terms of enzyme elaboration, is the same. The reason for the differing results, as stated earlier, is the relatively higher sucrose consumption in the fed-batch fermentations

carried out by Pennell.

For the calculation of the yield coefficients the value used for the growth of the microorganism in the fermentation system was that of the maximum OD_{590} value, the value of the enzyme activity that of the maximum enzyme activity obtained and the value of the sucrose consumed that of the total consumed up to the termination of the fermentation, i.e. until the time of maximum enzyme activity.

The procedure for these calculations was a follows. The maximum OD_{590} value was converted to cell dry weight (g/l) using a calibration curve drawn up for OD_{590} values against cell dry weight (g/l). Since, during a fed-batch fermentation, the broth volume increases from 6 litres at the start of the process to 8 litres at the end of the fermentation, the cell dry weight (g/l) was multiplied by 8 to give the value of the dry weight of the total biomass in the fermentation system at the end of the process. This value was divided by the total sucrose consumed over the whole fermentation period resulting in the value of $Y_{x/s}$.

For the determination of $Y_{p/s}$ the maximum enzyme activity

(DSU/cm^3) was multiplied by 1000 to give the concentration of dextransucrase in DSU/litre of fermentation broth and subsequently also multiplied by 8 to give the total amount of dextransucrase units in the total culture broth at the end of the fermentation, i.e. at the time of maximum enzyme activity. This value was divided by the amount of the total sucrose consumed, in grams, resulting in the value of $Y_{p/s}$.

Similarly, $Y_{p/x}$ was calculated as the ratio of the maximum total amount of dextransucrase units in the fermentation broth (8l) at the end of the process to the dry weight of the total biomass in the fermenter also at the end of the fermentation.

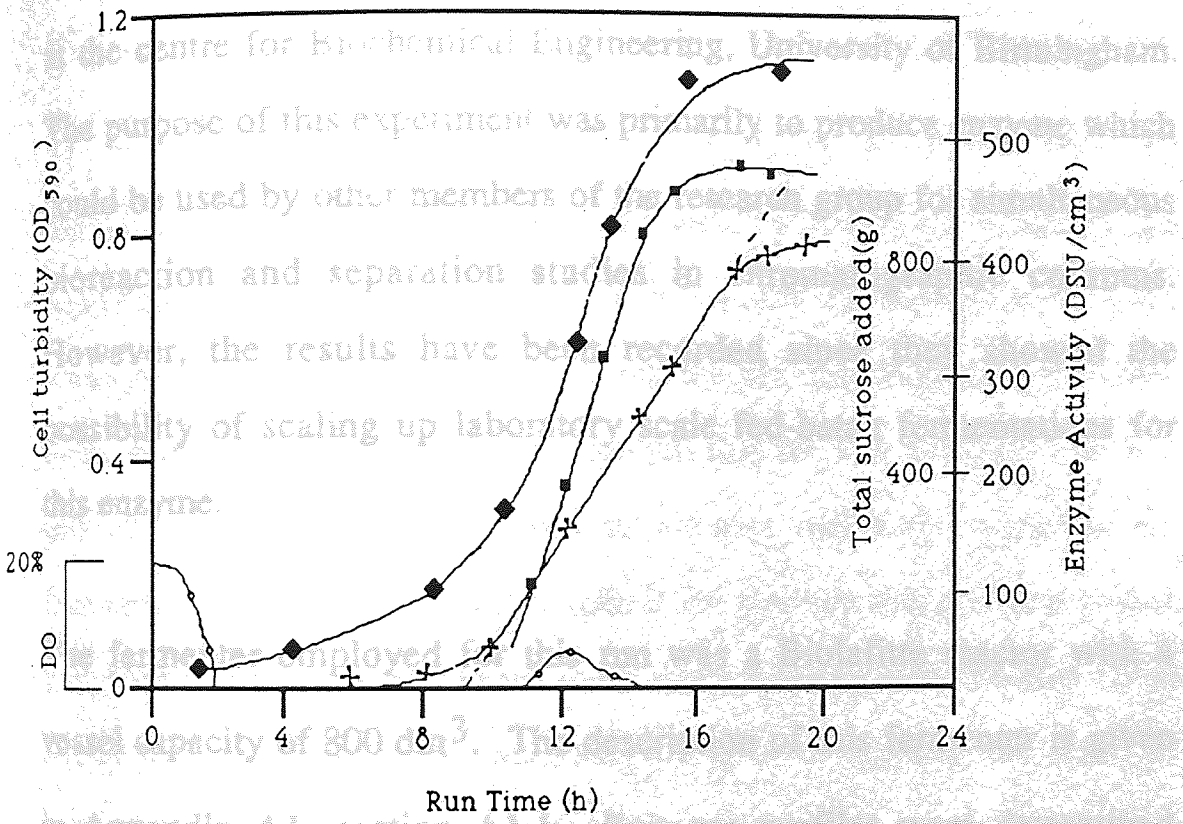


Figure 8.1: **Cell growth (♦), dissolved oxygen (o), total sucrose consumption (+) and (■) enzyme production during anaerobic fed batch fermentation.**

8.2 UNAERATED FED BATCH CULTURE IN A 800 dm^3 VESSEL AT BIRMINGHAM UNIVERSITY

One large scale fed batch fermentation was carried out during July 1990 at the centre for Biochemical Engineering, University of Birmingham. The purpose of this experiment was primarily to produce enzyme which could be used by other members of the research group for simultaneous bioreaction and separation studies in chromatographic columns. However, the results have been recorded since they showed the possibility of scaling up laboratory scale fed-batch fermentations for this enzyme.

The fermenter employed for this run was a Biolafitte reactor with a vessel capacity of 800 dm^3. The description of this fermenter is given in Appendix A1, section A1.1. Exit gas profiles were determined during the fermentation in terms of CO_2, N_2, O_2 and Ar.

Unfortunately, the actual fermentation based on enzyme activity achieved was not as successful as it was hoped it would be. The inoculum preparation procedure is given in Appendix A1, section A1.2. The inoculum was well grown on transfer to the main fermenter. The OD_{590} values of the three final inoculum bottles were 0.137, 0.126 and 0.127. The inoculum culture was fed into the fermenter by gravity and this process took up to 1.5 h to complete. The culture was in the lag

phase for 9 h and then it began to grow slowly exhibiting linear growth rather than exponential, indicating that it was substrate limited. Figure 8.2 shows the dextransucrase activity and OD_{590} profiles recorded for the fermentation. From this figure it can be seen that the maximum OD_{590} value achieved was 0.722 and the maximum enzyme activity was 133.2 DSU/cm^3. It was noticed that after 17 hours into the fermentation foam formation occurred which filled up the whole of the head space of the fermenter. Up to this point the fermentation broth was agitated at a rate of 100 r.p.m. and the pH was controlled at values in the range of 6.3 to 6.9. It was thought that the low agitation rate was providing inadequate mixing and the sucrose which was added to the fermenter was not dispersed throughout the medium and consequently it was not readily accessible to the cells. This resulted in the culture being substrate limited, with sucrose being the growth limiting substrate, as demonstrated by the linear growth curve experienced.

After 19 hours into the fermentation the agitation rate was increased to 250 r.p.m. to provide better mixing of the fermentation broth, in an attempt to boost growth and enzyme activity. The response of the whole system to the increase in this parameter was immediate. The pH began to be controlled much more accurately between the values of 6.64 and 6.66 indicating a more uniform environment within the bioreactor. CO_2 evolution rate was also increased demonstrating increased cell growth. Furthermore, the foam was quickly dispersed and the cells began to grow at a higher rate elaborating more enzyme. However this

treatment, i.e. the increase in agitation rate and consequent improved sucrose transfer to the cells, did not result in the recovery of microorganisms in terms of metabolic activity, since the maximum values of growth and enzyme activity of the fermentation were low. These values as stated earlier were 0.722 and 133.2 DSU/cm^3 respectively. After 23.5 hrs from the initial inoculation the fermentation was terminated.

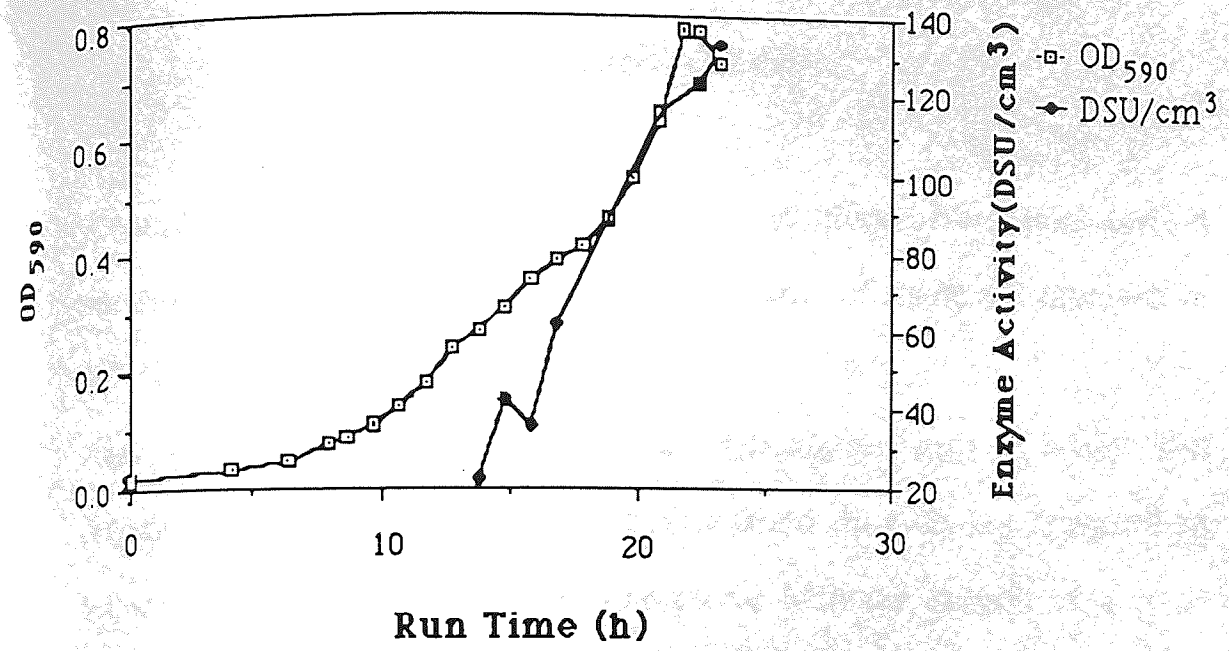


Figure 8.2: **Results from the production of dextransucrase in the 800 dm^3 fermenter.**

CHAPTER 9:

CHEMOSTAT CULTURE RESULTS AND DISCUSSION

9.1 CONVENTIONAL CONTINUOUS UNAERATED FERMENTATIONS

A number of conventional continuous fermentations have been carried out during the course of this project and some of them are reported in this chapter.

Also, the results of two continuous fermentation runs in which the fermentation broth was sparged with carbon dioxide are reported in section 9.2. These results are compared with the results of a non-aerated continuous fermentation experiment which was run under comparable conditions.

The experimental apparatus employed in these experiments, i.e. the MBR "mini" bioreactor, associated equipment and materials, have been described in section 6.3.1. The fermentation medium is specified in section 6.3.3 and the procedure for inoculum preparation is given in section 6.3.2. Finally, the sterilization and aseptic techniques employed in this part of the experimental work are described in section 6.3.4.

In these experiments, which were run with a 4.5% w/v sucrose feed, the effect of varying the dilution rate was investigated. In particular, the intention was to find operating conditions which would result in higher enzyme activity levels in continuous culture. Consequently, the dilution rate profiles with respect to enzyme activity and OD_{590} values were obtained over dilution rate values ranging from 0.375 h^{-1} to 0.55h^{-1}.

High dilution rates, in excess of $0.5h^{-1}$, were also employed to determine the region where cell wash-out occurs.

The results of these non-aerated conventional continuous fermentations are summarized in Table 9.1.

TABLE 9.1

RESULTS OF NON-AERATED CONTINUOUS FERMENTATIONS.

RUNS: CF-1, CF-2, CF-3 and CF-4.

Dilution rate (h^{-1}).	OD590 values.	$\tilde{x}_{EXP.}$ (1) (g/l)	$\tilde{x}_{CALC.}$ (2) (g/l)	Enzyme activity, (DSU/cm^3).	Run No.
0.375	0.417	3.8	3.86	54.2	CF-4
0.39	0.408	3.737	3.79	73.8	CF-4
0.40	0.368	3.416	3.74	62.5	CF-4
0.41	0.362	3.368	3.68	60.1	CF-4
0.43	0.425	3.873	3.54	55	CF-1
0.44	0.440	3.993	3.46	45.86	CF-1
0.45	0.346	3.24	3.37	32	CF-4
0.46	0.33	3.11	3.27	34.4	CF-1
0.47	0.355	3.312	3.16	43.6	CF-2
0.48	0.315	2.99	3.02	32.85	CF-2
0.49	0.287	2.767	2.9	29.4	CF-3
0.50	0.276	2.68	2.7	28.5	CF-3
0.52	0.221	2.24	2.2	15.0	CF-3
0.55	0.159	1.74	0.83	10.1	CF-3

(1): $\tilde{x}_{EXP.}$ = Experimentally determined biomass steady-state concentrations expressed as cell dry weight (g/l)

(2): $\tilde{x}_{CALC.}$ = Theoretically calculated biomass steady-state concentrations expressed as cell dry weight (g/l)

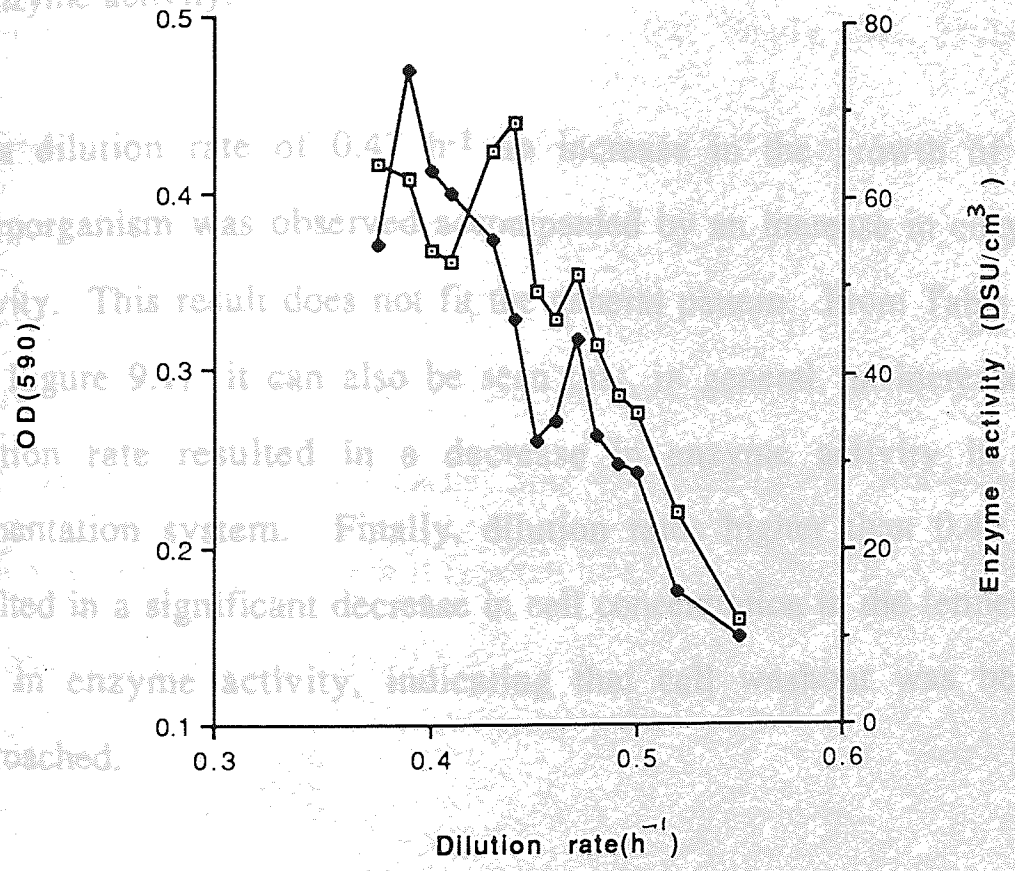


Fig.9.1 Results of the non-aerated continuous fermentations
Dilution rate profiles of enzyme activity (◈) and OD_{590} values (⊡).

From this Table and also from Figure 9.1 it can be seen that the maximum enzyme activity of ~ 74 DSU/cm^3 was obtained at the dilution rate of 0.39 h^{-1}. It will also be noted that the highest OD_{590} values were recorded at dilution rates of 0.43 h^{-1} and 0.44 h^{-1}; this increase in the growth of the microorganism was not accompanied by an increase in enzyme activity.

At a dilution rate of 0.47 h^{-1} an increase in the growth of the microorganism was observed accompanied by an increase in enzyme activity. This result does not fit the general pattern. From Table 9.1 and Figure 9.1, it can also be seen that, in general, an increase in dilution rate resulted in a decrease in enzyme activity in the fermentation system. Finally, dilution rates higher than 0.49 h^{-1} resulted in a significant decrease in cell concentration in the fermenter and in enzyme activity, indicating that cell washout was being approached.

With respect to the calculation of $\tilde{x}_{EXP.}$ values, is should be noted that the OD_{590} values given in Table 9.1 and which represent the steady-state cell concentration in the fermenter at the various dilution rates employed were converted to cell dry weight (g/l) using a calibration curve drawn up for OD_{590} values against cell dry weight (g/l).

It must also be mentioned that the procedure for the calculation of

$\tilde{x}_{CALC.}$, i.e. the theoretical values of steady-state cell concentrations expressed as g/l dry matter, using the chemostat theory was as follows.

The $Y_{X/S}$ was determined using the data given in Table 9.2 and found to be 0.1g cell dry matter/g sucrose consumed. It is also known from chemostat theory that at "wash-out", the steady-state biomass concentration $\tilde{x} \longrightarrow$ o and the steady-state residual growth-limiting substrate concentration, $\tilde{s} \longrightarrow s_r$

i.e. $\tilde{s} = K_S D/(\mu_m - D) \longrightarrow s_r$ Eqn. 9.1

where s_r = the growth-limiting substrate concentration in the nutrient feed, which as mentioned earlier was 45g/l. From the experimental work reported in this section it is taken that $D_{wash\text{-}out}$ (not μ_m) ~ $0.56h^{-1}$ and consequently from Eqn. 9.1

$$45 \longrightarrow \frac{K_S \times 0.56}{\mu_m - 0.56} \qquad \text{Eqn. 9.2}$$

Subsequently, using D and $\tilde{x}_{EXP.}$ values based in Table 9.1 and solving simultaneously with Eqn. 9.2 leads to μ_m ~ $0.61h^{-1}$ and K_S ~ 4.0 g/l. Inserting these values in Eqn. 2.18 (given in Chapter 2) we have

$$\tilde{x} = 0.1 \left[45 - \left(\frac{4D}{0.61 - D} \right) \right]$$ from which by inserting

the values of D employed, we obtain the values of $\tilde{x}_{CALC.}$ which appear

in Table 9.1.

The validity of this treatment is based on the assumption that the Monod model is applicable to this fermentation, i.e. the various fermentation parameters are as described in section 2.5 although it must be noted that this is not always the case in fermentation processes.

As we can observe from Table 9.1 there is a reasonable match between the predicted and the experimental values of $\tilde{x}$, but at the dilution rate of $0.55h^{-1}$ where $D_{wash\text{-}out}$ is approached the deviation of $\tilde{x}_{CALC.}$ from the $\tilde{x}_{EXP.}$ is more pronounced.

From the data given in Table 9.1 the $Y_{p/x}$ chemostat value was calculated and found to have a mean value of 12470 DSU/g cell dry weight, while in the fed-batch system as reported in Section 8.1 the value of $Y_{p/x}$ fed batch was 49400 DSU/g cell dry matter.

Consequently the ratio $\frac{Y_{p/x} \text{ fed-batch}}{Y_{p/x} \text{ chemostat}} = 3.96$ indicating that the productivity of biomass in terms of enzyme elaboration is almost four times higher in the fed-batch system than in the chemostat. Is is also interesting to note that in the fed-batch system the μ_{max} is lower than the μ_{max} in the chemostat; in the former case it has a value of $0.3h^{-1}$ while, in the latter, a value of $0.61h^{-1}$ derived from theoretical

calculations. In the chemostat the dilution rate where "wash-out" occurs ($D_{wash-out}$) is approximately 0.56h^{-1}.

9.2 CONTINUOUS FERMENTATIONS USING CO_2 TO SPARGE THE FERMENTATION BROTH

During the course of the chemostat studies, research work was carried out by other members of the Biochemical Engineering Research Group to determine the reason for the higher yields of dextransucrase in non-aerated fed-batch fermentations in comparison with aerated ones; this involved the investigation of the effect of sparging the fermentation broth in the fed-batch system with various gases (CO_2, N_2, O_2)[9.3]. In connection with the author's research program it was thought worthwhile to also investigate the effect of sparging the fermentation broth with CO_2, in continuous culture. For this purpose two continuous fermentations, CF-CO_2-R1 and CF-CO_2-R2, sparged with pure CO_2 at a rate of 10 l/h = 0.166 vvm were carried out and are reported in this section.

The results are compared in Table 9.3 and Figure 9.2 with the results of CF-4, a conventional non-aerated continuous fermentation operated under comparable conditions: data for CF-4 are summarized in Table 9.2.

TABLE 9.2

CF-4

Steady State Results

Dilution rate (h^{-1}).	OD_{590} values.	Enzyme activity, (DSU/cm^3)	Residual sucrose, (% w/v).
0.375	0.417	54.2	0.99
0.39	0.408	73.8	-
0.4	0.368	62.5	-
0.41	0.362	60.1	0.96
0.45	0.346	32	1.27

TABLE 9.3

Dilution rate (h^{-1})	0.35		0.375		0.39		0.4		0.41		0.45	
Run No.	A1	B	A1	B	A1	B	A2	B	A2	B	A2	B
OD_{590} values.	0.349	—	0.356	0.417	0.385	0.408	0.375	0.368	0.306	0.362	0.269	0.346
Enzyme activity, (DSU/cm^3).	54.6	—	47.6	54.2	49.13	73.8	50	62.5	46.8	60.1	30.3	32
Residual sucrose, (% w/v).	0.41	—	0.39	0.99	0.36	—	0.38	—	1.06	0.96	1.54	1.27

A: Results of the continuous fermentations purged with CO_2 at a rate of 10 l/h = 0.166 vvm.
A1 ≡ CF - CO_2 - R1, A2 ≡ CF - CO_2 - R2.

B: Results of conventional non-aerated continuous fermentation - CF-4.

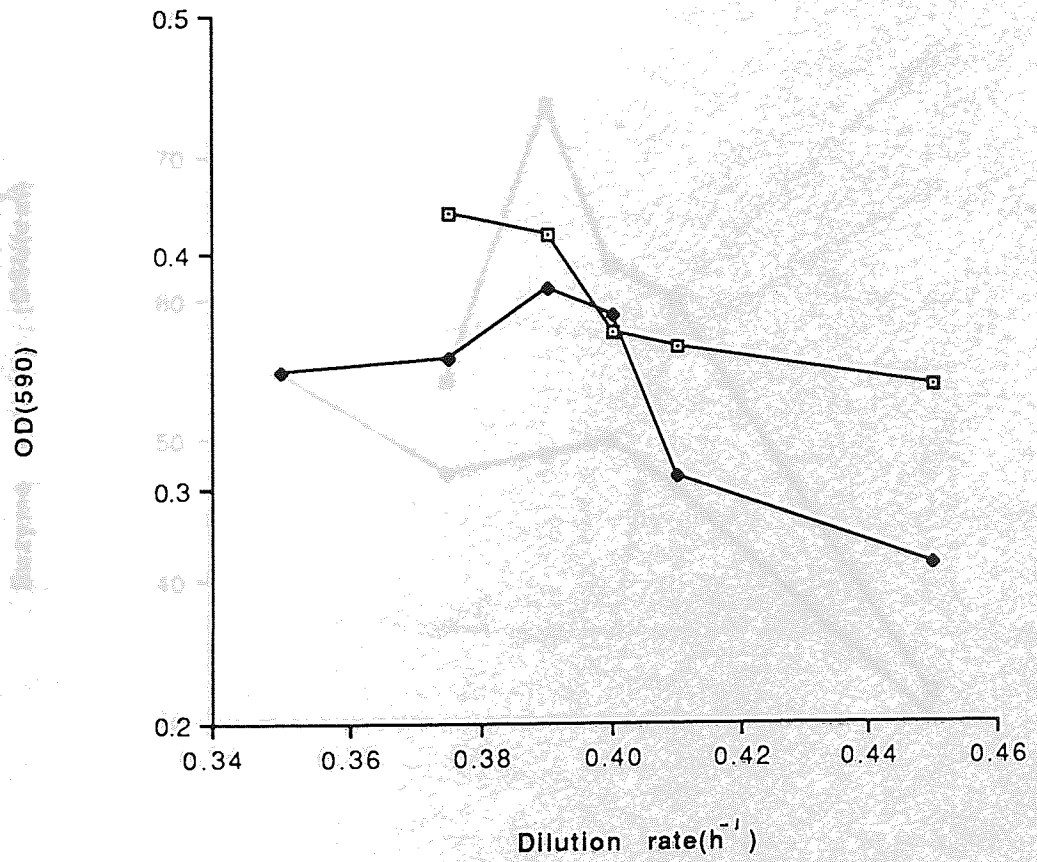


Fig.9.2(a) Comparison of the results of unaerated and CO_2 sparged continuous fermentations. Dilution rate profiles of OD_{590} values. Unaerated (▫), CO_2 sparged (*).

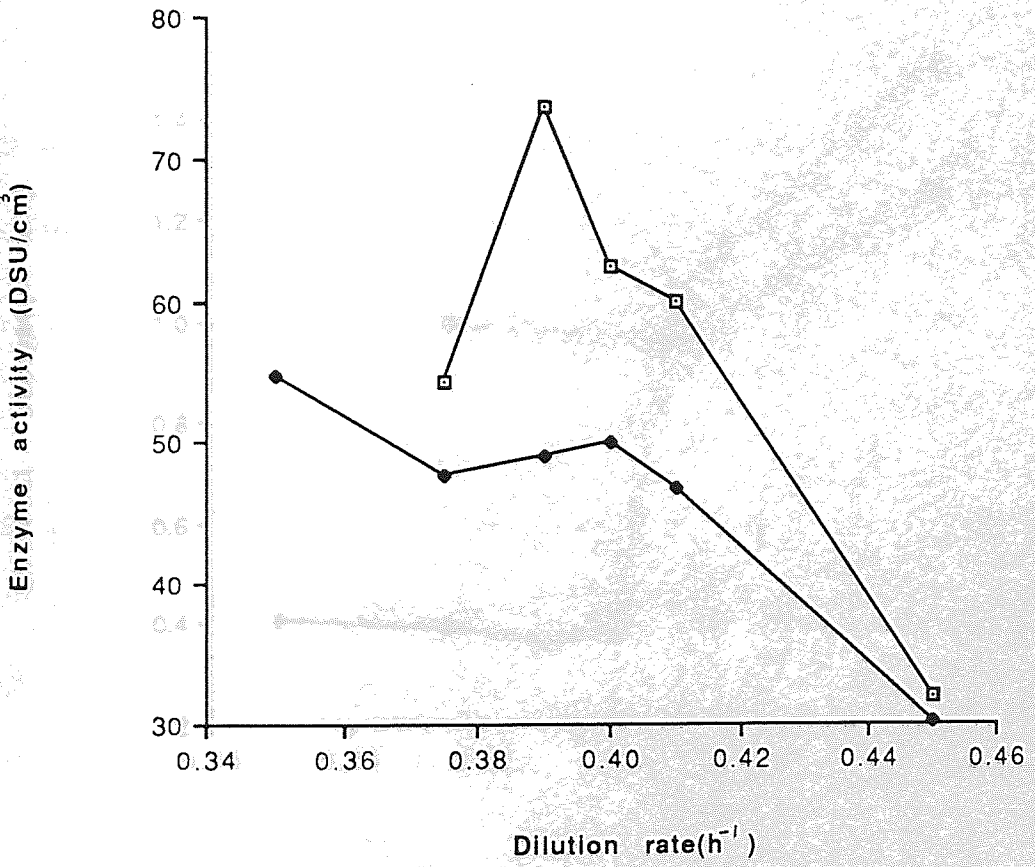


Fig.9.2(b) Comparison of the results of unaerated and CO_2 sparged continuous fermentations.
Dilution rate profiles of enzyme activity.
Unaerated (▫), CO_2 sparged (⁕).

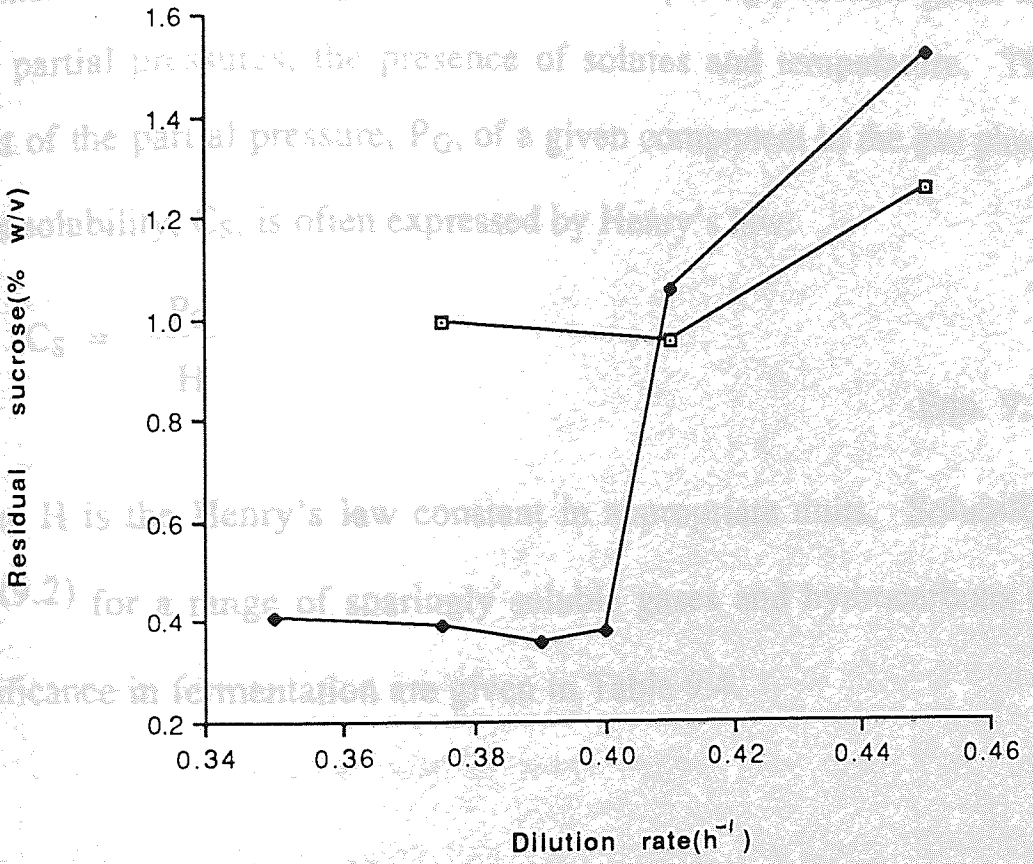


Fig.9.2(c) Comparison of the results of unaerated and CO_2 sparged continuous fermentations.
Dilution rate profiles of residual sucrose concentration. Unaerated (⊡), CO_2 sparged (◈).

Before discussing the results and introducing the conclusions drawn from them, a very brief account concerning some basic aspects of the solubility of gases in aqueous systems is given.

The main factors affecting the solubilities of sparingly soluble gases are their partial pressures, the presence of solutes and temperature. The effect of the partial pressure, P_G, of a given component in the gas phase on its solubility, C_S, is often expressed by Henry's law:

$$C_S = \frac{P_G}{H} \qquad \text{Eqn. 9.3}$$

where H is the Henry's law constant in appropriate units. Solubility data[9.2] for a range of sparingly soluble gases and hydrocarbons of significance in fermentation are given in Table 9.4.

TABLE 9.4

SOLUBILITIES OF GASES IN WATER AT 20°C AND 1 ATM

GAS	SOLUBILITY (mmol dm^{-3})
Butane	6.7
Carbon dioxide	40.0
Ethane	2.1
Methane	1.5
Nitrogen	0.76
Oxygen	1.4
Propane	2.9

From the above Table it can be seen that the solubility of CO_2 in water, at 20°C and 1 atm., is almost 29 times higher than that of 0_2 and approximately 53 times higher than that of N_2. The same Table also shows that the solubility of O_2 in water is 1.8 times higher than that of N_2 under the same conditions of temperature and pressure.

During production of the enzyme dextransucrase using non-aerated batch, fed-batch or continuous fermentations, carbon dioxide evolution is always observed. Furthermore, the very small bubbles observed dispersed throughout the fermentation broth contain carbon dioxide produced by the cells as a result of their metabolic activities. Analysis of the fermenter exit gases during non-aerated continuous fermentations

showed it to contain mainly CO_2. Similar observations have been made about the composition of the fermenter exit gases in non-aerated fed batch fermentations (9.3).

As can be seen from Table 9.3 and Figure 9.2 sparging of the fermentation broth with pure carbon dioxide in the continuous culture experiments, resulted in lower steady state values of growth and enzyme activity.

The fact that a lower growth of the microorganism is obtained in carbon dioxide sparged, continuous fermentations is in accordance with published results (9.3) from fed-batch experiments. The specific growth rate was the same in both cases, but the lower cell concentration in the CO_2 sparged fermentations resulted in lower volumetric cell growth rates, i.e. growth rates per unit volume of the reactor, which account for the lower steady state enzyme activity values obtained.

However, by contrast, sparging with carbon dioxide in fed batch fermentations led to higher maximum values of enzyme activity and lower maximum cell dry weight values in comparison with the maximum enzyme activity and maximum cell dry weight values obtained in non-aerated, fed batch fermentations. A possible reason for this difference is that in the fed-batch fermentations reported in the literature the vessel and gas in the head space were under pressure resulting in increased carbon dioxide solubility in the fermentation

broth. On the contrary, in the continuous fermentations reported in this section, no pressure build up was possible since the fermenter was open to atmosphere. This was due to the fact that in the continuous fermenter employed in this work constant culture volume was achieved by an overflow weir.

CHAPTER 10:

CONTINUOUS FERMENTATIONS WITH TOTAL RECYCLE OF BIOMASS RESULTS AND DISCUSSION

Three total cell recycle fermentation runs, TCRF-R1, TCRF-R2 and TCRF-R3, are reported in this chapter.

The procedure for inoculum preparation was given in section 6.3.2, the experimental apparatus for the total cell recycle experiments was described in section 6.4.2 and the fermentation medium and environmental conditions reported in section 6.4.3.

The agitation rate in these experiments was maintained at the relatively low value of 400 r.p.m. to prevent foam formation of the fermentation broth. Additional mixing in the fermentation system was also provided by the culture recirculation system.

The experiment TCRF-R1 was carried out using an acrylic cross-flow filtration cell, model number FS008K01, manufactured by Filtron Technology Corporation, Massachusetts, U.S.A. and supplied by Flowgen Instruments Ltd., Sittingbourne, Kent, U.K. This cell was not steam sterilizable so chemical means were used for its sterilization.

The sterilization procedure involved the recirculation of a 0.06% w/v distilled water solution of NaN_3 for two hours in the minisette and associated tubing. Subsequently 60 dm^3 of sterile distilled water was passed through the cross-flow filtration module to flush away the

chemical sterilization agent.

The total cell recycle continuous culture experiments were considered to have attained steady state when biomass and residual sucrose concentrations reached constant values.

In these experiments, after inoculation of the fermenter, the microorganism was cultivated batchwise under pH controlled conditions. The minisette was connected to the fermenter and the experiment commenced.

The results of the experiment TCRF-R1 at a 4.5% w/v sucrose concentration in the nutrient feed are given in Table 10.1.

In this experiment the effect of increasing the dilution rate, at constant substrate concentration, on the outcome of the fermentation i.e. enzyme activity and biomass concentration was investigated. Shortly before applying total cell recycle a sample was taken from the batch culture to determine enzyme activity and growth of the microorganism. The results of the analyses were: enzyme activity 55.54 DSU/cm^3 and $OD_{590} = 0.449$.

As can be seen from Table 10.1 high biomass concentrations were

achieved at both the steady state dilution rates used in this experiment. At a dilution rate of 0.05 h^{-1} the biomass steady state concentration had an OD_{590} value of 1.4 while at a dilution rate of D = 0.1 h^{-1} the steady state biomasss concentration had increased to an OD_{590} value of 2.08. As discussed in chapter 4, in a total cell recycle fermentation system, the biomass will accumulate in the bioreactor at a decreasing growth rate until finally no further growth occurs and steady state biomass concentration is reached. Once this occurs the continuous rate of limiting nutrient addition will only meet the maintenance energy requirements of the total concentration of the cells in the fermenter.

TABLE 10.1

STEADY-STATE DATA OF RUN TCRF-R1

Dilution rate (h^{-1}).	OD_{590} values.	Enzyme activity, (DSU/cm^3). fermenter.	permeate.
0.05	1.4	18.45	6.88
0.1	2.08	22.8	4.2

The second steady state was reached 6.5 hrs after setting the value of the second dilution rate. The system was operated continuously for 100 hrs.

Even under these non-growth conditions Leuconostoc Mesenteroides NRRL B-512(F) cells preserved to some extent their fermentation activity with respect to dextransucrase production as can be seen from Table 10.1, which also gives the enzyme activity values at both biomass steady states attained. Under these conditions the cells produced enzyme demonstrating that to some extent dextransucrase production is not strictly linked with cell growth.

Observations, under a microscope, of cell suspensions withdrawn from the fermenter showed that the cells had some morphological differences compared with cells cultivated without cell recycle. With the latter, the observations showed that the physical separation of cells was depressed. This phenomenon caused the formation of cell aggregates.

As can be seen from Table 10.1 and also from Tables 10.2 and 10.3 for the experiments TCRF-R2 and TCRF-R3 respectively, the enzyme transmission through the membrane was low. The reasons for this effect are: 1) the suitability and performance of the membrane employed in this particular application. 2) the effect of process time on membrane performance, 3) the effect of high cell concentrations and 4) dextran formation that could lead to fouling of the membrane. High cell concentrations and especially for prolonged fermentations, as in the case of continuous fermentations, affect permeate flux and in general membrane performance resulting in poor enzyme transmission.

Furthermore, during dextransucrase production there is dextran formation as well as association of the enzyme with dextran, facts which also may contribute to low enzyme transmission through the membrane.

For the three experiments reported in this chapter, at each biomass concentration steady-state sucrose utilization was complete.

In experiment TCRF-R2 the effect of the growth limiting substrate concentration in the nutrient feed on the outcome of the fermentation, i.e. its effect on the steady-state biomass concentration and enzyme activity, was investigated. This experiment was run at a dilution rate of 0.06 h^{-1}. In the first phase of the experiment which lasted 52 hrs the sucrose concentration in the feed was 4.5% w/v. The steady-state data of this phase was obtained and then the fermentation was run at the same dilution rate for another 43 hrs but with 6% w/v sucrose concentration in the nutrient feed. The steady-state results of this experiment are given in Table 10.2.

Before applying total cell recycle to the pH controlled batch culture a sample was taken for enzyme activity determination and OD_{590} measurements. The results of these analyses were: OD_{590} = 0.448 with respect to growth of the microorganism and 52.8 DSU/cm^3 with respect to enzyme activity.

As can be seen from Table 10.2 in both phases of the experiment high steady-state biomass concentrations were achieved. Increased feed substrate concentration resulted in a higher level of steady-state biomass concentration. As in the case with the TCRF-R1 experiment treated earlier and TCRF-R3 which follows, even at these non-growth, total cell recycle, steady-state conditions the cells produced enzyme indicating to some extent that dextransucrase is not strictly a growth associated product.

As can also be seen from the same Table a decrease in enzyme transmission through the membrane, at these high cell concentrations, was obtained. Deterioration of enzyme transmission was more pronounced in the second phase of the experiment due to higher cell concentrations and prolonged fermentation time at these high biomass levels causing gel formation and concentration polarization effects on the membrane surface.

It was observed, from samples taken from the fermenter and examined under a microscope, that the cells did not separate from each other as when cultivated without cell recycle but formed aggregates. This effect was also observed in the TCRF-R3 fermentation experiment.

As can be seen from the experiments reported in this chapter (see

Tables 10.1, 10.2, 10.3) the responses of the whole system to changes in substrate feed concentration, S_o, and dilution rate were very sensitive. An increase in these parameters resulted in a rise in biomass concentration until a new steady-state was reached.

The duration of the continuous operation of the fermentation experiment TCRF-R2 was 95 hrs.

TABLE 10.2

STEADY-STATE DATA FOR RUN TCRF-R2

First Phase

4.5% w/v sucrose concentration in the feed.

Dilution rate (h^{-1}).	OD_{590} values.	Enzyme activity, (DSU/cm^3). fermenter.	permeate.
0.06	1.63	20.1	13.55

Second Phase

6% w/v sucrose concentration in the feed at constant feed rate.

Dilution rate (h^{-1}).	OD_{590} values.	Enzyme activity, (DSU/cm^3). fermenter.	permeate.
0.06	1.9	22.5	5.88

In run TCRF-R3 dilution rates higher than those used in TCRF-R1 were employed. Sucrose concentrations in the feed were the same in both these experiments (S_0 = 4.5% w/v). As is evident from Tables 10.1 and 10.3, higher dilution rates resulted in higher biomass steady state concentrations.

With respect to product formation at biomass steady state, enzyme transmission through the membrane and cell physiology in this total cell recycle fermentation system, the same comments made for runs TCRF-R1 and TCRF-R2 are applicable and verified in this third total cell recycle fermentation experiment.

TABLE 10.3

STEADY-STATE DATA OF RUN TCRF-R3

Dilution rate (h^{-1}).	OD_{590} values.	Enzyme activity, fermenter.	(DSU/cm^3). permeate.
0.09	1.81	24	18
0.14	2.30	26.85	10

The total duration of the continuous operation of the fermentation system was 122 hrs. The length of the experiment at the first dilution rate was 62 hrs while the fermentation was run at the second dilution rate for 60 hrs.

In each of the total cell recycle experiments up to two dilution rates were employed.

The employment of a higher number of dilution rates per experiment, was limited by the performance of the filtration unit, in terms of permeate flux rates, at high cell concentrations. A considerable decrease in permeate flux rates with process time is common with microfiltration membranes owing to membrane fouling and polarization effects.

High values of dilution rate are also limited by the permeate flux rates of the cross-flow filtration cell.

Using the data given in Tables 10.1, 10.2 and 10.3 the value of the yield coefficient $Y_{p/s}$ was calculated and found to be ~ 230 DSU/g sucrose consumed. Using also the data of Table 9.2 (Section 9.2) the value of $Y_{p/s}$ in the chemostat system was found to be $Y_{p/s\ chemostat} = 1450$ DSU/g sucrose consumed while in the fed-batch system, as reported in

Section 8.1, $Y_{p/s}$ fed-batch = 4840 DSU/g sucrose consumed. Comparing the values of the yield coefficient $Y_{p/s}$ in these three fermentation systems we observe that in the fed-batch system more enzyme is elaborated per g sucrose consumed, indicating a more efficient fermentation in terms of substrate utilization. On the contrary, the value of the $Y_{p/s}$ in the total cell recycle system is very low, in particular the lowest in these three fermentation systems. This is due to the fact that in the total cell recycle system at steady-state, the growth rate of the biomass is as discussed earlier in this chapter approximately zero ($\mu = 0$) and since dextransucrase is mainly a growth-associated product little enzyme is produced.

Finally, the results of the total cell recycle fermentation experiments are summarized in Table 10.4.

TABLE 10.4

Summary of the Results[1] of the Total Cell Recycle Fermentation Experiments

Dilution rate, D (h^{-1})	OD_{590} values	Enzyme activity (DSU/cm^3) Fermenter.	Permeate.	Run No.
0.05	1.4	18.45	6.88	TCRF-R1
0.06	1.63	20.1	13.55	TCRF-R2
	1.9[2]	22.5[2]	5.88[2]	TCRF-R2
0.09	1.81	24	18	TCRF-R3
0.1	2.08	22.8	4.2	TCRF-R1
0.14	2.30	26.85	10	TCRF-R3

(1): Obtained with 4.5% w/v sucrose in the nutrient feed.

(2): Data obtained from the phase of the TCRF-R2 experiment which was run with 6% w/v sucrose in the nutrient feed.

CHAPTER 11:

PARTIAL CELL RECYCLE EXPERIMENTS

RESULTS AND DISCUSSION

Two partial cell recycle fermentation experiments PCRF-R1, PCRF-R2 are reported in this chapter. The results and the behaviour of the system together with the problems encountered with the membrane and the cross-flow filtration unit employed are also discussed.

The procedure for inoculum preparation is given in section 6.3.2 and the description of the experimental apparatus in section 6.5. The fermentation medium and environmental factors are reported in section 6.4.3.

In these experiments an autoclavable 0-6 bar Anderson pressure gauge, (supplied by Tanshire Equipment Ltd, Surrey, England), with a 1/2 inch triclover sanitary diaphragm was mounted on the stainless steel cross flow filtration cell by means of a suitable fitting.

Agitation in these experiments was controlled at the relatively low value of 500 r.p.m. which ensured good mixing of the fermentation broth and at the same time prevented foaming of the culture. The recirculation of the culture also provided additional mixing of the components in the fermentation vessel.

Partial cell recycle was applied to a sucrose excess steady-state chemostat culture operated at a dilution rate of 0.29 h^{-1}.

EXPERIMENT PCRF-R1

In the PCRF-R1 experiment the sucrose concentration in the feed was 4.5% w/v and the steady-state data obtained from the conventional chemostat is given in Table 11.1.

TABLE 11.1

PCRF-R1

RESULTS OF CONVENTIONAL CHEMOSTAT

Dilution rate, D	= 0.29 h^{-1}
OD_{590}	= 0.407
enzyme activity	= 41.27 DSU/cm^3
residual sucrose	= 0.9% w/v.

When the conventional chemostat reached a steady state, cell viability was determined and found to be 64.5%.

The operating conditions of the partial cell recycle phase of the experiment PCRF-R1 were 0.29 h^{-1} with respect to dilution rate and R=0.5 with respect to recycle ratio.

The initial mean residence time the culture spends outside the fermentation vessel, in the recycle loop, was calculated and found to be 4 seconds. This determination was carried out with water before the run began. The pump and the pump speed employed for this determination was the same as in the partial cell recycle phase of run PCRF-R1.

The cross-flow rate under actual fermentation conditions decreases considerably with process time, as further determinations demonstrated, which were carried out after the fermentation terminated. The performance of the membrane and filtration unit in general and its consequent suitability for cell recycle fermentation , and especially for cell recycle dextransucrase fermentation, will be discussed in detail later in this chapter.

In the conventional chemostat the volume of the broth at the agitation rate of 500 r.p.m. was 1036 cm^3. The hold-up of the recycle loop was found to be 165 cm^3 and consequently the total culture volume in the partial cell recycle fermentation experiments was (1036 + 165) cm^3 = 1201 cm^3.

Table 11.2 gives the data obtained from the fermentation run PCRF-R1 after switching to partial cell recycle operation. The length of this phase was 87 h.

TABLE 11.2

PCRF-R1

RESULTS OF PARTIAL CELL RECYCLE PHASE

Total Fermentation time measured from the start of the conventional continuous fermentation (h)	Fermentation time under partial cell recycle conditions (h)	Enzyme Activity, DSU/cm^3		Growth (OD_{590} Values)	Sucrose analyses, results, % w/v	
		Permeate	Bleed of cells (Fermenter)		Permeate	Bleed of cells (Fermenter)
175.0	20.5				0.43	0.56
175.5	21.0	51.3				
177.0	22.5		49.9			
178.0	23.5					
191.5	37.0					
195.5	41.0				0.233	0.502
196.5	42.0			0.381		
198.0	43.5	49.5				
200.0	45.5		49.16			
217.5	63.0			0.403		
220.5	66.0					
223.5	69.0	26.35				
225.0	70.5				0.486	0.498
225.5	71.0		26.6			
226.5	72.0			0.402		
241.5	87.0	25.7	27.1	0.4	0.32	0.46

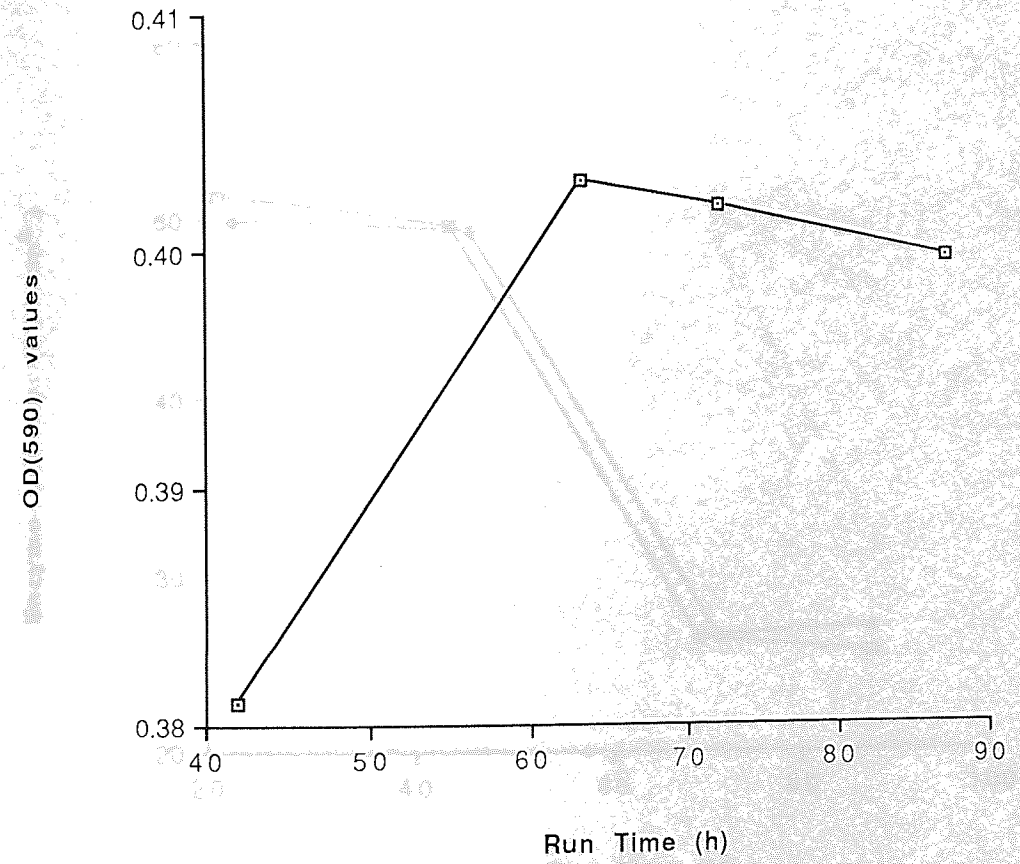


Fig. 11.1 (a) Results of the partial cell recycle phase of run PCRF-R1.
OD_{590} values profile.

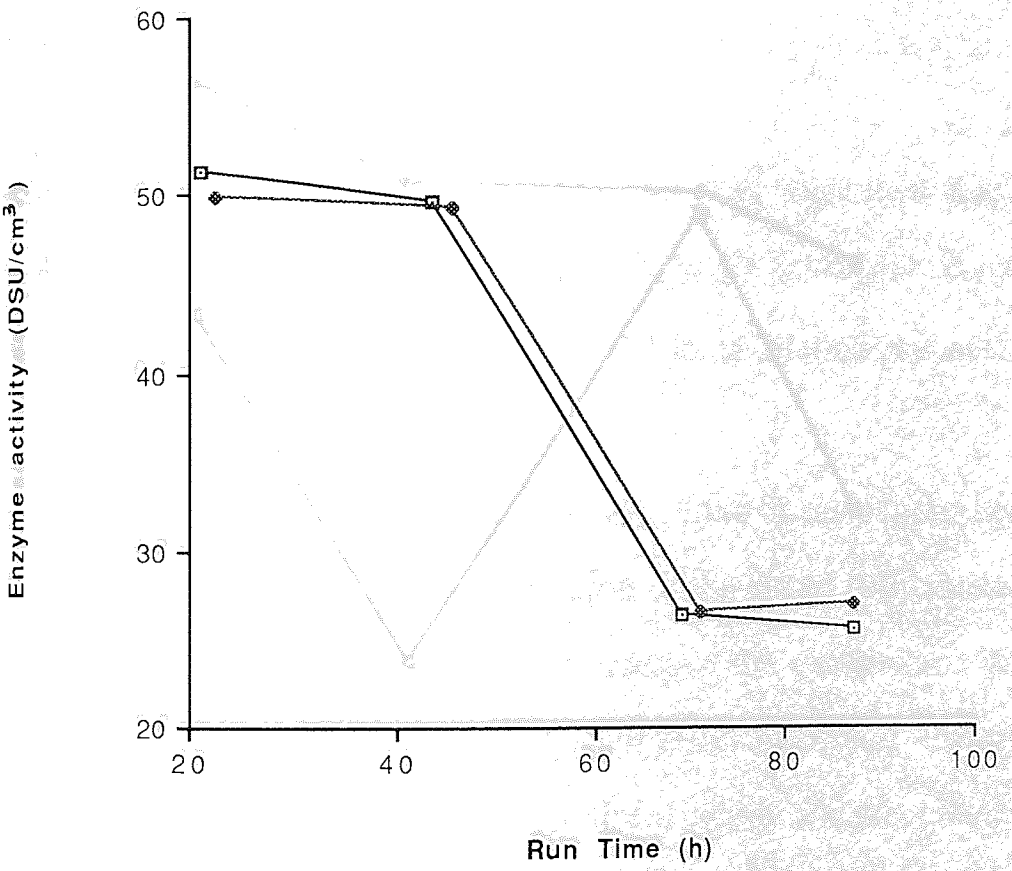


Fig. 11.1 (b) Results of the partial cell recycle phase of run PCRF-R1.
Enzyme activity profiles. Permeate (⊡), Fermenter (⋇).

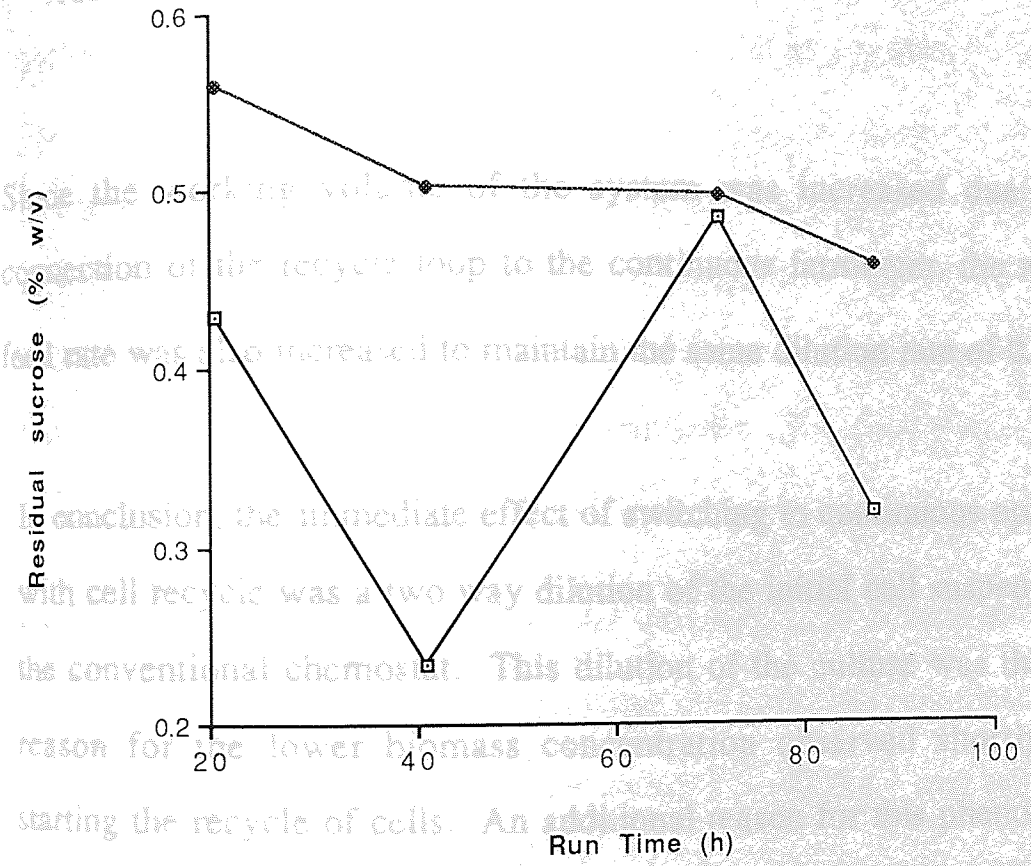


Fig. 11.1 (c) **Results of the partial cell recycle phase of run PCRF-R1.**
Residual sucrose concentration profiles.
Permeate (▫), Fermenter (⁕).

The hold-up of the recycle loop was 165 cm^3; consequently, when partial cell recycle was applied to the sucrose excess steady-state chemostat, there was a dilution effect experienced by the culture of a factor of

$$\frac{1036}{1036 + 165} = \frac{1036}{1201}$$

Since the working volume of the system was increased due to the connection of the recycle loop to the continuous fermenter, the nutrient feed rate was also increased to maintain the same dilution rate of 0.29 h^{-1}.

In conclusion, the immediate effect of switching to continuous operation with cell recycle was a two way dilution of the initial cell suspension of the conventional chemostat. This dilution of the culture was the main reason for the lower biomass concentration observed shortly after starting the recycle of cells. An additional reason for this phenomenon was a to an extent biomass deposition within the cassette membrane resulting in less cells being in suspension in the culture fluid in the fermenter, thus causing lower OD_{590} values from samples withdrawn from this vessel.

As can be seen from Table 11.2 and also from Figure 11.1 biomass reached a constant level of an OD_{590} value of 0.4 and there was not any further increase in cell concentration in the fermentation system.

A recycle ratio of R = 0.5 was employed and the concentrating effect experienced by the culture should have been 1/[1-R], i.e. a two-fold increase in the biomass concentration over that of the conventional chemostat. This unfortunately did not happen because of the poor performance of the membrane and filtration unit as a whole.

Observations of the recycle loop during the fermentation showed that cross-flow rates declined rapidly. The cross-flow rate of the micro-filtration unit was measured at the end of the run using as feedstock the fermentation broth which was in the fermenter. The determination gave a very low value of 0.08 dm^3 min^{-1} which is very low compared with the optimum cross flow rate of 3 - 3.5 dm^3 min^{-1} recommended by the manufacturer. This is especially so when one considers that these values are optimum for processing volumes of 1 - 20 dm^3 batches which typically take 6 - 10 min. Obviously for cell recycle continuous fermentations which last many days higher cross flow rates must be used to minimize biomass deposition inside the membrane and polarization effects.

During continuous fermentation with partial cell recycle, it was also noticed that the pressure in the feed line of the cross-flow filtration unit

was very high and reached the maximum output pressure of the broth recirculation peristaltic pump, (model 603 S/R, Watson and Marlow, Falmouth, Cornwall, U.K.), which is ~ 2 bar. The fact that the pump was running at its maximum output pressure together with the fact the cross flow rate was very low demonstrated that biomass deposition between the layers of the membrane was severe; the pump did not have the capacity in terms of output pressure to cause this material to be swept along with the culture fluid and thereby enable high cross flow rates to be attained.

A number of different type of pumps were considered for this part of the project. A suitable pump for continuous cell recycle fermentation should meet a number of requirements. Among these are that the pumphead should be steam sterilizable and in particular autoclavable. One should also be able to maintain aseptic conditions during operation eliminating any risk of contamination of the culture due to its recirculation through the pumphead. This requirement necessitates the pumphead to be detachable so as to be able to be disconnected from the pump main body and sterilized separately in an autoclave.

During the operation of the pump there must not be heat evolution in the pumphead since it would result in destruction or damage to the cells and denaturing of dextransucrase, which occurs at temperatures above 30° C. Contact of the culture fluid with hot surfaces of the fermentation

system must also be avoided for the same reasons.

A suitable pump for broth recirculation should not have any mechanical impact on the cells, as for example when using gear pumps. Elevated temperatures are also common with gear pumps during operation.

Another aspect which must be considered in selecting a suitable pump for cell recycle fermentation is that of the shear generated within the pumphead. This must not be very high because it would cause destruction or damage to the microorganisms.

Another requirement for the broth recirculation pump to meet, for cell recycle fermentation, is that it must provide a high output pressure and high cross-flow rates, hence minimizing biomass deposition in the filtration unit and thus enabling the membrane to perform satisfactorily throughout an entire continuous fermentation experiment.

As discussed in section 6.5, a peristaltic pump was used for broth recirculation in the cell recycle experiments reported in this thesis. Peristaltic pumps meet all the requirements for cell recycle fermentations apart from the fact that they have low maximum output pressures. In the case of the 603 S/R Watson and Marlow peristaltic pump used for partial cell recycle experiments, the maximum output pressure was ~ 2 bar.

As discussed earlier in this chapter, during partial cell recycle experiments biomass deposited firmly between the layers of the membrane and could not be removed by the action of the pump due to its relatively low maximum output pressure. The pumphead was rotating at its set speed, without being able to circulate substantially the fermentation broth in the membrane and consequently the recycle loop did not increase the cell concentration in the fermentation system.

Intensive cleaning of the membrane after the end of a fermentation run did not increase substantially the cross flow rate, indicating that the deposited biomass could not be washed away from the membrane by the action of the peristaltic pump because of its low output pressure.

In the PCRF-R1 fermentation experiment, as can be seen from Table 11.2 and Figure 11.1, there was good enzyme transmission through the membrane in contrast to the poor enzyme transmission in the total cell recycle continuous fermentation runs. This difference was due to the high cell concentrations experienced in total cell recycle experiments, while in the PCRF-R1 continuous partial cell recycle run the cell concentration was much lower, having an OD_{590} value of 0.4.

It is also evident from the same Table and Figure that there was a decline in enzyme activity with process time in both permeate and the "cell-bleed" stream. This was due to prolonged foam formation and

also because of "whipping" of the fermentation broth due to high pressure build-up in the filtration cell, which caused the formation of a jet returning the culture fluid back to the reactor.

In Table 11.2 and Figure 11.1 it is also shown that the values of the residual sucrose concentration in the permeate product stream were lower than those in the fermenter or "cell-bleed" stream. This was due to further sucrose consumption by the cells because of the significant residence time they spent in the recycle loop. For the determination of the residence time in the recycle loop, which was found to be 2.06 min, the value of the cross-flow rate employed was 0.08 dm^3 min^{-1}. This value was measured at the end of the run using as feedstock the whole broth which was in the fermenter vessel. It was not possible to measure cross flow rates during the experiments because of the lack of an autoclavable flow-meter incorporated in the feed line of the cross-flow filtration unit. Cross-flow rates, as has already been mentioned, were determined before a run began using water and at the end of a run using as feedstock the fermentation broth which was in the bioreactor vessel.

Since the fermentation broth did not recirculate in the filtration unit it was decided to terminate the experiment after 87 hrs under partial, (R=0.5), cell recycle conditions. Subsequent intensive cleaning of the membrane did not improve cross-flow rates. It was therefore decided to purchase a new membrane, to be employed for another partial cell recycle fermentation run.

EXPERIMENT PCRF-R2

The experiment PCRF-R2 consisted of two parts, as with the PCRF-R1 run. The first part was a conventional continuous fermentation experiment with a dilution rate of $D=0.29\ h^{-1}$. In the second part, partial cell recycle was applied.

In this experiment the sucrose concentration in the feed and also in the initial medium in the fermenter was 6% w/v. Table 11.3 gives the details of the fermentation medium and environmental conditions.

TABLE 11.3

RUN PCRF-R2 - CONVENTIONAL CHEMOSTAT. FERMENTATION MEDIUM AND ENVIRONMENTAL CONDITIONS.

sucrose	6% w/v
yeast extract	4% w/v
KH_2PO_4	2% w/v
R* salts	0.5 v/v
Temperature controlled at	23.4° C
pH controlled at	6.65 (5M NaOH)
Agitation controlled at	500 r.p.m.

The steady-state data obtained from this first part of the experiment is given in Table 11.4.

TABLE 11.4

PCRF-R2

RESULTS OF CONVENTIONAL CHEMOSTAT

Dilution rate	= 0.29 h^{-1}.
Enzyme activity	= 80.65 DSU/cm^3.
OD_{590}	= 0.504.
Residual sucrose	= 0.6% w/v.

The duration of this part of the experiment PCRF-R2 was 128 hrs. Then partial cell recycle was applied, the operating conditions of which were, R=0.5 with respect to the recycle ratio and D=0.29 h^{-1} with regards to the dilution rate.

Table 11.5 gives the data obtained from the partial cell recycle part of the PCRF-R2 experiment and in particular the values of enzyme activity, growth of the microorganism (OD_{590} values) and background sucrose concentration. The total duration of this part of the experiment was 241.5 hrs while the total duration of the PCRF-R2 fermentation was 370 hrs.

TABLE 11.5

PCRF-R2

RESULTS OF PARTIAL CELL RECYCLE PHASE

Fermentation time under partial cell recycle conditions (h)	Enzyme Activity, DSU/cm3		Growth (OD_{560} values)	Sucrose analysis results, % w/v	
	Bleed of cells	Permeate		Bleed of cells	Permeate
14.5				0.43	0.27
18				0.39	
19					0.32
21.5			0.441		
22	40				
23.5		43.1		0.46	0.36
40.5			0.464		
42				0.51	0.48
44			0.455		
45.5	36.2				
47		44.66			
48.5	38				
66			0.444		
71.5			0.421	1.83	1.65
73.5	32.7				
74.5		48.6			
88.5				2.18	1.4
94.5	33.4				
96.5					
97.5	74.2	33.46			
112	58.24	58.2		3.67	
113			0.399		
137.5	63.2	59		4.3	3.87
140			0.348		
161			0.176		
162			0.173		
168.5			0.296		
169.5	33	33		4.89	4.81
187			0.457		
193.5	70.8	66.3	0.422	2.55	2.34
210.5					
211.5		49.3			
215.5			0.45		
217	40				
218.5		41		2.9	2.66
237.5			0.453		
239	27.7	27			
240				2.85	
240.5					1.95
241.5			0.452		

Sucrose concentration in the feed, w/v: 6% ↑ / 8% ↓ (6% ↑ / 8% ↓)

Agitation rate: 500 rpm ↑ / 600 rpm ↓ (500 rpm ↑ / 600 rpm ↓)

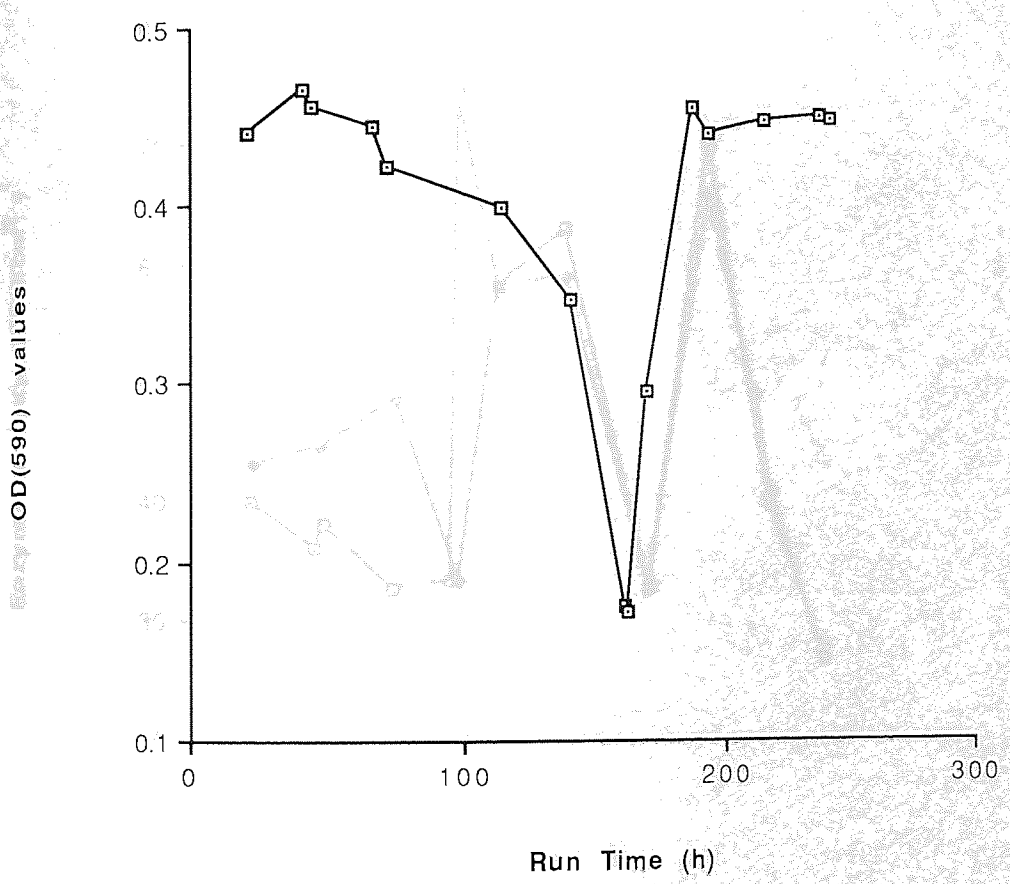


Fig. 11.2 (a) Results of the partial cell recycle phase of run PCRF-R2.
OD_{590} values profile.
Fermenter (●).

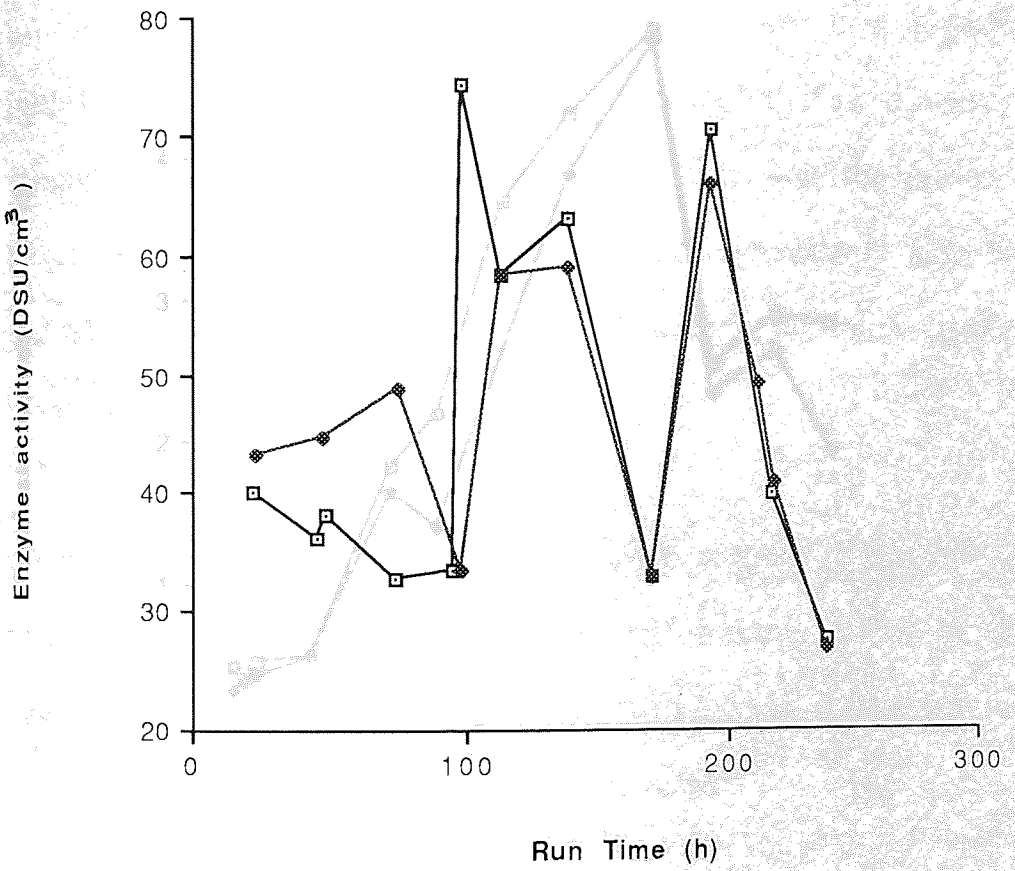


Fig. 11.2 (b) Results of the partial cell recycle phase of run PCRF-R2.
Enzyme activity profiles. Permeate (⁕), Fermenter (⊡).

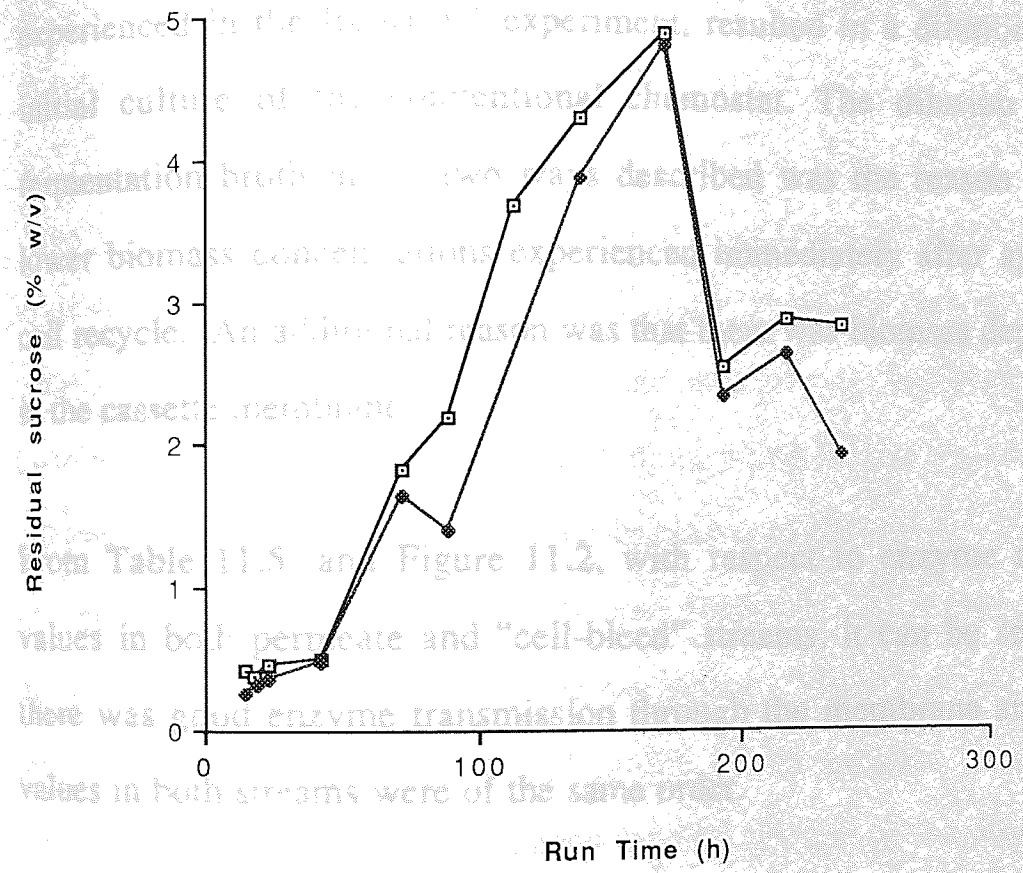


Fig. 11.2 (c) Results of the partial cell recycle phase of run PCRF-R2.
Residual sucrose concentration profiles.
Permeate (◈), Fermenter (⊡).

After applying cell recycle to the sucrose excess steady-state chemostat of the first part of the run the working volume of the fermentation system was increased due to the connection of the recycle loop to the continuous fermenter. The nutrient medium feed rate was increased to maintain the same dilution rate of 0.29 h^{-1}. These two effects, already experienced in the RCRF-R1 experiment, resulted in a dilution of the initial culture of the conventional chemostat. The dilution of the fermentation broth in the two ways described was the reason for the lower biomass concentrations experienced immediately after applying cell recycle. An additional reason was that there was biomass deposition in the cassette membrane.

From Table 11.5 and Figure 11.2, with respect to enzyme activity values in both permeate and "cell-bleed" streams, it can be seen that there was good enzyme transmission through the membrane since the values in both streams were of the same order.

High enzyme transmission was also observed in the PCRF-R1 fermentation experiment reported earlier in this chapter. The high enzyme flux in both partial cell recycle fermentations was in contrast with poor enzyme transmission observed in the continuous total cell recycle runs reported in chapter 10. The only reason for this phenomenon was the effect of the high cell concentrations encountered in the total cell recycle fermentation experiments.

During the second part of run PCRF-R2 there was foaming of the fermentation broth. Foaming became more intense after 8 days or approximately 195 hrs of continuous partial cell recycle operation, due to high pressure build-up in the cross-flow filtration cell causing "whipping" of the culture in the same way as in run PCRF-R1. The intense foam formation after 195 hrs of operation correlates with a drop in enzyme activity towards the end of the run.

After 140 hrs of partial cell recycle a drop in biomass concentration was noticed. The degree of mixing was thought to be the reason. It was observed that the incoming nutrient feed was leaving the fermentation zone via the level immersion tube without being mixed well with the culture. The agitation rate was then increased to 600 r.p.m. This was carried out after 170 hrs of partial cell feedback and resulted in an increase in the growth of the microorganism. This increase in the biomass concentration was accompanied by an increase in enzyme activity, which subsequently declined towards the end of the run, as can be seen from Table 11.5 and Figure 11.2, due to foam formation and "whipping" of the fermentation broth.

From sucrose analysis results obtained throughout the experiment it can be seen that in general the values of residual sucrose in the permeate product stream were a little lower than those in the fermenter or "cell-bleed" stream. This was due to further sucrose consumption by the cells because of the significant residence time they had in the recycle loop.

This value of residence time was estimated under conditions described in the presentation and discussion of the PCRF-R1 experiment, given earlier in this chapter, and found to be 2.06 min.

HPLC analyses of samples taken from the fermenter and cell-free product stream during the first 42 hrs of partial cell recycle operation showed a decline in residual sucrose which dropped to about 0.5% w/v. Since high levels of biomass were expected, which unfortunately did not occur, sucrose levels needed to be maintained to meet the growth requirements of the cells without sucrose becoming growth limiting. In addition, the presence of sucrose was needed for inducing enzyme elaboration. It was therefore decided to increase the sucrose concentration in the nutrient feed to 8% w/v. This change was introduced after 42 hrs of partial cell recycle operation.

From Table 11.5 and also from Figure 11.2 it can be seen that the growth of the microorganism reached a maximum level of an OD_{590} value of 0.45. The membrane system did not increase the cell mass although for the recycle ratio of R=0.5 a doubling in biomass concentration should have been observed. The reasons for this phenomenon have already been discussed in run PCRF-R1 and the comments made for that run can also be applied to this second partial cell recycle fermentation experiment. The pumphead of the broth recirculation pump was rotating at its set speed and at its maximum

output pressure without being able to circulate the fermentation broth in the filtration module. Consequently the recycle loop did not increase the biomass levels in the fermentation zone. The reason for this effect was, as already stated in the discussion of PCRF-R1 fermentation experiment, the firm deposition of biomass between the layers of the membrane which could not be removed by the action of the broth recirculation pump due to its low maximum output pressure.

Because of the problems with the cell recycle system, consisting of the broth recirculation pump and membrane and filtration cell, it was decided to abandon the cell recycle fermentation part of the research project and carry out experimental work on "enzyme activity enhancement" that the whole broth exhibits when the microorganism is cultivated further in a batch mode without pH control.

CHAPTER 12:

ENZYME ACTIVITY ENHANCEMENT EXPERIMENTS.

12.1 INTRODUCTION

From previous work of another colleague (12.1) in the same laboratory on the continuous production of dextransucrase and also from continuous fermentations carried out during the course of this project, it had been observed that the broth in the reservoir where the product stream of the continuous fermenter was collected, exhibited enhancement in enzyme activity upon hold-up. No agitation or temperature control was provided to the fermentation broth of this container.

The purpose of this part of the experimental work was to study and to use this effect in a more controlled manner, to increase the enzyme activity of the product stream fermentation broth of a continuous fermenter.

To study this phenomenon in a precise and scientific manner a second fermenter was needed or alternatively a vessel with a number of specific facilities as follows. Among them are that this vessel should provide the fermentation broth with controlled agitation; it should also be suitably equipped to enable accurate pH monitoring of its contents and in addition to be able to maintain aseptic conditions throughout an entire, prolonged experiment. In this vessel the whole broth of the steady state chemostat could be retained to enable further investigations or treatments to be performed.

It was considered that a solution to this problem was to employ the same fully equipped continuous bioreactor, described in section 6.3.1, in a way described later in this section and to split each of these fermentation experiments into two phases.

The first phase was a conventional continuous fermentation which was run until steady state was attained. After obtaining the steady-state data the fermentation system was switched to the second phase.

The second phase was batch operation without pH control cultivation of the cell suspension which was in the continuous fermenter during continuous operation. In this phase the fermentation was allowed to proceed in a controlled environment under aseptic conditions. During this phase controlled agitation was provided to ensure a homogenous environment within the reaction vessel. The temperature was also controlled.

The switching, in practical terms, of the fermentation system to the second phase involved the shut down of the pH control unit and nutrient medium addition by a peristaltic pump. In addition, the product stream line of the continuous fermenter was closed to prevent any air from entering the fermentation vessel and causing contamination.

The fermentation medium and environmental parameters of the continuous fermentations comprising the first phase of these experiments, with the exeption of EAE-R1 run, are given in Table 12.1.

TABLE 12.1

First phase of enzyme activity enhancement experiments.

Fermentation conditions.

a. Fermentation medium.

Sucrose	6% w/v.
Yeast extract	4% w/v.
KH_2PO_4	2% w/v.
R* salts	0.5% v/v.

b. Culturing conditions.

Dilution rate	0.29 h^{-1}.
Temperature controlled at	23.4°C.
pH controlled at	6.65, (5M NaOH).
Agitation controlled at	700 rpm.

The process conditions in the second phase, in all "enzyme activity enhancement" experiments are given in the following Table.

TABLE 12.2

Second phase of enzyme activity enhancement experiments.

Process conditions.

Temperature controlled at 23.4°C

Agitation controlled at 700 rpm.

In some of the "enzyme activity enhancement" experiments a sterile sucrose solution was added to the steady-state fermentation broth, immediately before switching to the second phase. This was performed to give a certain initial sucrose concentration for the second phase of these runs. The purpose of such additions was to increase sucrose levels in the fermentation broth to boost the enzyme activity by providing enough sucrose for the cells for their metabolism and for product induction and formation.

The procedure for the preparation and addition of a sucrose solution has been reported in section 6.6.

12.2 RESULTS AND DISCUSSION OF ENZYME ACTIVITY ENHANCEMENT EXPERIMENTS.

In the first phase, i.e. in the conventional chemostat, of the first enzyme activity enhancement experiment, EAE-R1, the sucrose concentration in the initial medium in the fermenter and also in the nutrient feed was 4.5% w/v. In this fermentation experiment the conventional chemostat comprising the first phase was run at a dilution rate of 0.47 h^{-1}. All the other components of the medium, environmental and operating conditions were the same as those given in Table 12.1.

The purpose of carrying out the EAE-R1 experiment was to verify again that there was enzyme activity enhancement upon "hold up" conditions, which have been described earlier. The steady state results of the first phase are given in Table 12.3.

TABLE 12.3

Run EAE-R1

Steady state results of the first phase.

Dilution rate	0.47 h^{-1}.
OD_{590} value	0.331
Enzyme activity	32.5 DSU/cm^3
Residual sucrose	2.65% w/v.

The duration of this phase of the experiment was 80.5 hours.

After obtaining the steady state data the system was switched to the second phase of the run. Table 12.4 and Figure 12.1 illustrate the changes of pH, enzyme activity, OD_{590} values and residual sucrose in the fermentation broth occurring with hold up time.

The initial enzyme activity for the second phase, at $t = 0$, was 32.5 DSU/cm^3 while the maximum value of enzyme activity obtained under hold up conditions was 55.3 DSU/cm^3. Consequently there was an enhancement of 70.1%.

Towards the end of this part of the run there was a decline in enzyme activity. These activity losses were due to instability of dextransucrase with time at a temperature of 23.4°C, and due to the prevailing low pH values where inhibition of the enzyme takes place. Irreversible denaturation of the enzyme occurs at pH $\leq$ 4.5.

The application of the hold up conditions to the steady state chemostat culture of the first phase was accompanied by a rapid drop in pH which was due to further cell metabolism. Under these conditions the cells consumed the residual sucrose for their growth and product formation. Sucrose is also consumed for dextran synthesis. Since in the culture supernatant there is dextransucrase and sucrose there will be action of the enzyme on sucrose resulting in dextran and fructose formation.

This reaction becomes more pronounced or enhanced due to the drop of pH towards values which are more favorable for this reaction. A pH value of 5.2 is considered to be the optimum for dextran synthesis.

From Table 12.4 and Figure 12.1 (b) it can be seen that there was a decrease in OD_{590} values towards the end of this second phase of the experiment. This decrease demonstrated that cell autolysis occurred. The duration of the second phase of the experiment EAE-R1 was 76 hours.

TABLE 12.4

Run EAE-R1

Results of the second phase.

Time (h)	pH	Enzyme Activity. (DSU/cm^3)	OD_{590} Values	Residual Sucrose (% w/v)
0	6.65	32.53	0.331	2.65
1	6.21			
1.5	5.98			
2	5.56	47.1		
2.5	5.26			
3	5.14			0.576
3.5	5.04	55.34		
4	4.99			0.453
4.5	4.97	54		
5	4.96			
5.5	4.96			
6	4.96			nil
6.5	4.96			
7	4.96	51.6		
7.5	4.95			
8	4.95		0.488	
8.5	4.95			nil
9	4.95	51.65		
9.5	4.94			
10	4.94			
23.5	4.89			
24	4.89			nil
25	4.89	39.32		
27	4.89	37.82		
28.5	4.89		0.46	
55	4.89	33.39		
55.5	4.89		0.434	nil
76	4.89	30.88		

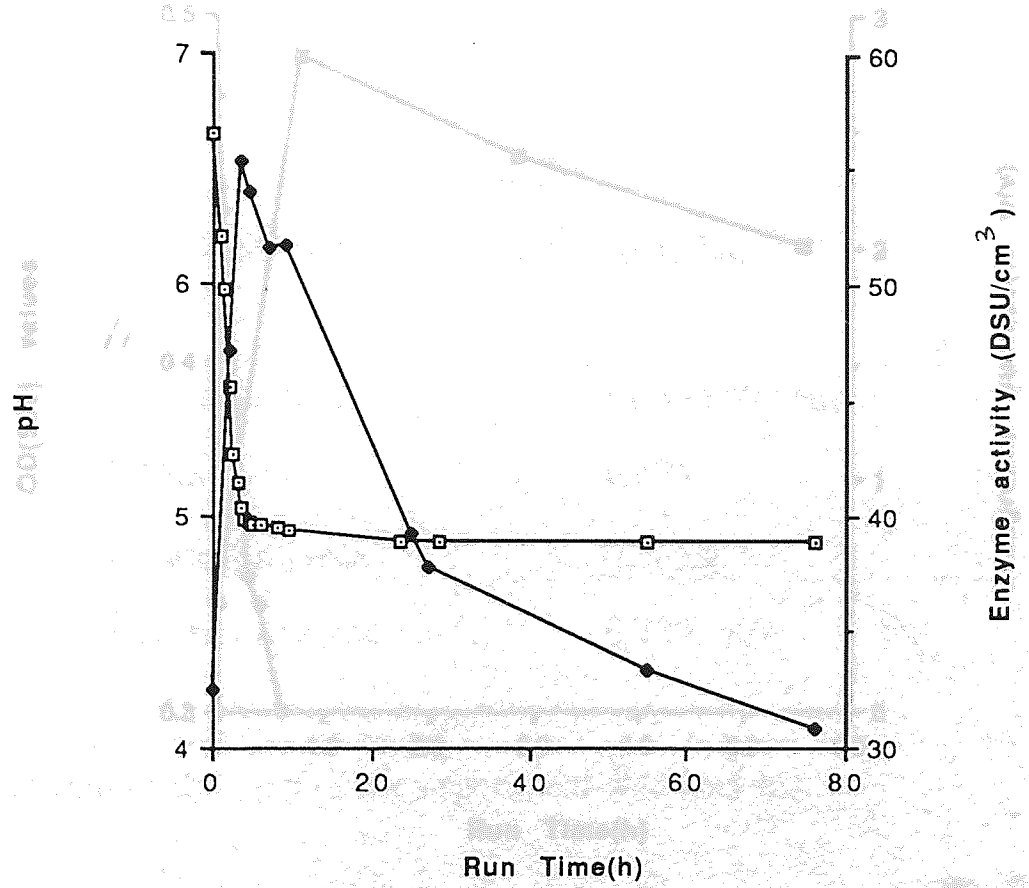


Fig.12.1(a) Results of the second phase of run EAE-R1.
pH (▫) and enzyme activity (◆) profiles.

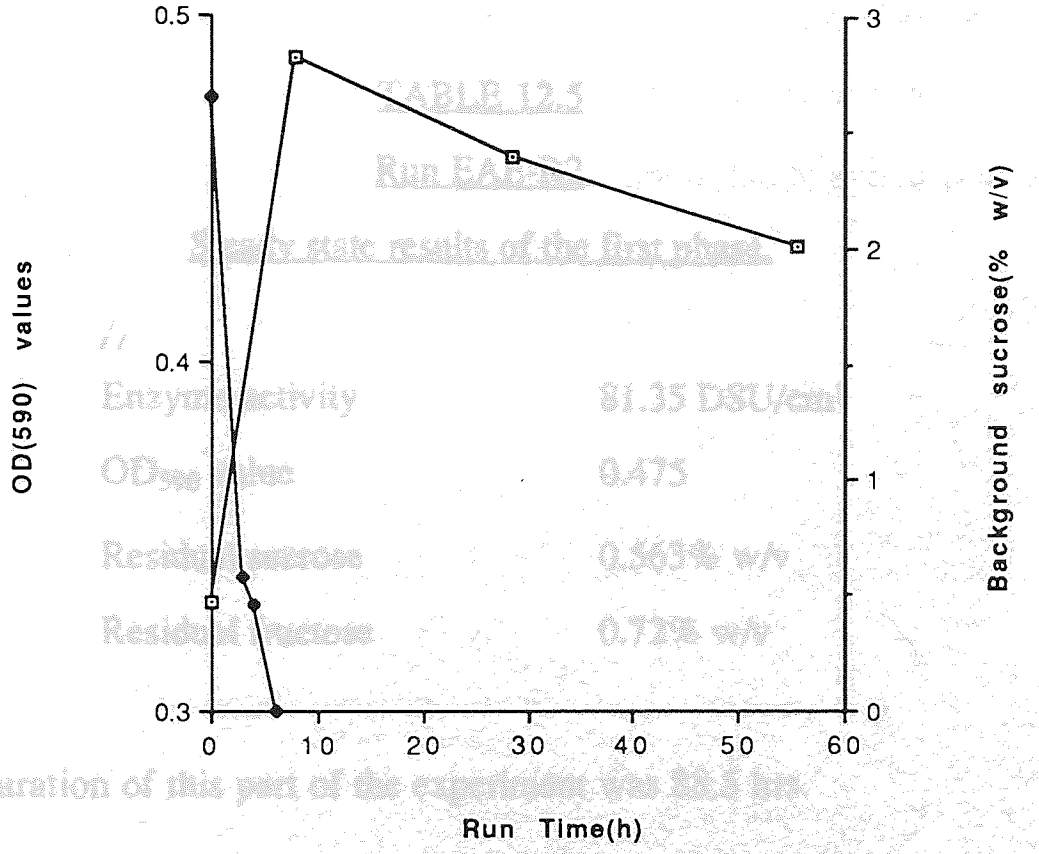


Fig.12.1(b) Results of the second phase of run EAE-R1.
OD_{590} values (▫) and background sucrose concentration (◆) profiles.

RUN EAE-R2

Table 12.5 gives the steady-state data of the first phase of the experiment, i.e. the conventional chemostat run at a dilution rate of 0.29 h^{-1}, as described in section 12.1.

TABLE 12.5

Run EAE-R2

Steady state results of the first phase.

Enzyme activity	81.35 DSU/cm^3
OD_{590} value	0.475
Residual sucrose	0.563% w/v
Residual fructose	0.72% w/v

The duration of this part of the experiment was 88.5 hrs.

The system, subsequently, was switched to the second phase. The results are given in Table 12.6.

Table 12.6 and Figure 12.2 (a) illustrate that the highest value of the enzyme activity reached was 96.4 DSU/cm^3 which is 18.5% higher than the initial value, at t = 0, of the enzyme activity.

Towards the end of this second phase of the experiment there were

enzyme activity losses, as indicated by a decline in enzyme activity values. These must be due to the instability of dextransucrase with time at the temperature of 23.4°C. After 122 hours under hold-up conditions the enzyme activity had dropped to 29.34 DSU/cm^3.

It is also evident from Table 12.6 and Figure 12.2 (b) that there was a decline in OD_{590} values with time which was due to biomass autolysis. Certainly this effect was caused by cell starvation due to exhaustion of all nutrients from the fermentation broth.

TABLE 12.6

Run EAE-R2

Results of the second phase.

Time (h)	pH	Enzyme Activity. (DSU/cm^3)	OD_{590} Values	Residual Sucrose (% w/v)
0	6.65	81.35	0.475	0.563
0.25	6.48			
0.5	6.33			
0.75	6.15			
1	6.01	90.17		0.288
1.5	5.8			
1.75	5.76			
2	5.72			
2.25	5.7			0.11
2.5	5.68	95.8		
3	5.66			
3.5	5.64		0.48	
4	5.62			
4.5	5.62	96.4		0.02
5.5	5.6			
6	5.6			Nil
6.25	5.6	96.28		
21.5	5.52			
22	5.52			
22.5	5.52			
22.75	5.5	78		
23.5	5.5		0.413	
47	5.5	80.27		
48	5.5		0.332	
76	5.5	48.95		
76.5	5.5		0.283	
95	5.5	37.47		
96.5	5.5		0.267	
98.5	5.5	33.34		
122	5.5	29.34		
123	5.5		0.244	

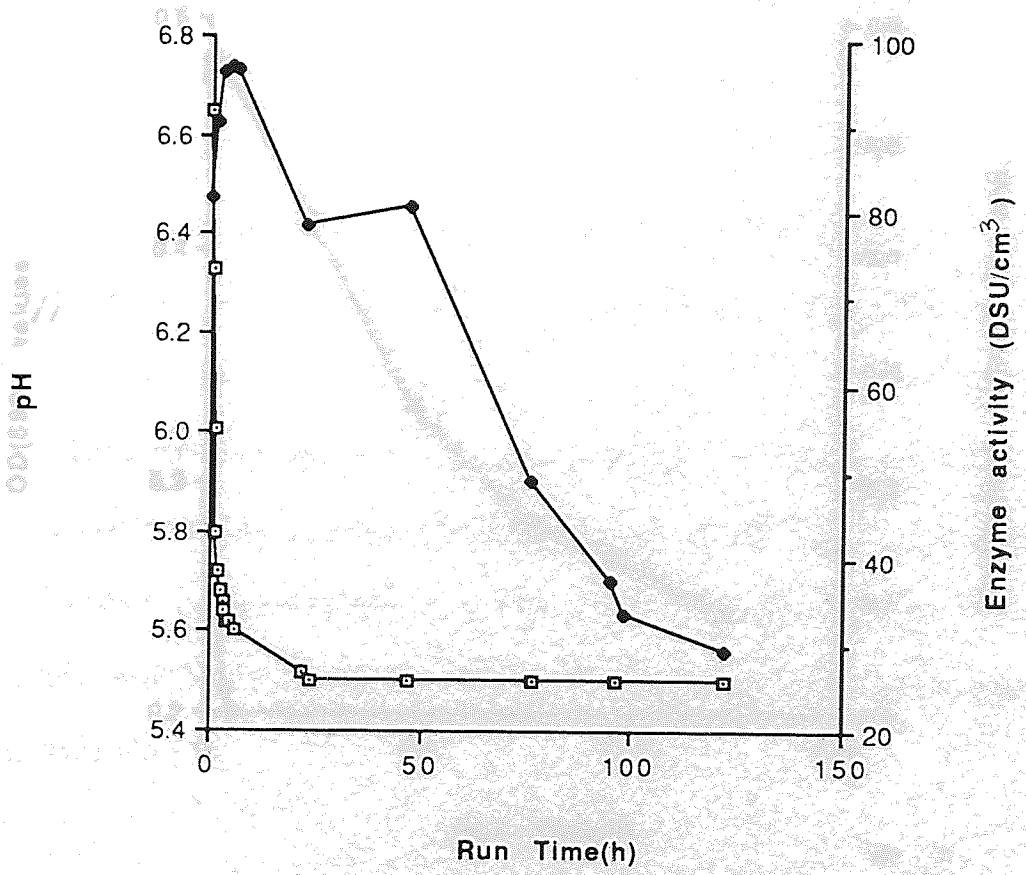


Fig.12.2(a) Results of the second phase of run EAE-R2.
pH (▫) and enzyme activity (◆) profiles.

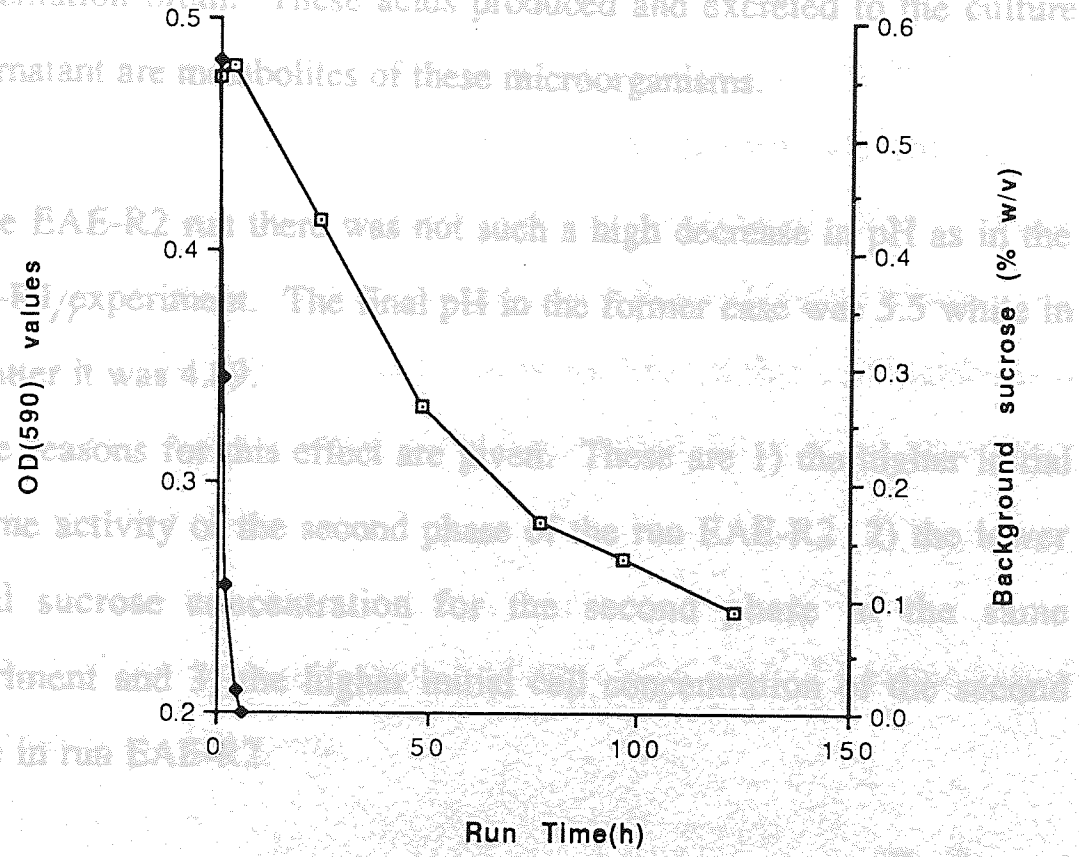


Fig.12.2(b) **Results of the second phase of run EAE-R2.**
OD_{590} values (▫) and background sucrose concentration (◆) profiles.

From the same Table and Figure 12.2 (a) it can be seen that the final pH was 5.5. As mentioned in the discussion of the EAE-R1 the drop in pH is due to cell metabolism which results in acid production. Leuconostoc mesenteroides is one of the lactic acid bacteria which when cultivated produce acids which are responsible for the drop of pH of the fermentation broth. These acids produced and excreted to the culture supernatant are metabolites of these microorganisms.

In the EAE-R2 run there was not such a high decrease in pH as in the EAE-R1 experiment. The final pH in the former case was 5.5 while in the latter it was 4.89.

Three reasons for this effect are given. These are 1) the higher initial enzyme activity of the second phase of the run EAE-R2 2) the lower initial sucrose concentration for the second phase in the same experiment and 3) the higher initial cell concentration of the second phase in run EAE-R2.

Higher initial enzyme activity in the EAE-R2 experiment in comparison to that of the EAE-R1 experiment resulted in a higher sucrose consumption by the enzyme which converted it to dextran and fructose.

In the EAE-R2 run the initial sucrose concentration of the second phase was lower than the corresponding initial sucrose concentration in the EAE-R1 experiment. Consequently sucrose was consumed earlier due to its utilization by the cells and its conversion by the enzyme.

Finally, the higher initial cell concentration in the second phase of the EAE-R2 experiment compared to the corresponding value in the EAE-R1 run resulted in faster consumption of sucrose by the cells for their metabolism.

For these three reasons the sucrose in the second phase in the EAE-R2 run was exhausted earlier from the fermentation broth than the sucrose in the second phase of the EAE-R1 run and consequently did not enable prolonged cell metabolism to take place which would result in obtaining a lower final pH value. It must also be mentioned that cell metabolism takes place as long as the pH of the fermentation broth is not lower than 5 when growth ceases.

The total duration of the second phase of the EAE-R2 fermentation experiment was 123 hours.

Since, in the second phase, sucrose is consumed in its reaction with the enzyme to dextran and fructose and also for cell growth and enzyme elaboration, it was thought desirable to increase the initial sucrose concentration in the second phase so as to provide more substrate for the cells in order to boost the enzyme activity. The presence of sucrose is also needed to induce enzyme elaboration by the microorganism.

RUN EAE-R3

The steady-state data for the first phase of this run is given in Table 12.7.

TABLE 12.7

Run EAE-R3

Steady state results of the first phase.

Enzyme activity	73.1 DSU/cm^3
OD_{590} value	0.450
Residual sucrose	0.523% w/v

The duration of the continuous fermentation comprising the first phase of the EAE-R3 run was 121.5 hrs.

The volume of the fermentation broth in the continuous fermenter, at the agitation rate employed, was 985 cm^3. The residual sucrose concentration at steady state was 0.523% w/v and consequently 100 cm^3 of 27.4% w/v of sterile sucrose solution was needed to be added to the fermentation broth to bring the initial sucrose concentration for the second phase to 3% w/v.

Because of the addition of the sucrose solution, the fermentation broth was diluted by a factor of;

$$\frac{985}{985 + 100} = \frac{985}{1085}$$

Consequently the initial value of the enzyme activity for the second phase was:

$$73.1 \times \frac{985}{1085} = 66.36 \text{ DSU/cm}^3.$$

As can be seen from Table 12.8 the maximum enzyme activity value of the second phase was 93.4 DSU/cm^3, which is 40.7% higher than the initial value of enzyme activity of the same phase. In comparison with the enzyme activity of the steady state chemostat of the first phase the maximum enzyme activity of the second phase was 27.8% higher.

As can also be seen from Table 12.8 and Figure 12.3 (a), there were significant enzyme activity losses with process time although the final pH was nearly optimum for enzyme stability. These enzyme activity losses occured towards the end of the run and after the enzyme activity reached its maximum and must be due to the instability of dextransucrase with time at the temperature of 23.4°C.

In all enzyme activity enhancement experiments reported in this chapter, at the early stages of the second phase there was an increase in OD_{590} values. Subsequently a decrease in OD_{590} values was observed, especially towards the end of the second phase, indicating biomass autolysis. This was a consequence of cell starvation due to the exhaustion of nutrients from the fermentation broth.

With respect to the change of pH with time and the final pH in this phase and the factors which influence it, the same comments made in the discussion of EAE-R2 run are applicable to this experiment as well as to the other enzyme activity enhancement experiments reported in this thesis.

In all experiments reported in this chapter, the behaviour and outcome of the fermentation system of the second phase depends on the combined effect of the following three parameters: initial enzyme activity, initial sucrose concentration and initial cell concentration. The changes with process time of pH, enzyme activity, residual sucrose concentration and OD_{590} values are all interdependent.

TABLE 12.8

Run EAE-R3

Results of the second phase.

Time (h)	pH	Enzyme Activity. (DSU/cm^3)	OD_{590} Values	Residual Sucrose/ Fructose (% w/v)
0	6.65	66.36		3 / –
0.25	6.57			
0.5	6.38			
0.75	6.16			
1	5.93	73.8		
1.25	5.7			
1.5	5.63			
1.75	5.58			2.1 / 1.81
2	5.52			
2.5	5.45			
2.75	5.4	81.76		
3	5.4			
3.5	5.36	87.6		
3.75				1.45 / –
4	5.32			
4.5	5.31		0.520	
5	5.3			0.89 / –
5.25	5.3	92.8		
5.5	5.28			
6	5.28			
6.5	5.28			0 / 0
6.75	5.28	93.4		
7	5.28			
7.5	5.28		0.580	
21.25	5.32			0.148 / 0.08
21.75	5.32	46.32		
23	5.32		0.501	
24.75	5.32	44.06		
28.25	5.32	42.93		
29.5	5.32			0 / 0.748
30.5	5.32	42.18		
31.25	5.32		0.500	
47	5.32	37.66		0 / 0.746
50	5.32	37.66		
53	5.32		0.502	0 / 0.758
53.5	5.32	38.4		

TABLE 12.8 *Continued*

Time (h)	pH	Enzyme Activity. (DSU/cm^3)	OD_{590} Values	Residual Sucrose/ Fructose (% w/v)
69.5	5.32			
70.5	5.32		0.500	0 / 0.760
95	5.32	24.1		
96	5.32		0.486	
121	5.32	12.55		
122	5.32			0 / 0.798
124	5.32		0.473	
125	5.32	11.57		
142	5.32	8.66		
143	5.32		0.467	
144	5.32			0 / 0.88
145.5	5.32	8.54		
147.5	5.32		0.463	

The initial sucrose concentration for the second phase was 3% w/v. As can be observed from Table 12.8 and Figure 12.3. during the early stages of the second phase there was a decline in the residual sucrose with process time. In an HPLC analysis, for sucrose and fructose, carried out after 6.5 h into the same phase no residual sucrose and fructose could be detected.

With respect to fructose production in the fermentation system the following must be noted.

Fructose is produced in the steady state chemostat of the first phase, due to the reaction of dextransucrase with sucrose, but not in significant amounts due to the fact that the pH is controlled at 6.65 which does not favour this reaction.

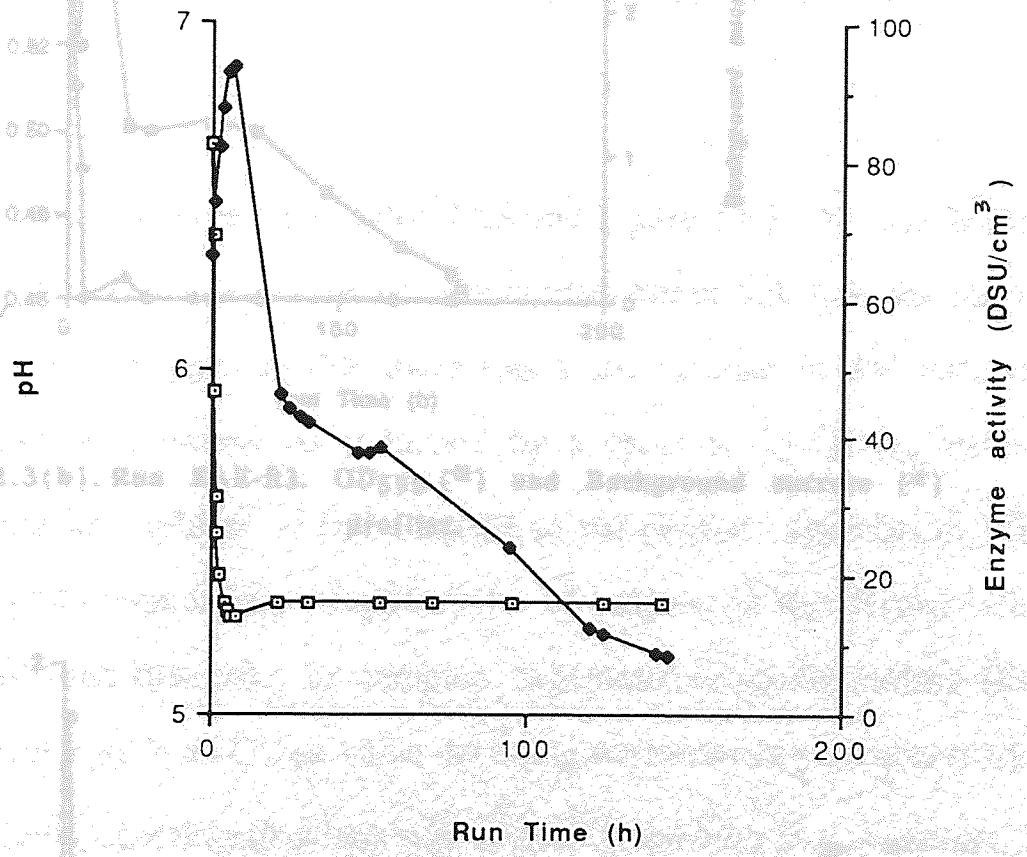


Fig.12.3(a). Run EAE-R3. pH (⊡) and enzyme activity (❖) profiles.

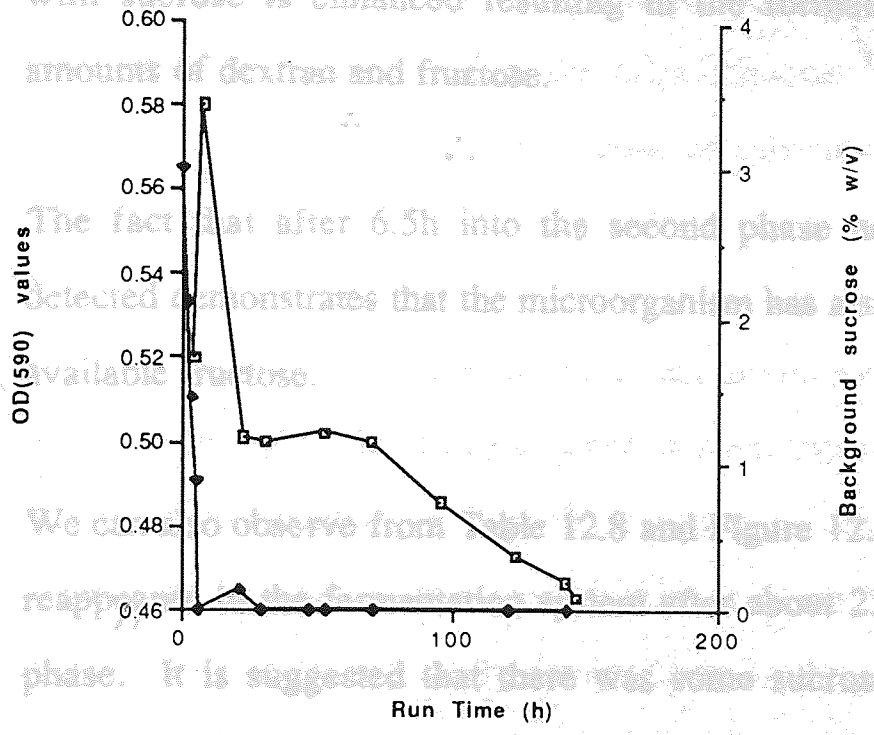


Fig.12.3(b). Run EAE-R3. OD_{590} (□) and Background sucrose (◈) profiles.

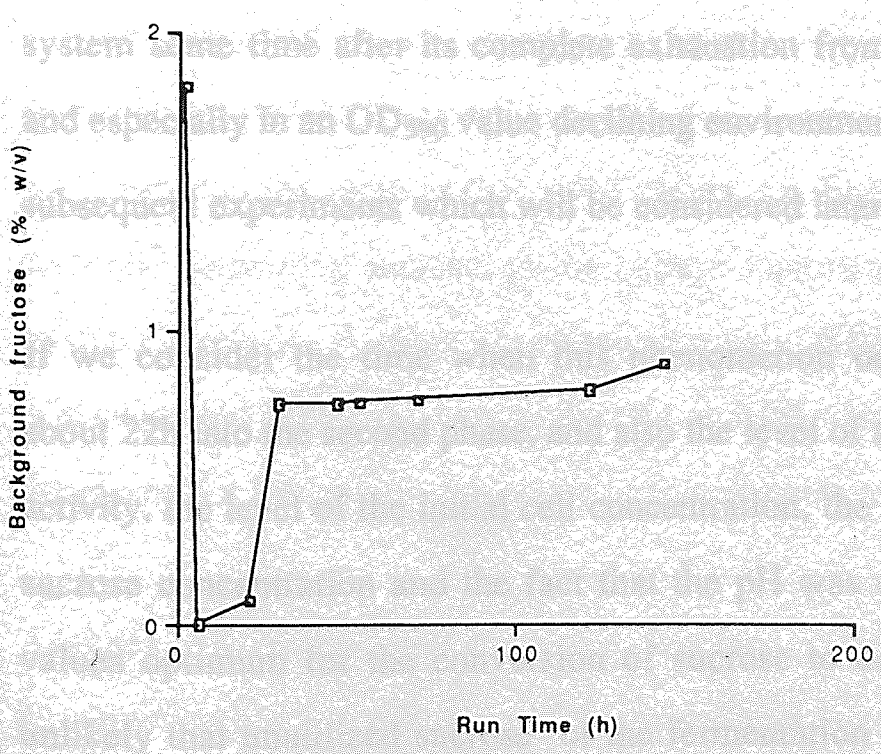


Fig.12.3(c). Run EAE-R3. Background fructose profile.

In the second phase, due to the drop of pH the reaction of the enzyme with sucrose is enhanced resulting in the formation of significant amounts of dextran and fructose.

The fact that after 6.5h into the second phase no fructose can be detected demonstrates that the microorganism has also consumed all the available fructose.

We can also observe from Table 12.8 and Figure 12.3 (b) that sucrose reappeared in the fermentation system after about 22h into the second phase. It is suggested that there was some sucrose in the cells and autolysis of biomass, as indicated by a decrease in OD_{590} values, resulting in a release of cell contents to the culture supernatant. The same phenomenon, i.e. reappearance of sucrose in the fermentation system some time after its complete exhaustion from the culture fluid and especially in an OD_{590} value declining environment was observed in subsequent experiments which will be considered later in this section.

If we consider the time when this phenomenon occurred, i.e. after about 22h into the second phase, and also the level of the intitial enzyme activity, the level of the initial cell concentration, the level of the initial sucrose concentration and the fact that the pH was declining towards values optimum for the conversion of sucrose to dextran, it appears unlikely that unutilized sucrose of the fermentation broth at t=0 was the cause.

A possible explanation for the reappearance of sucrose in the fermentation broth after about 22h into the second phase that can be put forward at the moment is that the cells contained some sucrose and autolysis of biomass resulted in release of cell contents to the culture supernatant.

Since in the fermentation broth there was dextransucrase, sucrose and the prevailing pH was nearly optimum for dextran synthesis there was action of the enzyme on sucrose resulting in dextran and fructose production. The occurrence of this reaction accounts for the reappearance of fructose in the fermentation broth.

Towards the termination of the second phase of this experiment there was a further decrease in OD_{590} values due to biomass autolysis which was accompanied by an increase in fructose concentration in the fermentation system. A possible explanation for this effect that can be put forward is the same as that given earlier, i.e. further release of cell contents, containing sucrose, to the culture supernatant due to further biomass autolysis. As soon as sucrose appears in the fermentation broth it is converted by the enzyme to dextran and fructose and this must be the reason for the observed increase in fructose concentration.

Another reason for the increased fructose concentration in the fermentation broth at this stage of the experiment could be that the cells contained fructose which was released to the culture supernatant when

biomass autolysis was occurring. This may happen at this stage of the experiment but this event is not in disagreement with the argument that was put forward to explain the reappearance of sucrose in the fermentation broth after ~ 22h into the second phase, since this occurred after about 15h of its complete exhaustion from the culture fluid.

The total duration of the second phase of the EAE-R3 fermentation experiment was 147.5h.

RUN EAE-R4

The steady-state data obtained from the first phase of this experiment is given in Table 12.9.

TABLE 12.9

Run EAE-R4

Steady state results of the first phase.

Dilution rate	=	0.29 h^{-1}
Enzyme activity	=	75.32 DSU/cm^3
OD_{590} value	=	0.479
Residual sucrose	=	1.438% w/v

The total duration of the conventional chemostat was 206 hrs.

In this experiment the initial sucrose concentration of the second phase was increased to 4% w/v by the addition of 100 cm^3 of a 29.236% w/v sterile sucrose solution to the steady state chemostat fermentation broth of the first phase, immediately before switching to the second phase of the experiment.

Because of the addition of this sucrose solution the fermentation broth was diluted by a factor of:

$$\frac{985}{985 + 100} = \frac{985}{1085}$$

as in the case of the EAE-R3 run and also in subsequent experiments. Due to this dilution the initial enzyme activity of the second phase was:

$$75.32 \times \frac{985}{1085} = 68.38 \text{ DSU/cm}^3$$

The data obtained from this phase of the experiment is given in Table 12.10 and graphically presented in Figure 12.4.

The maximum recorded value, with respect to enzyme activity, in this phase was 111.5 DSU/cm^3 which is 63.06% higher than the initial value of enzyme activity which demonstrates the ability of this system to enhance the enzyme activity.

In comparison with the enzyme activity of the steady state chemostat, there was an increase in enzyme activity of 48%.

The duration of this phase of the run was 32 hrs.

TABLE 12.10

Run EAE-R4

Results of the second phase.

Time (h)	pH	Enzyme Activity. (DSU/cm^3)	OD_{590} Values	Residual Sucrose/ Fructose (% w/v)
0	6.65	68.38		4 / –
0.166	6.59			
0.416	6.47			
0.5	6.36	73.31		1.02 / –
0.75	6.25			
1	6.12			
1.25	5.97			
1.5	5.82	108.45		0.415 / 1.18
1.75	5.62			
2	5.46			
2.15	5.31			
3.5	5.11			
3.75	5.08	111.5		
4	5.07			
4.15	5.05			
4.5	5.04			0 / 0.556
4.75	5.03			
5	5.02	106.2		
5.25	5			
5.5	4.99			
5.75	4.98		0.668	
6	4.97			0 / 0.421
6.5	4.96			
6.75	4.96	104.7		
7	4.95			
7.25	4.95			
7.75	4.94			
8	4.94	100.4		
8.75	4.94			
20	4.9			
21.5	4.9			0.127 / 0.826
23	4.9	108.45		
24	4.9		0.617	
27.5	4.9	97.16		
28.5	4.9		0.610	
31	4.9	93.4		
31.75	4.9		0.610	

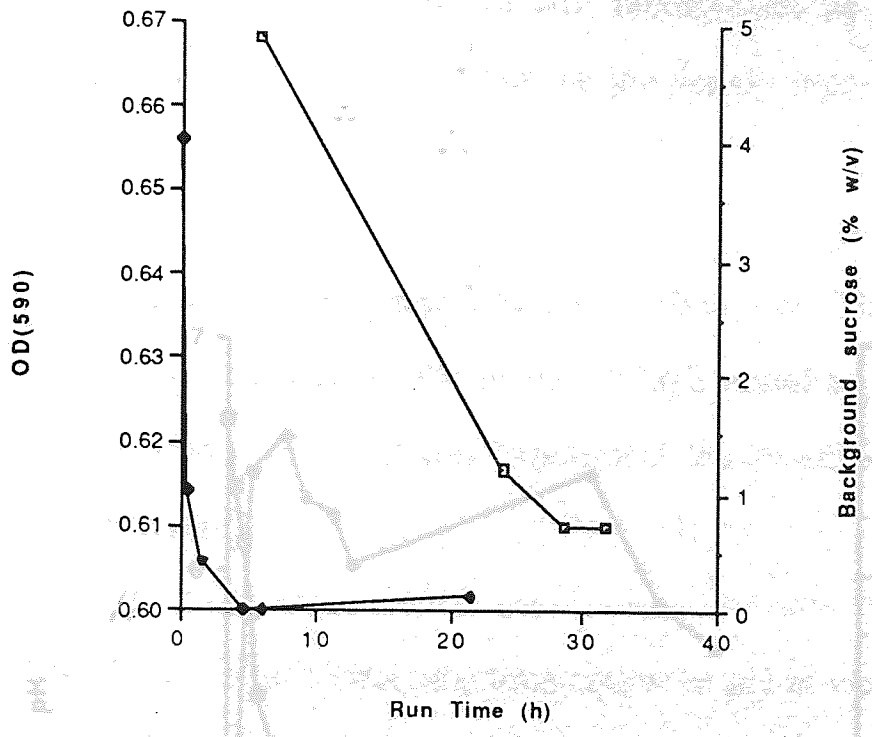


Fig.12.4(a)Run EAE-R4. OD_{590} (□) and background sucrose (◈) profiles

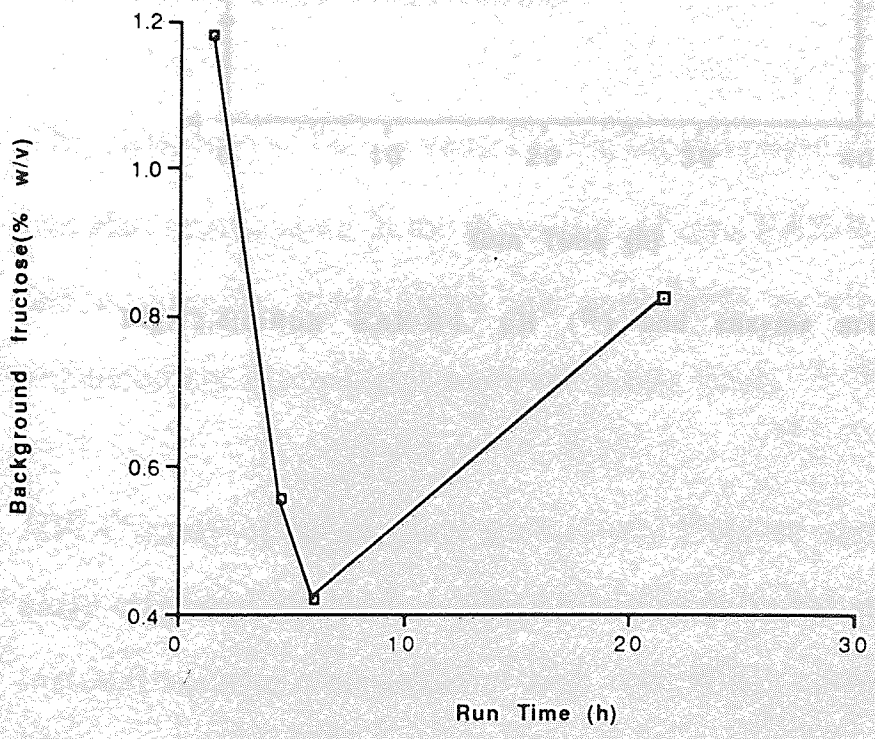


Fig.12.4(b) Run EAE-R4. Background fructose profile

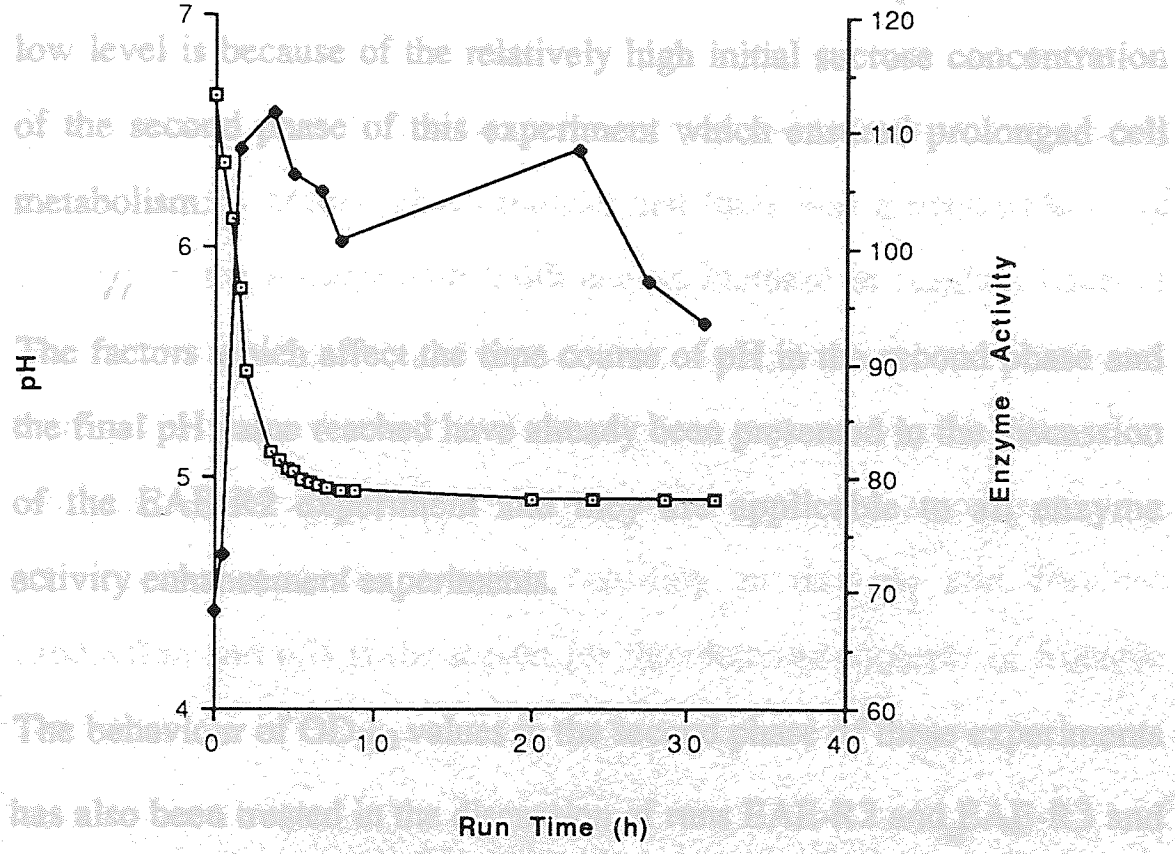


Fig.12.4(c)Run EAE-R4. pH (□) and enzyme activity (◆) profiles

As can be seen from Table 12.10 and Figure 12.4 (c) a drop of pH was observed which was due to cell metabolism as had already been mentioned in the discussion of the previously reported experiments of this chapter.

The final pH of this phase was 4.9 which is low. The pH fall to such a low level is because of the relatively high initial sucrose concentration of the second phase of this experiment which ensured prolonged cell metabolism.

The factors which affect the time course of pH in the second phase and the final pH value reached have already been presented in the discussion of the EAE-R2 experiment and they are applicable to all enzyme activity enhancement experiments.

The behaviour of OD_{590} values in the second phase of these experiments has also been treated in the discussion of runs EAE-R2 and EAE-R3 and the comments made there are applicable to all enzyme activity enhancement experiments reported in this thesis.

HPLC analyses for residual sucrose and fructose carried out during the early stages of the hold up phase of the experiment showed a decline in residual sucrose concentration with time which dropped to zero some time between 1.5 and 4.5 h into this phase. There was also a decline in residual fructose concentration in the same time period which fell from

1.18% w/v at 1.5h to 0.556% w/v after 4.5h, demonstrating that fructose was consumed by the cells. After 6h into the second phase of the run HPLC analysis results showed again the absence of sucrose from the fermentation broth and also a further decline in residual fructose concentration demonstrating again that fructose was consumed by the microorganism.

The results of an HPLC analysis for sucrose and fructose carried out at 21.5h into the second phase showed that there was a reappearance of sucrose in the fermentation broth and an increase in residual fructose concentration. A possible explanation for this phenomenon has already been given in the discussion of the previous experiment, EAE-R3. The reappearance of sucrose in the fermentation broth was accompanied by its reaction with the enzyme resulting in dextran and fructose production and this is the reason for the observed increase in fructose concentration, at this stage of the hold up phase of the experiment.

If the cell contents released to the culture supernatant due to biomass autolysis contained fructose, then the increase in fructose concentration at this stage of the second phase of the experiment was partly due to this fact and partly due to the production of dextran and fructose as a result of the action of dextransucrase on reappeared sucrose.

RUN EAE-R5

Table 12.11 gives the steady-state data of the first part of the experiment, i.e. from the conventional chemostat run at a dilution rate of $0.29h^{-1}$.

TABLE 12.11

Run EAE-R5

Steady state results of the first phase.

Enzyme activity	=	76.82 DSU/cm^3
OD_{590} value	=	0.488
Residual sucrose	=	1.46% w/v

The total duration of this continuous fermentation was 134 hrs.

In this experiment the initial sucrose concentration of the second phase was 4.6% w/v. This was accomplished by the addition of 100 cm^3 of a 35.53% w/v sucrose solution to the steady-state culture of the first phase immediately before switching to the second phase of the experiment.

The aim of this experiment was to boost the enzyme activity in the fermentation broth by increasing the initial sucrose concentration in the fermentation system.

Because of the addition of sucrose solution the fermentation broth was diluted by a factor of:

$$\frac{985}{1085}$$

as explained previously. As a consequence of this dilution the initial enzyme activity for the second phase of the run was:

$$76.82 \times \frac{985}{1085} = 69.74 \text{ DSU/cm}^3$$

The maximum value of enzyme activity obtained in the second phase was 131 DSU/cm^3 which is 87.84% w/v higher than the initial value of 69.74 DSU/cm^3.

In comparison with the enzyme activity of the conventional chemostat, there was an increase of 70.53%. The results of the second phase of the EAE-R5 run are given in Table 12.12 and graphically presented in Figure 12.5.

The enzyme activity in the second phase of the experiment, as can be seen from Table 12.12 and Figure 12.5 (a), was declining after reaching a maximum value. A possible explanation for these enzyme activity losses could be that the pH during the time period where these enzyme activity losses occurred, was declining to values not optimum for

enzyme stability and in particular to values where inhibition of dextransucrase starts taking place.

Enzyme activity losses occurring at pH values ≤ 5 were also experienced in the hold up phase of the EAE-R6 fermentation experiment which will be considered later.

In Table 12.12 and Figure 12.5 (b) is also shown that OD_{590} values were declining towards the end of the second phase of this experiment demonstrating biomass autolysis.

Sucrose concentration in the fermentation system was decreasing with time due to its consumption by the cells and its conversion by the enzyme to dextran and fructose.

During the early stages of this phase there was an increase in fructose concentration due to fructose production resulting from the reaction of the enzyme with sucrose. This reaction was enhanced by the decreasing pH of the fermentation broth towards values optimum for dextran synthesis.

The results of the HPLC analyses, given in Table 12.12 and plotted in Figure 12.5 (c), also demonstrate that fructose was consumed by the cells. The exact moment the cells started to take up fructose has not been determined. This may have occurred before or after the complete exhaustion of sucrose from the fermentation broth.

TABLE 12.12

Run EAE-R5

Results of the second phase.

Time (h)	pH	Enzyme Activity. (DSU/cm^3)	OD_{590} Values	Residual Sucrose/ Fructose (% w/v)
0	6.65	69.74		4.6 / –
0.5	6.46			
0.75	6.36			2.36 / 1.29
1	6.24	77.32		
1.25	6.12			
1.5	5.93			1.62 / 1.93
1.75	5.76			
2	5.61	105.44		
2.25	5.3			
2.5	5.3			0.74 / 1.95
3	5.19			
3.25	5.14	115.6		
3.5	5.1			
3.75	5.08			
4	5.06			0 / 0.64
4.5	5.03	131		
5	5			
5.25	4.99			0 / 0
5.5	4.98			
5.75	4.98	110.5		
6.75	4.95			
7	4.95		0.658	
7.5	4.93	96.4		
9	4.9			0 / 0
9.5	4.9	79.8		
10.25	4.9			0 / 0
23	4.86			
23.25	4.86			
23.5	4.86		0.612	
24	4.86	119		
27	4.86		0.602	
29	4.86	129.5		
32.5	4.86	94.9		
33.25	4.86		0.580	

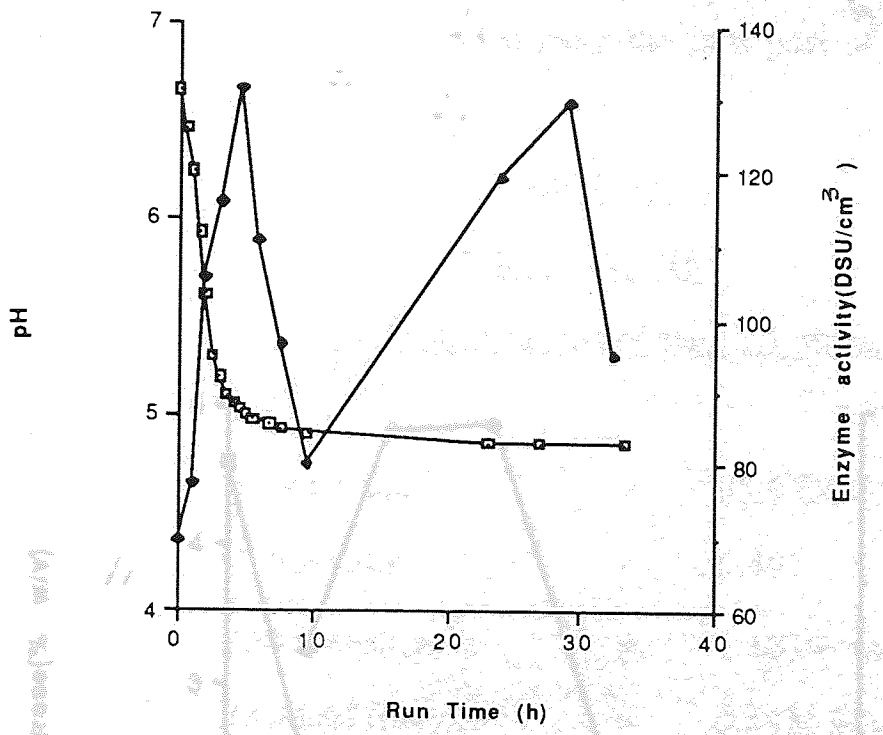


Fig.12.5(a) Run EAE-R5. pH(⊡) and enzyme activity (✱) profiles.

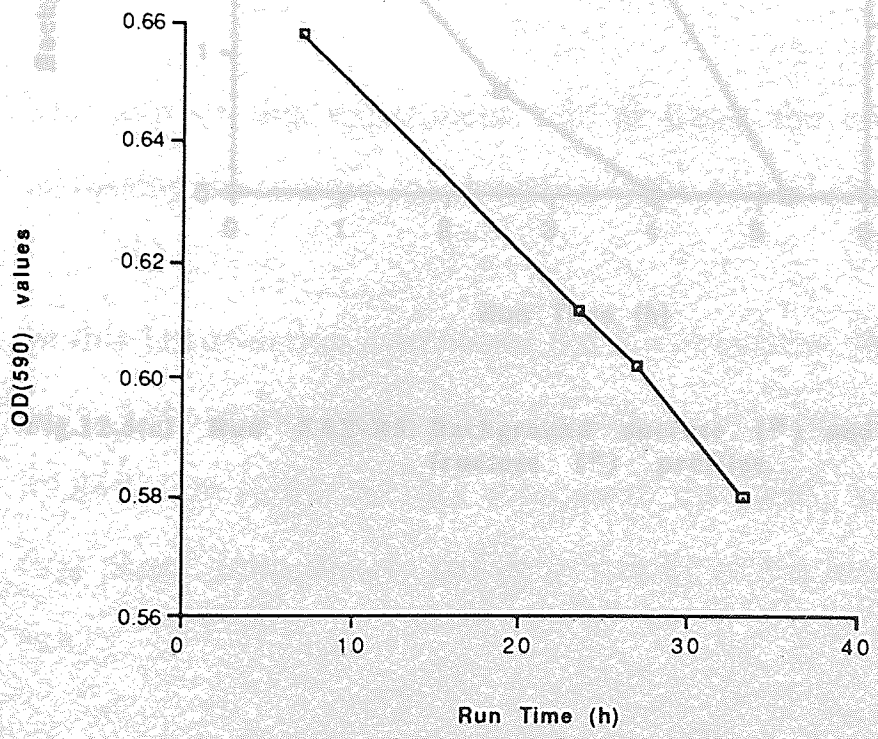


Fig.12.5(b) Run EAE-R5. OD_{590} values profile.

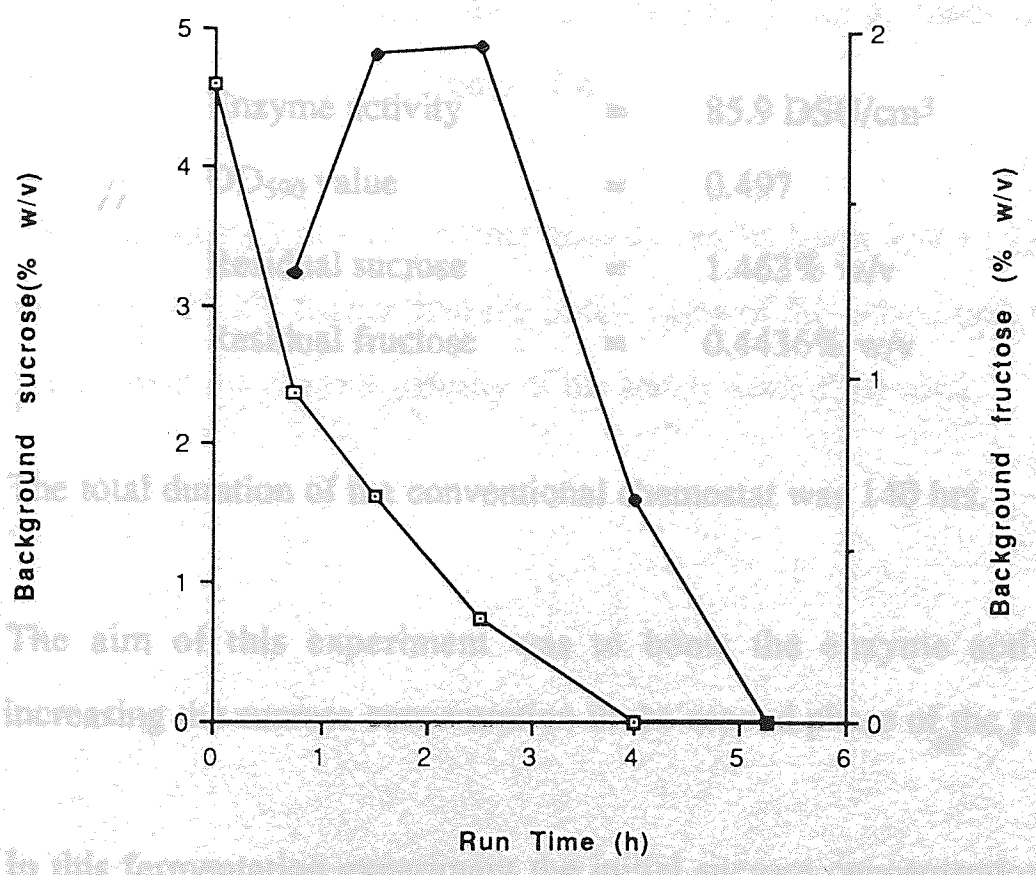


Fig.12.5(c) Run EAE-R5. Background sucrose (□) and Background fructose (◆) profiles.

RUN EAE-R6

The steady state data obtained from the first part of the experiment is given in Table 12.13.

TABLE 12.13

Run EAE-R6

Steady state results of the first phase.

Enzyme activity	=	85.9 DSU/cm^3
OD_{590} value	=	0.497
Residual sucrose	=	1.463% w/v
Residual fructose	=	0.4436% w/v

The total duration of the conventional chemostat was 140 hrs.

The aim of this experiment was to boost the enzyme activity by increasing the sucrose concentration in the second phase of the run.

In this fermentation experiment the initial sucrose concentration of the second phase was increased to 5% w/v by the addition of 100 cm^3 of a 39.84% w/v sterile sucrose solution to the steady state culture of the first phase, immediately before switching to the hold up phase of the run.

This addition resulted in dilution of the fermentation broth by the

factor $\frac{985}{1085}$ as previously discussed. As a consequence of this dilution the initial enzyme activity for the second phase of the experiment was:

$$85.9 \times \frac{985}{1085} = 77.98 \text{ DSU/cm}^3$$

The results of this phase of the experiment are given in Table 12.14 and graphically presented in Figure 12.6.

The maximum value of enzyme activity reached was 177.17 DSU/cm^3 which is 127.2% higher than the initial value of this phase and 106.25% higher than the enzyme activity of the steady-state chemostat.

TABLE 12.14

Run EAE-R6

Results of the second phase.

Time (h)	pH	Enzyme Activity. (DSU/cm^3)	OD_{590} Values	Residual Sucrose (% w/v)
0	6.65	77.98		5
0.25	6.55			
0.5	6.41	84.66		
0.75	6.27			
1	6.12			2.7
1.25	5.98			
1.5	5.81	107.91		
1.75	5.68			
2	5.51			
2.25	5.3			1.45
2.5	5.19			
2.75	5.13	177.17		
3	5.08			1.088
3.25	5.04			
3.5	5.01			
3.75	4.98	150.4		
4	4.96		0.631	0.98
4.25	4.93			
4.5	4.92			
4.75	4.9	93		
5	4.89			
5.25	4.88			
5.5	4.87			0.88
5.75	4.86			
6	4.85			
6.5	4.84			
6.75	4.84		0.640	
7	4.83			
7.5	4.83	92		
8	4.82			
8.5	4.81	87.5		
24	4.8	75.7		
25	4.8		0.568	
26	4.8			1.38
26.5	4.8	70.3		
28	4.8		0.557	0
30.5	4.8	82.14		
31.5	4.8		0.542	0

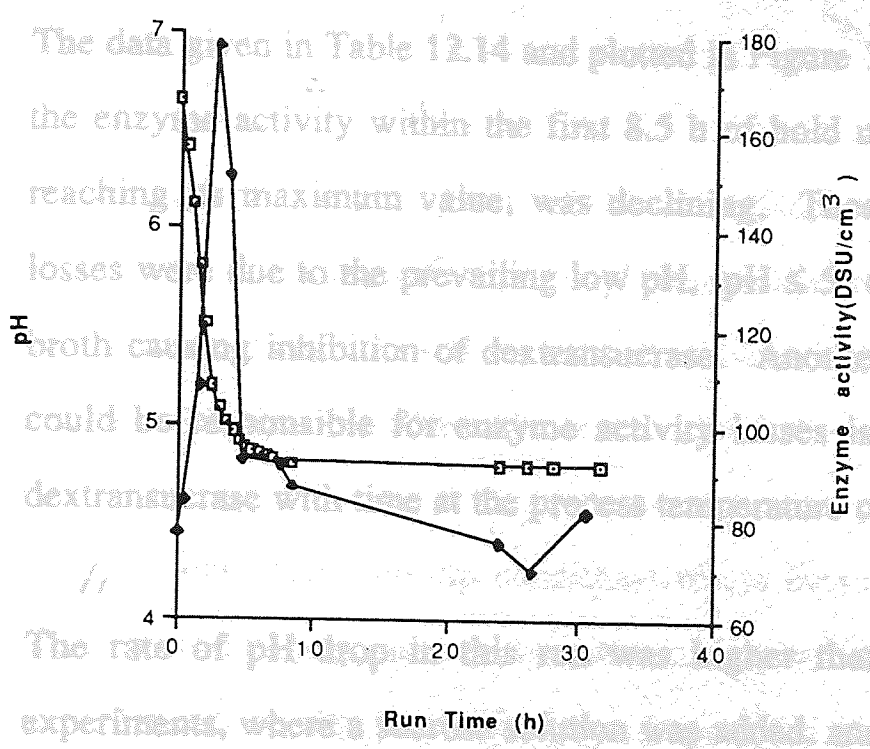


Fig.12.6(a) Run EAE-R6. pH(⊡) and enzyme activity (❋) profiles.

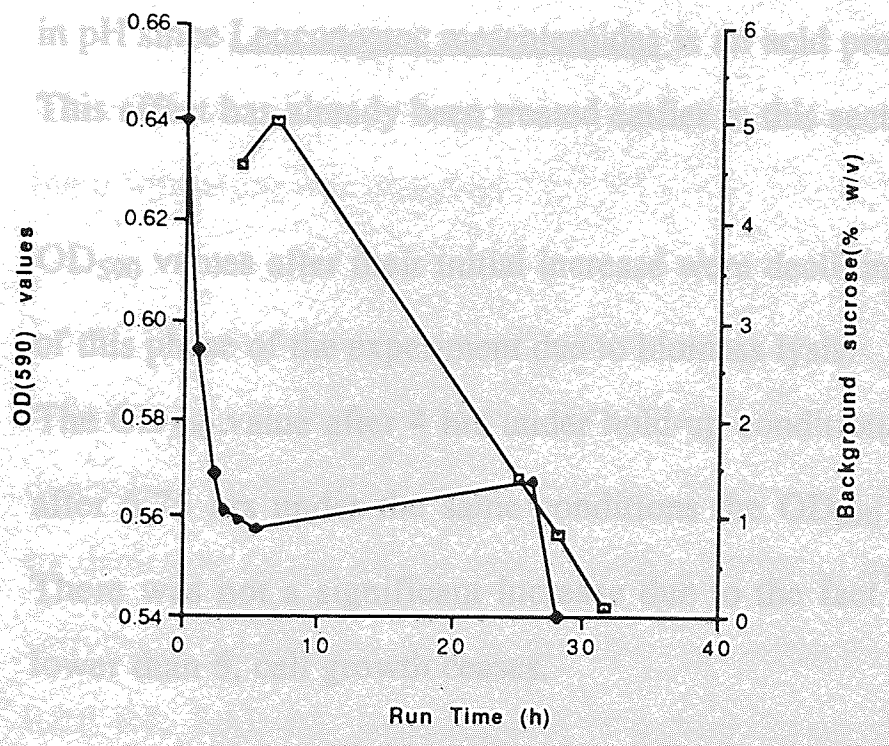


Fig.12.6(b) Run EAE-R6. OD_{590} (⊡) and background sucrose (❋) profiles.

The duration of the second part of the run was 32 hrs.

The data given in Table 12.14 and plotted in Figure 12.6 illustrates that the enzyme activity within the first 8.5 h of hold up conditions, after reaching its maximum value, was declining. These enzyme activity losses were due to the prevailing low pH, ($pH \leq 5$) of the fermentation broth causing inhibition of dextransucrase. Another parameter which could be responsible for enzyme activity losses is the instability of dextransucrase with time at the process temperature of 23.4°C.

The rate of pH drop in this run was higher than in the previous experiments, where a sucrose solution was added, and also the final pH was lower. This was due to high initial sucrose concentration which enabled prolonged cell metabolism resulting in faster and a further drop in pH since Leuconostoc mesenteroides is an acid producing bacterium. This effect has already been treated earlier in this section.

OD_{590} values after their initial increase were declining towards the end of this phase of the experiment due to biomass lysis.
The OD_{590} value after 4 hrs under hold-up conditions was 0.631 while after 6.75 hrs under the same conditions the OD_{590} value was 0.640. There was not a significant increase due to the fact that at pH values lower than 5, cell growth ceases.

HPLC analysis results showed a decline in residual sucrose

concentration due to the consumption of sucrose by the cells and also due to its reaction with dextransucrase resulting in dextran and fructose formation.

Following a period of high rate of sucrose utilization a drop in the rate of its consumption was observed at pH values lower than 5 both due to dextransucrase inhibition and a decline of bacterial growth because of the prevailing low pH of the fermentation broth.

After 26 hrs under hold-up conditions where biomass was dead and autolysis was taking place an increase in sucrose concentration in the fermentation system was recorded. A possible explanation for this phenomenon has already been given in the discussion of the other enzyme activity enhancement experiments.

After 2.25 hrs in the second phase of this fermentation experiment foam formation was observed.

From the experimental work carried out on the enzyme activity enhancement it was noticed that after 23-47 hours into the second phase, depending on the particular experiment, biomass autolysis as indicated by declining OD_{590} values was accompanied by an increase in enzyme activity in the fermentation broth. This was recorded in runs EAE-R2, EAE-R4, EAE-R5, EAE-R6 and in a non- reported experiment. A possible explanation for this phenomenon is that biomass autolysis

resulted in release of intracellular dextransucrase into the fermentation broth.

The results of the enzyme activity enhancement experiments are summarized in Table 12.15. Section 12.3 gives the conclusions drawn from this experimental work and section 12.4 presents a novel fermentation process for dextran production using the findings of the enzyme activity enhancement work reported in this chapter.

TABLE 12.15

Summary of the Results of the Enzyme Activity Enhancement Experiments.

	EAE[1] -R1	EAE -R2	EAE -R3	EAE -R4	EAE -R5	EAE -R6
Initial sucrose concentration for the seond phase. (% w/v)	2.65	0.563	3	4	4.6	5
Inital enzyme activity for the second phase. (DSU/cm^3)	32.53	81.35	66.36	68.38	69.74	77.98
OD_{590} value of the steady state chemostat of the first phase.	0.331	0.475	0.450	0.479	0.488	0.497
Final pH of the second phase.	4.89	5.5	5.32	4.9	4.86	4.8
Enzyme activity of steady state chemostat of the first phase. (DSU/cm^3)	32.53	81.35	73.1	75.32	76.82	85.9
Maximum enzyme activity obtained under hold-up conditions. (DSU/cm^3)	55.34	96.4	93.4	111.5	131	177.17
Enhancement in enzyme activity over that of conventional chemostat. (%)	70.1	18.5	27.8	48	70.53	106.25

(1) : In experiment EAE-R1 the conventional chemostat was run with 4.5% w/v sucrose in the nutrient feed and at $D = 0.47h^{-1}$.

12.3 CONCLUSIONS

This work has shown that under controlled conditions considerable enhancement in enzyme activity occurs with time when a dextransucrase whole broth from a continuous fermenter is held up in an agitated tank for between 1.5 - 4 hours.

Enzyme activity was also shown to increase with increase in the sucrose concentration of the whole broth at the commencement of the hold up phase.

The maximum enzyme activity value reached, under hold up conditions, was ~ 180 DSU/cm^3 representing an 106% increase over the enzyme activity of the steady state conventional chemostat.

Under hold up conditions sucrose was consumed in its reaction with the enzyme, favoured by a drop in pH towards values optimum for dextran and fructose production, and also by the cells for their metabolism and product formation.

High initial sucrose concentrations at the commencement of the second phase of the fermentation resulted in prolonged cell metabolism causing a more rapid decrease in pH.

The enzyme activity losses that occured were due to the instability of

dextransucrase with time at the process temperature of 23.4°C and/or due to the inhibition of the enzyme at pH values ≤ 5, in the runs where the pH dropped to such low levels. Dextransucrase is irreversibly denatured at pH ≤ 4.5.

To minimize activity losses caustic or a preservative/bacteriostat can be added to the holding tank to prevent the pH falling to values below 5. In addition or alternatively, the crude enzyme can be transferred to the main fermenter, where dextran is synthesized, before the pH drops to low values and activity losses occur.

If activity losses due to pH can be kept to a minimum, the instability of dextransucrase with time at the process temperature must be considered. To minimize this effect the crude enzyme must be transferred to the main fermenter as quickly as possible.

After the exhaustion of substrate from the fermentation broth and towards the end of the second phase of these experiments OD_{590} values were declining, indicating biomass autolysis. It is suggested that the cell contents released to the culture supernatant contained sucrose and probably fructose, which could be responsible for the reappearance of sucrose and fructose in the fermentation broth sometime after their complete exhaustion from the culture fluid.

Fructose is consumed by the microorganism under hold-up conditions.

Future work is needed to determine if this occurs before or after the complete exhaustion of sucrose from the fermentation broth.

12.4 A NOVEL FERMENTATION PROCESS FOR DEXTRAN PRODUCTION.

The current method for dextran production involves the growth of the microorganism, L.M. the production of the enzyme and dextran synthesis in the same vessel under continually changing pH conditions to which both enzyme production and dextran synthesis are very sensitive.

Sugar solutions fortified with suitable nutrients, growth stimulators, buffer salts and minerals are innoculated at around a pH value of 7 with the bacterial culture. Little further process control takes place and the fermentation is left to grow on its own.

Within this fermentation three processes occur:

1. A growth phase of the organism.
2. Enzyme-producing phase.
3. An enzyme / sucrose reaction phase.

pH 7 is around the optimum for cell growth, pH 6.6 - 6.7 for enzyme production and pH 5.2 for enzyme action and stability.

Interest has recently been shown in dividing the overall process into two stages, the enzyme producing stage and the dextran synthesis stage. The intention has been to optimize each one separately and consequently maximize dextran yields. One of the key objectives of this research has

been the optimization, in terms of maximum enzyme activity, of the continuous fermentation for the dextransucrase production stage.

In this section a novel process for dextran production is reported. In this process, the enzyme is produced in a continuous fermenter. The product stream of this reactor is collected in holding tanks where enzyme activity enhancement is achieved. At the moment the enzyme activity reaches a maximum, the whole broth of the holding tanks is transferred to the main fermenter where dextran is produced, see Figure 12.7, to react with a sterile sucrose solution.

The holding tanks, where the product stream of the continuous fermenter is collected, must provide means of agitation to ensure a uniform environment for their contents and accurate pH monitoring.

In addition, these holding tanks must be able to maintain aseptic conditions for a time period which is required for the maximum enzyme activity to be reached. The length of this time period, from the experimental work reported in this chapter, is not greater than 7h. The purpose of collecting the whole broth that leaves the continuous fermenter in holding tanks is to obtain enhancement in enzyme activity and then, before low pH deactivates the enzyme, to transfer the contents of the holding tanks to the main fermenter for dextran synthesis.

To prevent enzyme activity losses, due to low pH, from occurring a

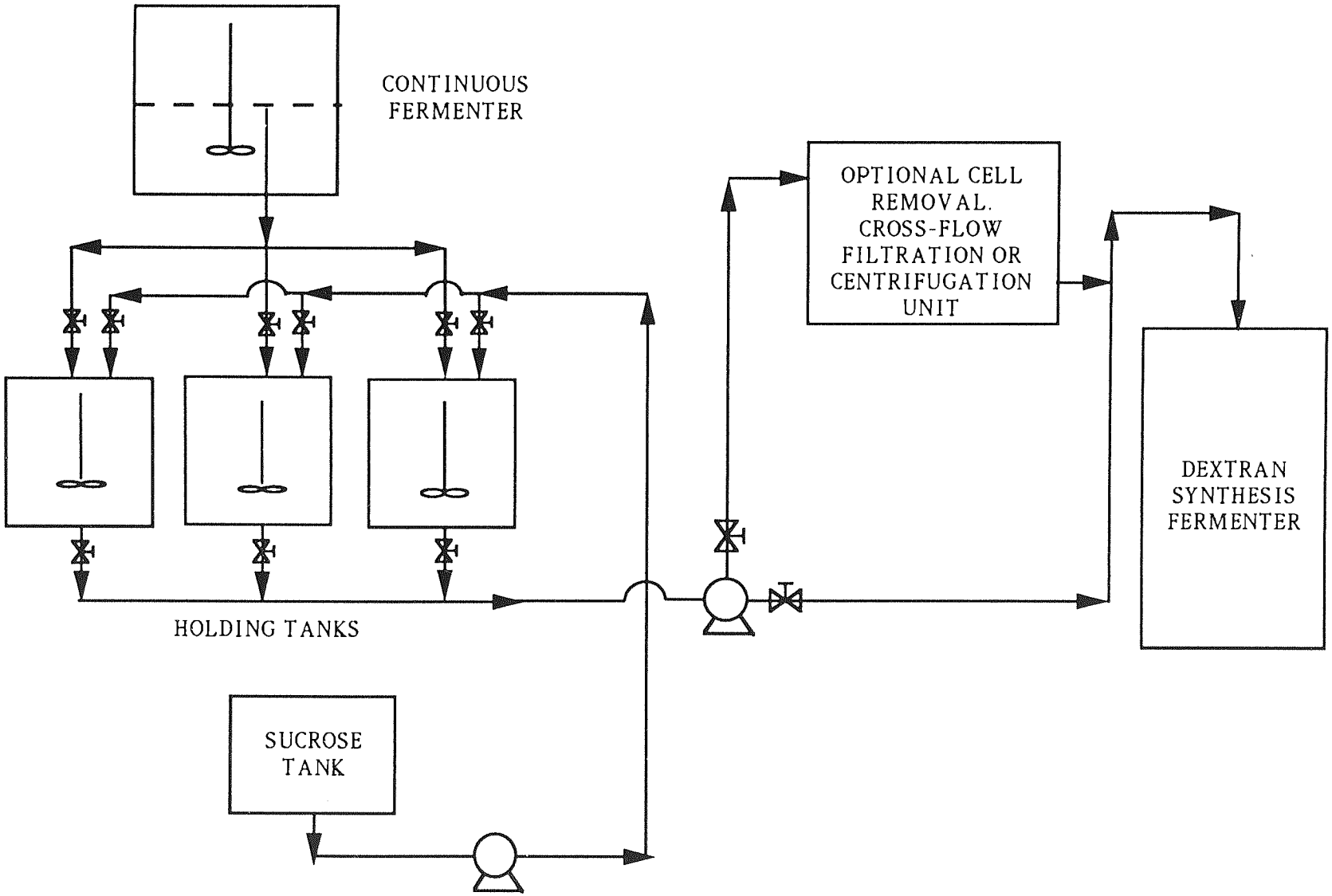


FIGURE 12.7 FLOW DIAGRAM FOR THE PRODUCTION OF DEXTRAN USING A CONTINUOUS FERMENTER AND INTERMEDIATE HOLDING TANKS TO OBTAIN ENZYME ACTIVITY ENHANCEMENT.

number of actions can be taken. pH control can be carried out with caustic addition. Alternatively, buffer salts can be used or preservatives/bacteriostats to retard or stop a pH decrease. pH monitoring, by incorporating pH probes in the holding tanks, is also important so that the broth can be transferred to the main fermenter before the pH drop to low levels, in particular to pH values below 5. Alternatively, the time required for the maximum enzyme activity value to be attained, before low pH inhibits or deactivates the enzyme, must be determined by experimentation and at the end of this time period the crude enzyme solution must be transferred immediately to the main fermenter before enzyme activity losses occur.

In these holding tanks a sterile sucrose solution can be added to boost the enzyme activity or alternatively the continuous fermentation for dextransucrase production can be run at high sucrose concentrations in the feed. This would result in high residual or unutilized sucrose in the fermentation broth which will boost the enzyme activity in the holding tanks, under the hold-up conditions specified in section 12.1.

The latter case requires experimentation to determine a suitable sucrose concentration for the nutrient feed and the enzyme activity production level for this concentration, before being applied on an industrial scale.

The cost of running the continuous fermentation at high sucrose concentrations must be considered and an optimum must be found as

regards the process economics.

After the enzyme activity enhancement has been achieved the whole broth can be transferred to the main fermenter to react with a sterile sucrose solution for dextran and fructose production.
The reaction vessel where dextran is synthesized can be a continuous or batch reactor. As regards the continuous production of dextran very limited work has been published so far and the realization of the industrial production of dextran in a continuous reactor will require laboratory, pilot plant and industrial scale development work.

Currently, dextran is produced in batch reactors typically of around 50,000 dm^3 in size.

The addition of the crude enzyme in the main fermenter causes a drop of pH of the reaction mixture. This, as has already been pointed out in this chapter, is due to cell metabolism. If the pH drops to unacceptably low levels it will cause deactivation of the enzyme resulting in low sucrose conversion and thereby low yields of dextran will be obtained.

For the maintenance of optimum pH levels a number of control actions can be taken. These include the control of pH with caustic addition which is the best method.

Alternatively, preservatives, bacteriostats and buffer salts can be used to

prevent or retard the pH decrease so its rate of decline does not outpace the dextran production rate and cause the enzyme to be deactivated before converting the sucrose to dextran to a maximum level.

Another method is to employ a sufficiently high overall enzyme activity in the dextran synthesis fermenter so that, as in the previous case, the enzyme is able to achieve maximum sucrose conversion before low pH deactivates it.

A drop in pH either in the holding tanks or in the main fermenter, where the enzyme is transferred to react with the sucrose solution for dextran synthesis, is due to cell metabolism. Cell removal from the crude enzyme solution by cross-flow filtration or centrifugation, prior to its transfer to the main fermenter, can eliminate the decrease in pH and facilitate the maintenance of an optimum value for this reaction.

After the completion of the dextran synthesis stage the crude dextran solution undergoes a number of treatments (12.2), which are the same as in the case of dextran produced by the conventional whole cell method, for the preparation of clinical dextran or other dextran products of commercial interest.

AN EXAMPLE ON THE DETERMINATION OF PROCESS PARAMETERS FOR A REQUIRED PROCESS THROUGHPUT.

For a chosen throughout of this process, in terms of native dextran, the various parameters of the process can be determined. These parameters include the size of the continuous fermenter and in particular its working volume, the concentration of sucrose in the sucrose solution in the main fermenter and the volume of the crude enzyme solution which must be added to the dextran synthesis fermenter to give a required overall enzyme activity, which will result in a complete sucrose conversion to products within a required period of time.

The procedure for all these determinations is illustrated in the following example based on the production of 2 tonnes of native dextran, from a dextran synthesis fermenter.

For these determinations, it is assumed that the pH in the dextran synthesis fermenter is controlled at the value of 5.2 which is the optimum for enzyme activity and stability.

The maintenance of pH at this value apart from the fact that it is the optimum for enzyme stability and activity is also necessary to enable a complete sucrose conversion by the enzyme. As mentioned earlier in this chapter, if no pH control is feasible in the reactor, then there is a high risk that the rate of pH decrease will outpace the rate of dextran production which will consequently result in deactivation of the

enzyme, before complete conversion of all the sucrose to products is achieved. Sucrose consumption by the cells, for their metabolism, in the dextran synthesis fermenter is considered to be insignificant.

The required throughput of native dextran of 2 tonnes requires the conversion of 4 tonnes of sucrose in the main vessel. For the purpose of the subsequent calculations it is also assumed that the capacity of the main vessel and in particular its working volume is 50,000 dm^3. Consequently the overall sucrose concentration in this vessel has to be 8% w/v.

It is also assumed that the desired overall enzyme activity for the complete conversion of the 4 tonnes of sucrose to dextran and fructose, at a pH value of 5.2, is 5 DSU/cm^3.

Higher or lower enzyme activity levels in the main fermenter only affect the time required for complete sucrose conversion.

The crude enzyme which is added to the main fermenter is transferred from the holding tanks, as can be seen from Figure 12.7, where enzyme activity enhancement is obtained.

This crude enzyme solution has an activity of 180 DSU/cm^3 which has been achieved in the laboratory, as detailed in section 12.2.

If Y is the volume, in dm^3, of the crude enzyme of 180 DSU/cm^3 which is added to the main fermenter and X is the volume, in dm^3, of the initial sucrose solution in the same vessel then after the addition of the Y dm^3 of crude enzyme the total volume of the reaction mixture will be 50,000 dm^3. This situation is represented by equation 12.1. Equation 12.2 is a mass balance on dextransucrase activity in the crude enzyme transferred from the holding tanks, and in the 50,000 dm^3 reaction mixture in the dextran synthesis fermenter.

$$X + Y = 50{,}000 \qquad \text{Eqn. 12.1}$$

$$Y \times 180 \times 1000 = 50{,}000 \times 5 \times 1000 \qquad \text{Eqn. 12.2}$$

From equations 12.1 and 12.2 Y = 1388.88 ≈ 1389 dm^3 of crude enzyme of 180 DSU/cm^3 to give an overall enzyme activity in the main fermenter of 5 DSU/cm^3. Consequently the initial sucrose solution in the same vessel must have a volume of (50,000 - 1389) dm^3 = 48,611 dm^3.

If Z is the concentration of sucrose in the initial medium in the main fermenter, prior to the addition of the crude enzyme solution, then:

$$48611 \times \frac{Z}{100} = 50{,}000 \times \frac{8}{100} \qquad \text{Eqn. 12.3}$$

From this equation it follows that Z = 8.23% w/v which reduces to 8% w/v after completing the addition of the 1389 dm^3 of crude enzyme.

The product stream of the steady-state chemostat is collected in holding tanks where it has a residence time of about 4 h, in which period of time the enzyme activity is boosted, under the hold up conditions specified in section 12.1, to a maximum level.

The operating conditions which resulted in the enhancement of the enzyme activity of the steady state chemostat, which was ~ 86 DSU/cm^3, to 180 DSU/cm^3 are given in section 12.2.

It has been assumed that the addition of the 1389 dm^3 of crude enzyme of 180 DSU/cm^3 to the main fermenter is accomplished in 4 charges carried out at time t=0, t=4, t=8 and t =12 with respect to the reaction time in the dextran synthesis bioreactor. Consequently the volume of each charge fed to the main fermenter is (1389/4) dm^3 = 347.25 dm^3 which is also the output of the continuous fermenter over a time period of 4 h.

As illustrated in section 12.2 the continuous fermentation was run at a dilution rate of 0.29 h^{-1} i.e.:

$$D = 0.29 = \frac{F}{V} \quad h^{-1}$$

Eqn. 12.4

where V is the fermentation broth volume, in dm^3, in the continuous fermenter and F is the medium feed rate, in $dm^3\ h^{-1}$, which is also the

flow rate of the product stream of the chemostat.

In 4 h the output of the chemostat is:

$$F_{4h} = 0.29 \times V \times 4 = 347.25 \text{ dm}^3. \qquad \text{Eqn. 12.5}$$

From this equation it follows that $V = 299.35 \text{ dm}^3 \approx 300 \text{ dm}^3$, which is the working volume of the continuous fermenter.

CHAPTER 13:
CONCLUSIONS AND SUGGESTIONS FOR FUTURE WORK

13.1 CONCLUSIONS

The following conclusions can be drawn from this research work.

1. Anaerobic fed batch fermentation for dextransucrase production under low agitation conditions resulted in obtaining yields of this enzyme in excess of 400 DSU/cm^3 which are higher than those reported in the literature and obtained under aerobic conditions. This is in accordance with the results of Ajongwen-Numfor(13.1).

2. From a fermentation conducted on a 800 dm^3 fermenter under anaerobic low agitation conditions it became evident that special attention must be paid to the degree of agitation of the fermentation broth. It is important to ensure good mixing of the sucrose/NaOH feed mixture with the culture so as to achieve a uniform environment in the vessel, accurate pH control and a sucrose feed readily accessible to the cells thus preventing the culture from being substrate limited. To facilitate this mixing process the sucrose/NaOH mixture can be introduced to the bioreactor via a submerged pipe in the fermentation broth.

3. In continuous fermentations, conducted on a 2 dm^3 MBR "mini" bioreactor, sparging the fermentation broth with CO_2 resulted in lower yields of enzyme activity and growth of the microorganism in the

fermentation system. In these experiments, as detailed in chapter 9, the contents of the bioreactor, both culture fluid and gas in the headspace, were not under pressure which could have caused increased solubility of CO_2 in the fermentation broth.

4. Fermentation experiments carried our under total cell recycle conditions showed that the fermentation environment affected the physiology of the microorganism. The physical separation of cells was depressed and this caused the formation of cell aggregates.

5. In the total cell recycle fermentation experiments an increase in dilution rate resulted in an increased steady state biomass concentration. Also higher values of sucrose concentration in the nutrient feed resulted in higher cell steady state concentrations.

6. In total cell recycle fermentation experiments, enzyme flux rates through the membrane deteriorated with process time and increased cell concentrations.

7. In the total cell recycle fermentation system high steady state biomass concentrations were achieved. In such a fermentation system the biomass accumulates in the bioreactor at a decreasing growth rate until finally no further growth occurs and a steady state biomass concentration is reached. Once this occurs the nutrient medium which is fed into the bioreactor will only meet the maintenance energy

requirements of the total concentration of the cells in the fermentation system. Even under these non-growth conditions Leuconostoc Mesenteroides NRRL B-512(F) cells produced dextransucrase, as detailed in chapter 10, demonstrating that the production of the enzyme is not linked with cell growth in very strict terms. From the results given in Chapter 10, enzyme elaboration in this system was very low, much lower than in the fed-batch and chemostat system as was indicated by the comparison of $Y_{p/s}$ values from these three systems.

8. The performance of the cell recycle system, consisting of the broth recirculation pump and membrane and filtration cell, was poor. This system proved to be unsuitable for the continuous cell recycle dextransucrase fermentations as discussed in chapters 10 and 11.

9. The enzyme activity enhancement experiments have shown that under suitable hold up conditions there was a considerable increase in enzyme activity. The maximum value of enzyme activity obtained was ~180 DSU/cm^3 representing a 106% increase over the enzyme activity of the steady state conventional chemostat.

10. In the enzyme activity enhancement work it was also shown that the enzyme activity obtained under hold up conditions increased with increase in the sucrose concentration in the whole broth at the commencement of the hold up phase.

11. The enzyme activity enhancement work also demonstrated that under hold up conditions fructose was also consumed by the microorganism.

12. A new process for dextran production has been proposed. According to this process, dextransucrase is produced in a continuous fermenter the product stream of which is collected and kept without pH control in holding tanks where agitation is provided and sterile conditions can be maintained.

A sterile sucrose solution can be added to the holding tanks to boost the enzyme activity, if necessary. Having reached the peak enzyme activity, the enzyme is then transferred to a fermentation vessel to react with a sterile sucrose solution to produce dextran and fructose.

13.2 SUGGESTIONS FOR FUTURE WORK

1. In the conventional continuous fermentations carried out in this project the sucrose concentration in the feed was 4.5% w/v. It is recommended that further experimental work be carried out, employing higher sucrose feed concentrations whilst operating the chemostat at relatively high dilution rates. The presence of sucrose in the cell environment, i.e. in the fermentation broth, is essential if dextransucrase production is to be induced.

2. From the work that has been carried out on cell recycle fermentation, it is evident that another cell recycle system must be developed. Probably another membrane in a different configuration would perform better, for example a hollow fiber membrane cartridge. A different type of pump for broth recirculation in the external recycle loop should also be considered.

It is recommended that fermentation experiments be carried out under partial cell recycle conditions varying the process parameters, which as mentioned in Chapter 2, are: the recycle ratio, R, the dilution rate, D, and the sucrose concentration in the nutrient feed, S_o. Dilution rates in excess of μ_{max} at various recycle ratios and $S_o$values should be used to optimize the yield of dextransucrase.

3. With respect to the enzyme activity enhancement work, it is

suggested that the sucrose concentration at the commencement of the hold-up phase should be increased further. It is stressed once again that the presence of sucrose is required to induce dextransucrase production.

Also, the continuous fermentation for dextransucrase production could be run at high sucrose concentrations in the nutrient feed which would result in high residual sucrose concentrations. These elevated residual sucrose levels would boost the enzyme activity during the hold-up phase. With this procedure, the addition of extra sucrose to the holding tanks could be avoided and thus the whole process for dextran production, suggested in section 12.4, would be more simple, straightforward and cost effective.

Further experimental work is needed to study the effect of keeping the pH >5 during the hold-up phase; this could be achieved by the addition of caustic or preservatives/bacteriostats and should help to minimize the loss in enzyme activity.

As mentioned in Chapter 12, Section 12.2 biomass lysis, as indicated by a decrease in OD_{590} values was accompanied by an increase in enzyme activity in the fermentation broth. This phenomenon was recorded in runs EAE-R2, EAE-R4, EAE-R5, EAE-R6 and in a non-reported experiment. A possible explanation for this effect is that lysis of biomass resulted in release into the fermentation broth of intracellular dextransucrase. Future work investigating this effect could possibly

lead in the attainment of even higher enzyme activity in the holding tanks, prior to the transfer of the broth to the "main" fermenter for dextran biosynthesis. In this proposal the biomass is allowed to lyze and to minimize the loss in enzyme activity until biomass lysis occurs the pH is kept to values above 5.

4. It is suggested that dextran can be produced according to the process outlined in section 12.4. In this proposal, a continuous fermenter is used for dextran synthesis with the fermentation broth from the holding tanks providing the feed, probably in a continuous mode. The feasibility of this process scheme requires further evaluation and this will involve both experimental and design studies.

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NOMENCLATURE

A	Arrhenius constant.
Avg. TMP	Average transmembrane pressure.
B	Constant depending on wall boundary conditions in cross-flow filtration.
C_a	Concentration of component a.
C_s	Substrate concentration.
D	Dilution rate.
D	Diffusion coefficient.
D_c	Critical dilution rate.
DOC	Dissolved oxygen concentration.
d	Dilution factor of enzyme.
DSU	Dextransucrase unit.
E	Enzyme.
[E]	Enzyme concentration.
ES	Enzyme substrate complex.
$[E_o]$	Total enzyme concentration.
E_a	Activation energy.
Eqn.	Equation.
F	Medium flow rate.
ΔG	Free energy change.
GPC	Gel permeation chromatography.
H	Henry's law constant.
HPLC	High performance liquid chromatography.

I	Inhibitor molecule.
[I]	Inhibitor concentration.
J	Solvent flux.
K_m	Michaelis-Menten constant.
K_i	Inhibitor constant.
K_s	Saturation constant.
K	Rate constant of a chemical reaction.
K_{eq}	The equilibrium constant of an enzyme catalyzed chemical reaction.
L	Length.
ln	Logarithm to base e.
log	Logarithm to base 10.
M	Molarity.
m	Maintenance coefficient.
MW	Molecular weight.
OD_{530}	Optical density at 530 nm.
OD_{590}	Optical density at 590 nm.
P	Pressure.
p	Product concentration.
[P]	Product concentration.
P	Total amount of product in culture.
P	Productivity.
R	Gas constant.
R	Recycle ratio.
R	Biomass output rate in a chemostat.

rpm	Revolutions per minute.
s	Substrate concentration.
[S]	Substrate concentration.
S	Total amount of substrate in culture.
s_r	Substrate concentration in medium feed.
S_o	Substrate concentration in medium feed.
t	Time.
T	Temperature.
U	SI unit of enzyme activity.
V	Culture volume.
vvm	Volumetric air flow rate per unit volume.
v/v	Volume per volume.
w/v	Weight per volume.
x	Biomass concentration, g/l cell dry weight
x_o	Initial biomass concentration, g/l cell dry weight
$Y_{x/s}$	Growth yield (- dx/dt).
$Y_{a/b}$	Yield coefficient of component (a) with respect to (b).
>	Is greater than.
>>	Is much greater than.
<	Is less than.
<<	Is much less than.
≈	Approximately equals.
~	(Over a symbol) denotes steady-state value.

Greek Symbols

$\dot{\gamma}$	Fluid shear rate at a membrane surface (cross-flow filtration).
μ	Specific growth rate, 1/x . dx/dt.
μ_m	Maximum specific growth rate.
μm	Microns.
υ	Enzyme reaction rate.
υ_{max}	Maximum enzyme reaction rate.

APPENDIX A1

FED BATCH FERMENTATION IN 800 LITRE VESSEL AT BIRMINGHAM UNIVERSITY

A1.1 DESCRIPTION OF APPARATUS

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 circuity 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 pinholes, 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 170mm 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 80mm 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 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 ± 0.2°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 ± 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 longitudinal viewing windows, with a three valve set being fitted to the base of each vessel to allow separate sterilization of the feed line.

A1.2 INOCULUM PREPARATION

MRS broth was used as the medium in each stage of inoculum development. Initially, six McCartney bottles, with a volume of 10 cm^3 broth, were inoculated with cells from two slopes of culture. These cells were incubated at a temperature of 25°C for approximately 12 hrs, after which time the cells were transferred to six flasks, each containing 250 cm^3 broth. This inoculum was then transferred after 13 hrs to three flasks, each containing approximately 16 litres of MRS broth, with the culture from two 250 cm^3 flasks being added to each 16 litres of sterile growth medium.

This final inoculum was incubated for 15hrs, 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 OD_{590} value of about 0.1. The inoculum was transferred to the main fermenter using a simple gravity feed technique. The three-stage inoculation procedure for this fermentation experiment is given diagramatically in Figure A1.1.

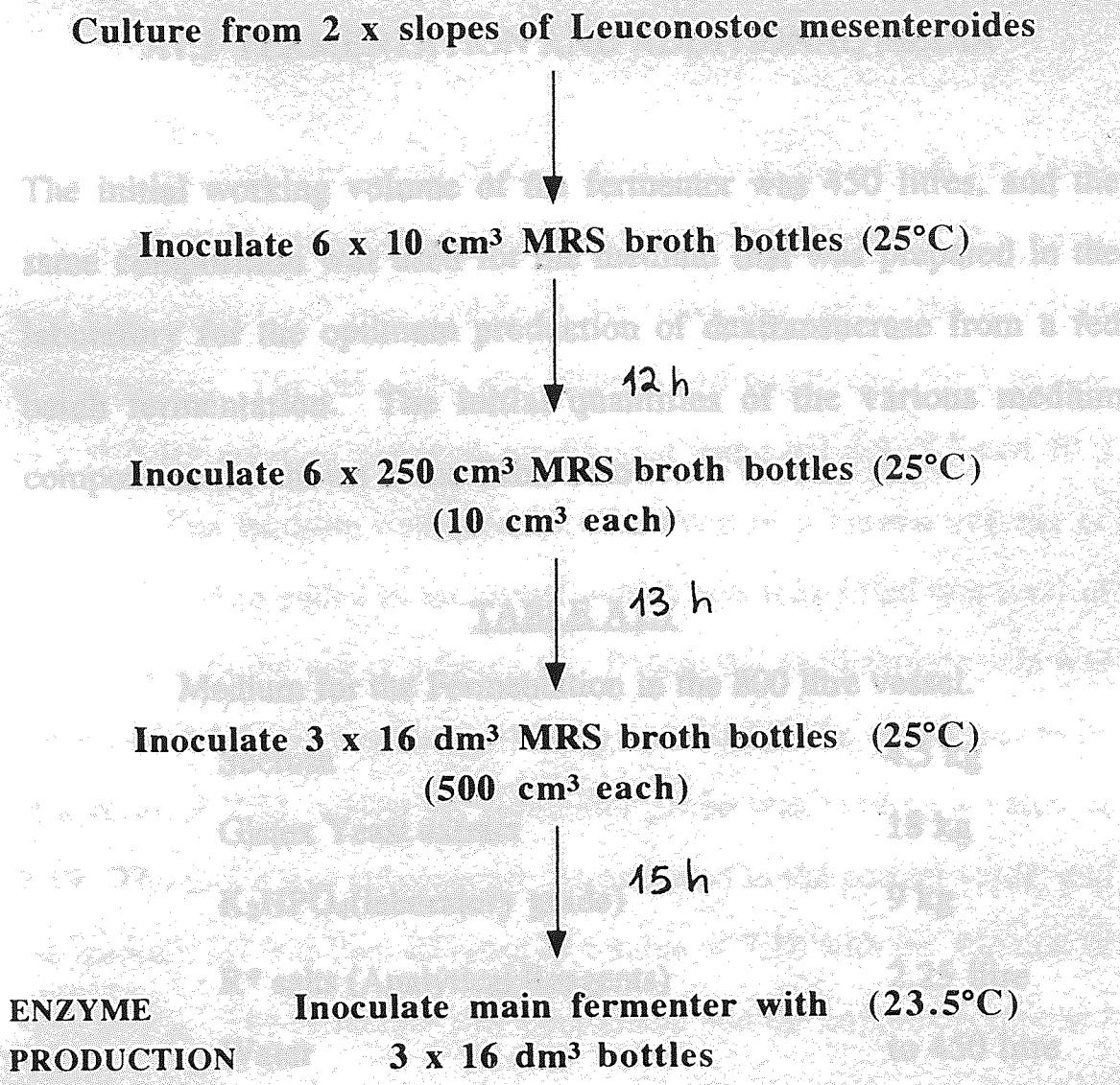

Figure A1.1 Inoculum Development Process for 800 litre Fermentation

A1.3 FERMENTATION AND ADDITIONAL MEDIA

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 the Table below.

TABLE A1.1

Medium for the Fermentation in the 800 litre vessel.

Sucrose	4.5 kg
Gistex Yeast extract	18 kg
K_2HPO_4(laboratory grade)	9 kg
R* salts (Analytical Reagents)	2.25 litre
Water	to 450 litre
H_2S0_4 (98%)	~0.25 litre (to pH 7.1)
R* salts	
$MgSO_4 . 7H_2O$	90 g
NaCl	4.5 g
$FeSO_4 . 7H_2O$	4.5 g
$MnSO_4 . 4H_2O$	4.5 g

The sucrose/NaOH solution was prepared according to the procedure reported in section 6.2.3.

A1.4 FERMENTER PREPARATION AND STERILIZATION

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% H_2SO_4. The fermenter was then sealed and the computer, supplied as an integrated part of the unit, was programmed with the details of the sterilization cycle required. The fermenter was sterilized at 121°C for 20 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.