# THE PRODUCTION OF THE ENZYME DEXTRANSUCRASE BY BATCH AND CONTINUOUS FERMENTATION TECHNIQUES

A thesis submitted by
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PhD NJ AJONGWEN-NUMFOR 1988

## SUMMARY

A review of the literature of work carried out on dextransucrase production, purification, immobilization and reactions has been carried out. A brief review has also been made of the literature concerning general enzyme biotechnology and fermentation technology.

Fed-batch fermentation of the bacteria *Leuconostoc mesenteroides* NRRL B512 (F) to produce dextransucrase has formed the major part of this research. Aerobic and anaerobic fermentations have been studied using a 16 litre New Brunswick fermenter which has a 3 - 12 litre working volume. The initial volume of broth used in the studies was 6 litres. The results of the fed-batch fermentations showed for the first time that yields of dextransucrase are much higher under the anaerobic conditions than during the aerobic fermentations.

Dextransucrase containing 300-350 DSU/$cm^3$ of enzyme activity has been obtained during the aerobic fermentations, while in the anaerobic fermentations, enzyme yields containing 450-500 DSU/$cm^3$ have been obtained routinely. The type of yeast extract used in the fermentation medium has been found to have 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. Studies have also been carried out on the effect of agitation rate and antifoam on the enzyme production during the anaerobic experiments. Agitation rates of up to 600 rpm were found not to affect the enzyme yield, however, the presence of antifoam in the medium led to a significant reduction in enzyme activity (less than 300 DSU/$cm^3$).

Scale-up of the anaerobic fermentations has been performed at up to the 1000 litre level with enzyme yields containing more than 400 DSU/$cm^3$ of activity being produced. Some of the enzyme produced at this scale was used for the first time to produce dextran on an industrial scale via the enzyme route, with up to 99 % conversion of sucrose to dextran being obtained.

An attempt has been made at continuous dextransucrase production. Cell washout was observed to occur at dilution rates greater than 0.4 $h^{-1}$. Dextransucrase containing up to 25 DSU/$cm^3$/h has been produced continuously.

**Keywords**: Dextransucrase, dextran, *Leuconostoc mesenteroides*, fermentation.

Dedicated to

**Mr and Mrs VAT ANTUNKA**

and

to the memory of my late uncle

**JOHN NUMFOR**

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# CHAPTER ONE

# INTRODUCTION

# CHAPTER ONE

# INTRODUCTION

## 1.1 DEXTRANSUCRASE

Dextransucrase is an extracellular enzyme which is responsible for the conversion of sucrose to dextran and fructose. It is an inducible enzyme that requires only sucrose for its induction. Dextransucrase is produced by a vast number of bacteria, the most widely used being *Leuconostoc mesenteroides* NRRL B512(F). The dextran produced by this enzyme is widely used in the pharmaceutical industry as a blood volume expander, a blood flow improver and as an iron complex for the treatment of anaemia. Dextran is also used in oil drilling muds, as a gelling agent in the food industry and for the manufacture of molecular sieves used for separation processes.

## 1.2 PRODUCTION OF DEXTRANSUCRASE

The enzyme is elaborated by growing bacteria under suitable conditions of pH, temperature and nutrient supply. pH of 6.7 and temperatures between 20 $^{o}$C and 30 $^{o}$C have generally been used for dextransucrase production. Sterile broth containing the required nutrients is inoculated with an actively growing bacterial culture where the cells grow under the specified process conditions and elaborate the enzyme into the growth medium. The enzyme is produced while the cells are growing. Once cell growth has ceased, the fermentation is terminated and cell separation carried out. The enzyme is then extracted and purified by a series of precipitation and acid hydrolysis processes. The pure enzyme can then be used either to study its properties or to produce dextran.

Traditionally, dextran is produced by whole cell fermentation. During this process, excess sucrose is present in the growth medium. The pH of the medium during the fermentation is normally not controlled, and as the cells grow, enzyme is elaborated into the medium where it converts the excess sucrose to dextran. The resulting broth at the end of the fermentation is very viscous and contains mainly dextran which can then be precipitated out of the

growth medium and purified. After purification, the crude or native dextran may then be fractionated to produce dextrans of particular molecular weight distributions.

Industrial production of dextran is carried out using the whole cell fermentation process. Enzymatic production of dextran has mainly been carried out using laboratory scale fermentations. This is partly due to the fact that the enzyme production processes have generally been carried out on a small scale, and partly because of the fermentation conditions that have been employed. The largest scale batch production of dextransucrase reported in the literature was by Schneider and co-workers[53]. They obtained up to 300 DSU/$cm^3$ of enzyme activity on the 300 litres scale. Paul *et al.*[64] obtained 70 DSU/$cm^3$/h of enzyme activity when they carried out the fermentation continuously.

Recent biotechnological developments have led to the need for large quantities of high activity cell-free enzyme preparations. The novel techniques that have led to this increased enzyme demand include enzyme immobilization[60,65,83], its use in chromatographic bioreactor/separators and the possibility of higher efficiency of dextran production by the enzymatic route. This project has thus been carried out to produce high activity dextransucrase on a large scale using *Leuconostoc mesenteroides* NRRL B512(F).

## 1.3 AIM OF THE PROJECT

The aim of this project is to investigate the best conditions for producing large amounts of high activity dextransucrase using batch and continuous fermentation techniques.

Included in the aim are:

(1) A review of the available techniques, especially the predominantly batch process which have led to the production of high activities of dextransucrase.

(2) An investigation into the different parameters that affect the yields of dextransucrase so as to determine those that need to be

studied more closely.

(3) A determination of the best conditions under which the selected parameters could be operated, so as to produce dextransucrase containing preferably in excess of 400 DSU/$cm^3$ of activity routinely.

(4) A strategy for scaling-up the process to a scale that would produce enzyme which may be used for industrial production of dextran.

The project has been carried out in a number of stages as described in the different chapters of this thesis:

A survey of the literature on general enzyme biotechnology, fermentation technology and on dextransucrase has been carried out in Chapters 2, 3 and 4 respectively;

Chapter 5 describes all the experimental and analytical techniques used throughout the course of this research work;

The results of the different investigations that were undertaken using aerobic batch fermentations are outlined in Chapter 6. Some of the studies included the use of different yeast extract types in the fermentation medium and a study of the effect of the aeration rate on the fermentation results;

Chapter 7 describes the anaerobic fermentations that produced dextransucrase containing in excess of 450 DSU/$cm^3$ of enzyme activity. Results of studies on the effects of agitation rate, antifoam and different yeast extract types are also presented in this chapter;

Scale-up of the anaerobic batch fermentation process, up to 1000 litres scale, has successfully been carried out. Chapter 8 describes how the scale-up processes were undertaken. An attempt was also made in this section to

model the batch processes using a computer simulation package. The chapter also contains the results of preliminary experiments on continuous dextransucrase production;

A comparative discussion of the results, especially those of the aerobic and the anaerobic processes, is detailed in Chapter 9. An outline of the conclusions drawn from the project is then presented, together with those aspects of the work that would require further attention.

# CHAPTER TWO

# LITERATURE SURVEY 1

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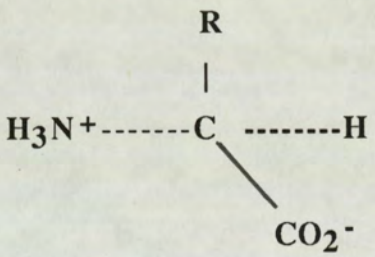
# LITERATURE SURVEY 1

# ENZYME BIOTECHNOLOGY

## 2.1 INTRODUCTION

Enzymes are organic substances produced by living cells[1]. They have the ability to catalyse specific chemical reactions. Enzymes are proteins that synthesise or breakdown chemical compounds (substrates), or transform them from one type to another (products) according to their ability to build them individually. They are highly specific in the type of reaction catalysed. There are two broad types of enzymes; intracellular (those that remain within the cells) and extracellular enzymes (those secreted out of the enzyme producing cells). Enzymes are either produced at all times (constitutive enzymes), or synthesised in response to an inducing agent stimulating their expression ( inducible enzymes ).

All enzymes consist primarily of a protein molecule which in essence is a linear polymer of amino acids of the general structure:




The central carbon is known as the α-carbon to which are linked an amino group ($NH_3^+$), a hydrogen atom, a carboxyl group ($CO_2^-$) and a fourth known as an R group or side chain. There are twenty different side chains of varying size, polarity and acid-base properties. Two or more amino acids can condense to form a linear polypeptide, in which the amino acids are linked together by amide bonds. The amino acids are linked in specific sequence as defined by the genetic information of the cell. This forms what is known as the primary structure of the enzyme and contains the active sites of the enzyme[1-3]. The active site consists of relatively few amino acids that actually have a direct role in binding the substrate and catalysing the reaction

characteristic of each particular form of enzyme molecule. The secondary structure of an enzyme are those sections of a polypeptide chain which assume certain well defined structures such as the α-helix. The tertiary structure refers to the overall coiled structure of the polypeptide as maintained by the secondary forces including hydrogen, ionic and hydrophobic bonds. The quartenary structure describes the way in which certain complex enzymes (usually intracellular) consist of a number of polypeptide chains associated by means of secondary forces to form multi-subunit enzymes.

Some enzymes consist only of polypeptides and contain no other chemical groups. Others require chemical compounds called cofactors for their activities. The cofactor may either be inorganic such as $Fe^{2+}$, $Mn^{2+}$, $Zn^{2+}$, or complex organic molecules called coenzymes[4]. In some enzymes the cofactors are loosely or transiently bound to the enzyme while in others, they are tightly or permanently bound and are known as prosthetic groups. A complete catalytically active enzyme together with its cofactors is known as a holoenzyme. The protein part is known as the apoenzyme.

## 2.2 CLASSIFICATION OF ENZYMES

Enzymes are classified by the reactions they catalyse[1-3,5-6]. Generally, they are divided into six different groups. These are oxidoreductases, transferases, hydrolases, lyases, isomerases and ligases.

**1) Oxidoreductases:** Catalyse oxidation-reduction reactions involving oxygenation or overall removal or addition of hydrogen atom equivalents.

**2) Transferases:** Mediate the transfer of various groups such as aldehydes, ketones, acyl sugar, and so on from one molecule to another.

**3) Hydrolases:** Catalyse a broad group of hydrolysable compounds including esters, amides and other C-N containing functional groups.

**4) Lyases:** Catalyse additions to, or the formation of, double bonds such as C=C, C=O, and C=N.

**5) Isomerases:** Catalyse various types of isomerization reactions.

**6) Ligases:** These are often termed synthetases. They catalyse the formation of C-S, C-O and C-N bonds.

Each group of enzymes is further subdivided into sub-classes, to specify the type of reaction more fully and to indicate the chemical nature of the reactant. This method of classification leads to enzymes being identified by a chemically meaningful Enzyme Commission (EC) number.

## 2.3 SOURCES OF ENZYMES

Enzymes are constituents of living cells. Cells normally produce just enough enzyme to meet their requirements, but some are capable of producing excess amounts of enzyme. As a result these can serve as a useful source for the biosynthesis of enzymes. They include cells of certain animal glands or mucus membranes, cells in certain plant tissues and cells of specific microorganisms. These have been isolated and utilised for the production of industrial enzymes.

## 2.4 MEASUREMENT OF ENZYMES

The amount of enzyme present in a system is determined by the number of units of its activity. The activity gives an indication of the amount of enzyme that will be required to achieve the conversion of a certain amount of substrate to a desired product. Enzyme activity is expressed in arbitrary units per milligram (mg) or unit volume of enzyme solution (the activity being related to the decomposition of a certain amount of substrate, or formation of a certain amount of product in a given period of time). It can also be expressed in terms of a velocity constant of the reaction/mg of enzyme, or as moles of substrate reacting/min/mg of enzyme. Units of activity are arbitrary and quite different for each enzyme, thus making it

difficult to compare enzyme activities[6].

The commission on enzymes of the International Union of Biochemistry defined an enzyme unit (U) as that amount that will catalyse the transformation of one micromole of substrate per minute under defined conditions. This was followed by the introduction of the S.I. unit of Katal (Kat) by the commission on Biochemical Numenclature. One Kat is defined as the amount of enzyme which will cause the transformation of one mole of substrate into products in one second under defined conditions[7]. The defined conditions include temperature (25 $^{o}$C wherever possible), and optimal pH and substrate concentration. The two units of activity widely used are:-

**Specific activity** - Units of activity per mg of protein.

**Molecular activity** - Units of activity per micromole of enzyme.

It is recommended that the measurements be based on the measurement of initial rates of reaction in order to avoid complications due, for example, to reversibility of reaction or the formation of inhibitory products (see Section 2.8).

## 2.5 PRODUCTION AND PURIFICATION OF ENZYMES

### 2.5.1 Production

Plant and animal tissue cells and microorganisms are important sources of industrial enzymes. The supply and cost of each source governs the selection of the particular source.

#### 2.5.1.1 Enzymes from animal and plant cells

Animal cell enzymes are obtained exclusively from animal tissues[1]. Highest concentrations are found in cells of glands such as pancreas, stomachs, and spleen. Extraction of the enzymes from enzyme-rich tissues is carried out immediately the animal is killed or the tissue extracted. Loss of activity is prevented by drying or freezing.

Plant cell enzymes require the supply, well in advance, of large amounts of plants. The production of enzymes from plant and animal cells involve a number of stages which include:

(1) Selection of cells actually or potentially rich in enzyme.

(2) Enrichment of the desired enzyme(s) in a carefully selected group of cells.

(3) Extraction of enzymes from the cells.

(4) Concentration and purification of the enzymes.

(5) Stabilisation of enzymes to prevent of loss of potency.

(6) Standardisation of the enzyme activity in the product.

#### 2.5.1.2 Enzymes from microbial cells

Fast growth and the ability of many microorganisms to produce enzymes in excess of their required amounts have made them most favoured as enzyme sources. Screening for the most suitable organism is the first stage. The most outstanding strain selected will be that which will give the highest enzyme activity, good cell yield, require inexpensive nutrient, is not sensitive to infection or mutation, etc. If necessary, strain improvement by techniques of genetic engineering (e.g. artificial mutation, hybridization, and cloning) will be carried out.

The culturing of the selected microorganism under suitable conditions leads to high enzyme production. The enzyme is then extracted from the growth medium and purified. The extraction process employed depends on whether the enzyme is extracellular or intracellular.

### 2.5.2 Enzyme extraction

#### 2.5.2.1 Introduction

Enzymes generally occur either within cells (intracellular) or outside cells (extracellular enzymes) and their extraction and purification depend on the type of enzyme produced. Figure 2.1 shows a generalised process for the extraction and purification of enzymes from cells .

#### 2.5.2.2 Extraction of enzymes

Extraction of enzymes from cells is influenced by a number of factors such as the nature of the source, the scale of operation, the stability of the enzyme, and the degree of purification. The choice of the process used depends on the actual cellular location of the enzyme . If extracellular, no

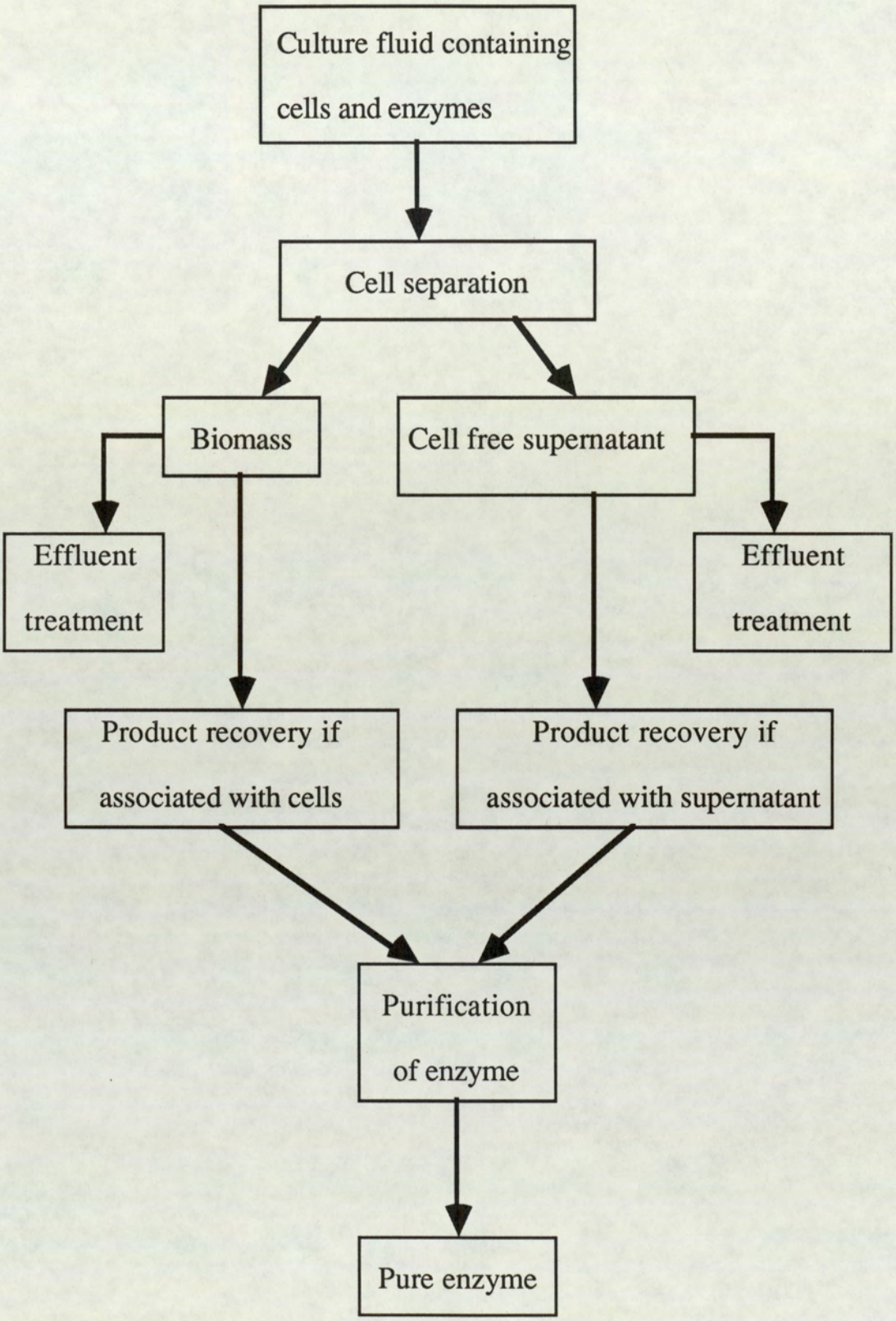



**Fig. 2.1: Generalised process for extraction and purification of enzymes ( ref 8 ).**

extraction is necessary. For intracellular enzymes a certain degree of cell disruption is required[5-7,9,12]. The methods of cell disruption and enzyme extraction widely used include:

## A) Physical methods

**1) Grinding or agitation**: With abrasives such as glass beads.

**2) Liquid shear:** A suspension of the cells is passed at high pressure through a small orifice into a chamber at atmospheric pressure. The sudden drop in pressure causes the cells to be literally blown apart.

**3) Sonication:** Ultrasonic treatment is applied to cause the cells to disintegrate.

**4) Freezing and thawing.**

**5) Solid shear :** This involves freezing a cell paste (usually to -20 $^{o}$C ) and forcing it through a small hole at high pressure. The hole acts as an abrasive agent, causing the cells to disrupt.

## B) Chemical methods

**1) Alkalis :** Most cells are disrupted under conditions of high alkalinity (pH 11.5 - 12.5). Thus alkalation of cells will be useful for extracting the enzyme if it is stable under these conditions.

**2) Lysosomes and EDTA:** Lysosome is an enzyme produced commercially from hen egg white. It catalyses reactions which lead to the breakdown of cell walls. If EDTA (ethylenediamine tetraacetic acid) is added, it causes the release of cell material.

**3) Detergent:** Ionic and non-ionic detergents (e.g. sodium lauryl sulphate and Tweens) dissolve lipoproteins of cells under appropriate pH and temperature and can thus be used for their extraction.

**4) Osmotic shock:** The cells are washed in buffer to free them from the growth medium and resuspended in, say, a 20 % sucrose

solution. This leads to loss of cell water. The cells are centrifuged and then resuspended in water at about 4 $^{o}$C. This causes an increase in the cell osmotic pressure and leads to the release of certain cell constituents. The large volumes of liquid involved, the number of centrifugation stages involved and the necessity to work at low temperatures makes large scale application of this method limited.

**5) Organic solvents:** Organic solvents, e.g. toluene, are effective for disrupting a multitude of cells. They are used both for extracting and purifying enzymes.

## 2.5.3 Enzyme Purification

### 2.5.3.1 Introduction

Enzymes occur in complex mixtures. They are usually present in mixtures containing cells (disrupted or not), growth medium components as well as other enzymes. Generally the cost of extracting and purifying an enzyme from its source is a major cost component in the overall cost of producing the enzyme. The concentration of the desired enzyme in the complex medium is usually very low. Various methods exist for purifying enzymes from their complex mixtures. The process adopted should be that which would not adversely affect the enzyme itself such as removing the cofactor of the enzyme, changing its properties or causing it to denature. The degree of purification depends on the enzyme's intended use.

During the purification process some of the enzyme activity is lost. It is thus important to be able to assess the recovery of the enzyme at the end of each purification stage. This requires a quick and accurate method of testing for the enzyme activity (see Section 2.7).

### 2.5.3.2 Removal of solids

The first step in the purification of an enzyme is the removal of cell matter which could be whole or disrupted cells. This is generally achieved by centrifugation. Filtration techniques such as microfiltration and ultrafiltration are also increasingly being used[13-14]. When the required enzyme is intracellular, removal of cell constituents (nucleic acids) should also be

included in the initial solids removal stages.

### 2.5.3.3 Enzyme isolation and concentration

Several methods are applied to isolate and concentrate the desired enzyme from enzyme containing supernatants. They include:

**I) Precipitation:** The precipitation technique could be such that either the enzyme or the impurities are precipitated. Due to the large volumes of supernatant involved, enzyme precipitation is generally preferred. Salts like ammonium sulphate which are cheap, non-toxic to most enzymes, highly soluble and in some cases have stabilising effects(5) are widely used for enzyme precipitation. Organic solvents, for example, polyethylene glycol (PEG) have also been employed. The problems with organic solvents include their tendency to denature proteins, and their high flamability.

**II) Adsorption:** Various amounts of adsorbent are stirred with the enzyme solution. If the enzyme is adsorbed, it can be separated (usually by centrifugation) and afterwards extracted or eluted from the adsorbent. If not, adsorption can be used to remove unwanted materials from the enzyme solution.

**III) Chromatography:** In recent years, purification of enzymes by column chromatography has become the most effective of all enzyme isolation methods. The mechanisms of separation depend in different cases on adsorption, ion exchange, specific affinity to immobilised ligands or molecular sieving effects. The main types of chromatographic operations employed in enzyme purification are gel chromatography, ion exchange chromatography and affinity chromatography.

Gel chromatography is carried out by means of diffusional partitioning of solute molecules between a readily mobile phase and the confined spaces within the porous particles that make up the stationary phase, i.e. the gels. High molecular weight compounds elute first, but because of interaction with the stationary phase, the low molecular weight compounds are retained longer.

Three types of ion exchange materials generally used are ion exchange resins, celluloses and gels[5]. Ion exchange chromatography is based on the selective removal of enzyme molecules onto a solid phase due to interaction with groups or ions on the solid phase. A change in pH, ionic strength of solvent (or a combination of both) will cause enzyme to be transferred back into solution.

Affinity chromatography depends on specific interaction of an enzyme with an immobilised ligand. Usually, non-specific adsorbents are used in affinity chromatographic columns. The drawback of such systems is that they are useful only for small scale preparatory systems such as the High Performance Liquid Chromatography (HPLC) systems.

**IV) Ultrafiltration:** Exploitation of the differences in molecular weight (size) of different proteins is the basis of operation of ultrafiltration. Permeable membranes are used to separate macromolecules from smaller molecules in solution. When a solution containing high and low molecular weight compounds is pumped at high pressure through a membrane, molecules which are smaller than the pore sizes of the membrane pass through it. The solution which passes through the membrane is known as the permeate and its rate of flow is the flux. The high molecular weight stream that does not pass through the membrane is called the retentate. Membrane filters of different configurations are generally used, e.g. hollow fibre and sheet membranes.

**V) Electrophoresis:** Enzymes can be purified by this technique because of their characteristic mobility (as electrolytes) when placed in an electric field. This method is applicable to work on a small scale. It has been used widely for the study of various ionising groups (-COOH, $-NH_2$, -OH, and -SH) on the enzyme molecules[10].

**VI) Crystallization:** Crystallization of the enzyme using various techniques can also be useful as a means of purifying it. This method is very expensive and often requires repeated crystallization to obtain the desired level of purity.

## 2.6 ENZYME KINETICS

Enzymes catalyse reactions by reducing the activation energy of the reaction. They do this by forming a complex between the enzyme and the subtrate. The activity of an enzyme is determined by the enzyme concentration, the presence and type of inhibitor, the ionic strength of the reaction medium, pH and temperature. The way in which these parameters affect the enzyme is determined by a study of the kinetics of the enzyme reaction. For a single substrate (S) being converted to a single product (P), the general reaction scheme is

$$S + E \underset{k_2}{\overset{k_1}{\rightleftharpoons}} ES \longrightarrow E + P \qquad \text{------2.1}$$

When the substrate concentration is low,the rate of reaction (V') is proportional to the substrate concentration, i.e. first order reaction. At high substrate concentrations the reaction becomes saturated, reaches a maximum rate ($V_{max}$) and the rate becomes independent of the substrate concentration until nearly all the substrate is consumed, i.e. zero order reaction. Figure 2.2 is a representation of the effect of substrate concentration on reaction rate.

The Michaelis-Menten relationship best described the kinetics of an enzyme reaction as described by the model above (Equation 2.1)

$$V' = \frac{K_2 [E] [S]}{[S] + K_m} \qquad \text{..... 2.2}$$

where $K_m = (k_1 + k_2)/k_1$

For a system where the substrate concentration is in excess and there is no inhibition,

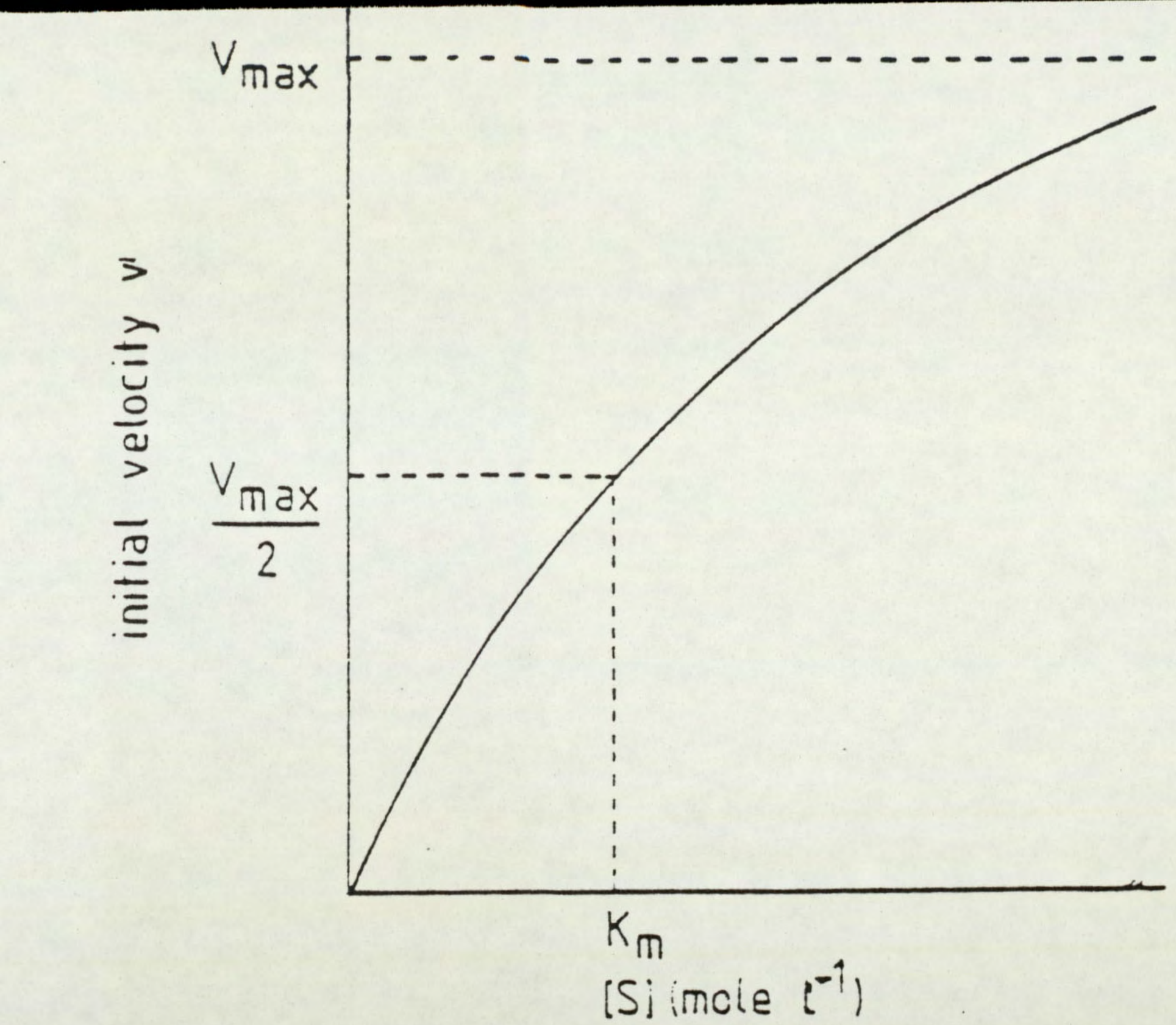



**Figure 2.2: Initial reaction rate as a function of substrate concentration**

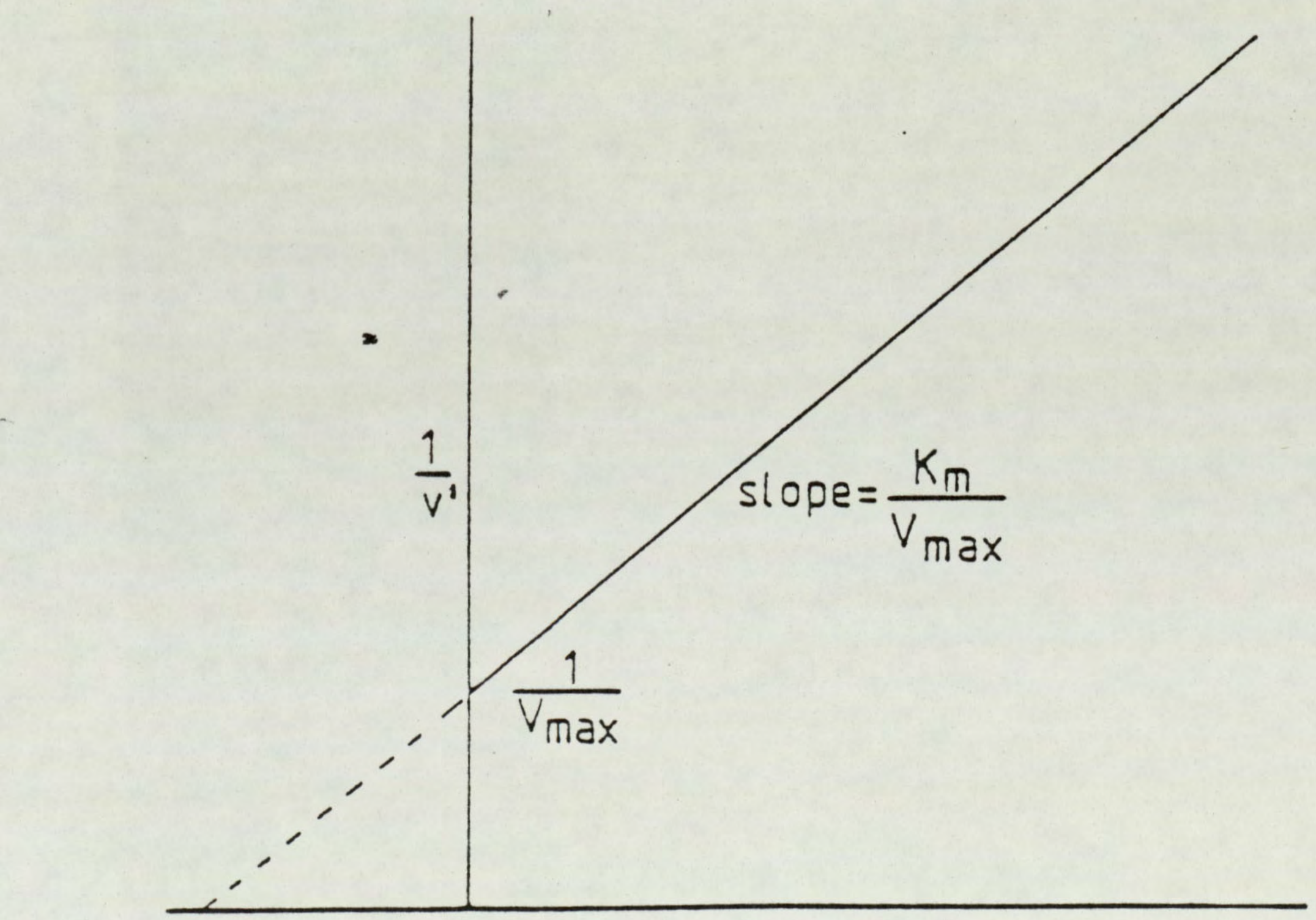



**Figure 2.3: Lineweaver- Burke plot**

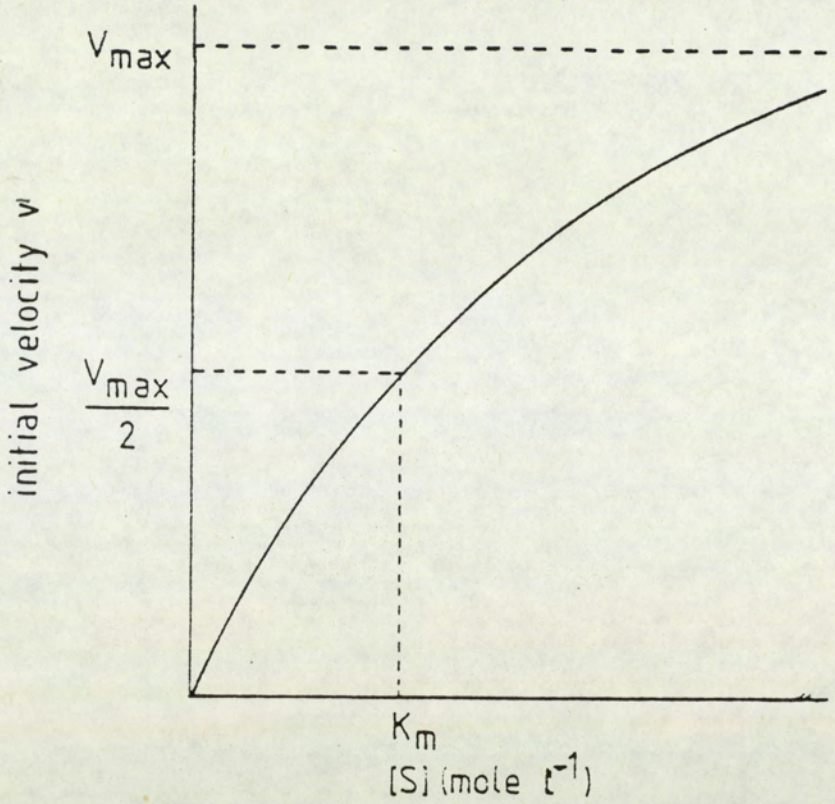



**Figure 2.2: Initial reaction rate as a function of substrate concentration**

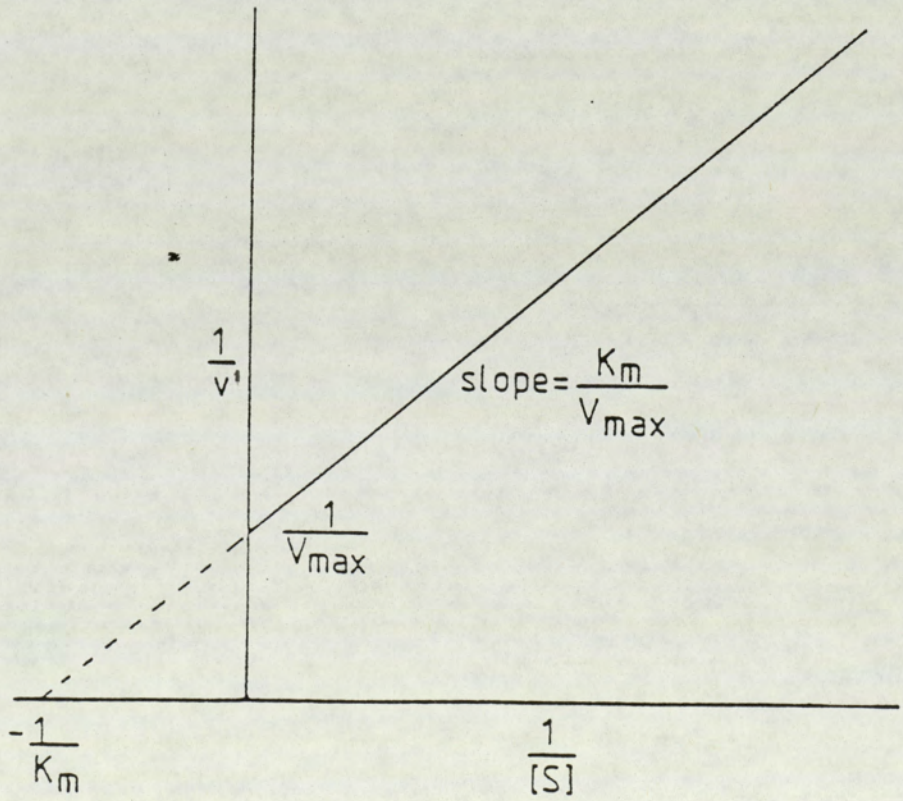



**Figure 2.3: Lineweaver- Burke plot**

$$V' = V_{max} = k_2 [E] \qquad \text{..... 2.3}$$

$$V' = \frac{V_{max} [s]}{[s] + K_m} \qquad \text{..... 2.4}$$

$K_m$ describes the affinity of the enzyme for its substrate and is generally known as the saturation constant or the Michaelis-Menten constant(7). The higher the value of $K_m$, the the lower the affinity of the enzyme for its substrate. Numerical values for $V_{max}$ and $K_m$ can be estimated from a plot of the reaction rate (V') against the substrate concentration as shown in Figure 2.2. $K_m$ is the substrate concentration when V' is equal to half of $V_{max}$.

More accurate values of $K_m$ and $V_{max}$ can be obtained by an inverted form of Eqn. 2.4. This is known as the Lineweaver-Burke equation.

$$\frac{1}{V'} = \frac{K_m}{V_{max}\, s} + \frac{1}{V_{max}} \qquad \text{..... 2.5}$$

This equation is a linear relationship of the form $y = mx + c$ . $K_m$ and $V_{max}$ can be obtained form the plot of this relationship as shown in Figure 2.3. This plot is also referred to as the double reciprocal plot.

The Lineweaver-Burke plot is also useful in determining the type of inhibition that affects a particular enzyme reaction. Figures 2.4 and 2.5 show the patterns of the Lineweaver-Burke plots for different inhibitor types.

Inhibition of enzymes occur when inhibitor chemicals bind either at the enzyme's active sites or at its control (allosteric) site. It can be reversible, i.e. competitive or non-competitive as shown in Figures 2.4 and 2.5, or irreversible. Irreversible inhibition occurs when the inhibitor reacts with some functional groups essential for the enzyme catalytic activity and either modify or destroy them(11). Competitive reversible inhibition occurs when the inhibitor (which usually resembles the substrate) competes with the substrate for binding to the active site but is not transformed by the enzyme. The effect can be reduced by increasing the ratio of substrate to inhibitor in the reaction

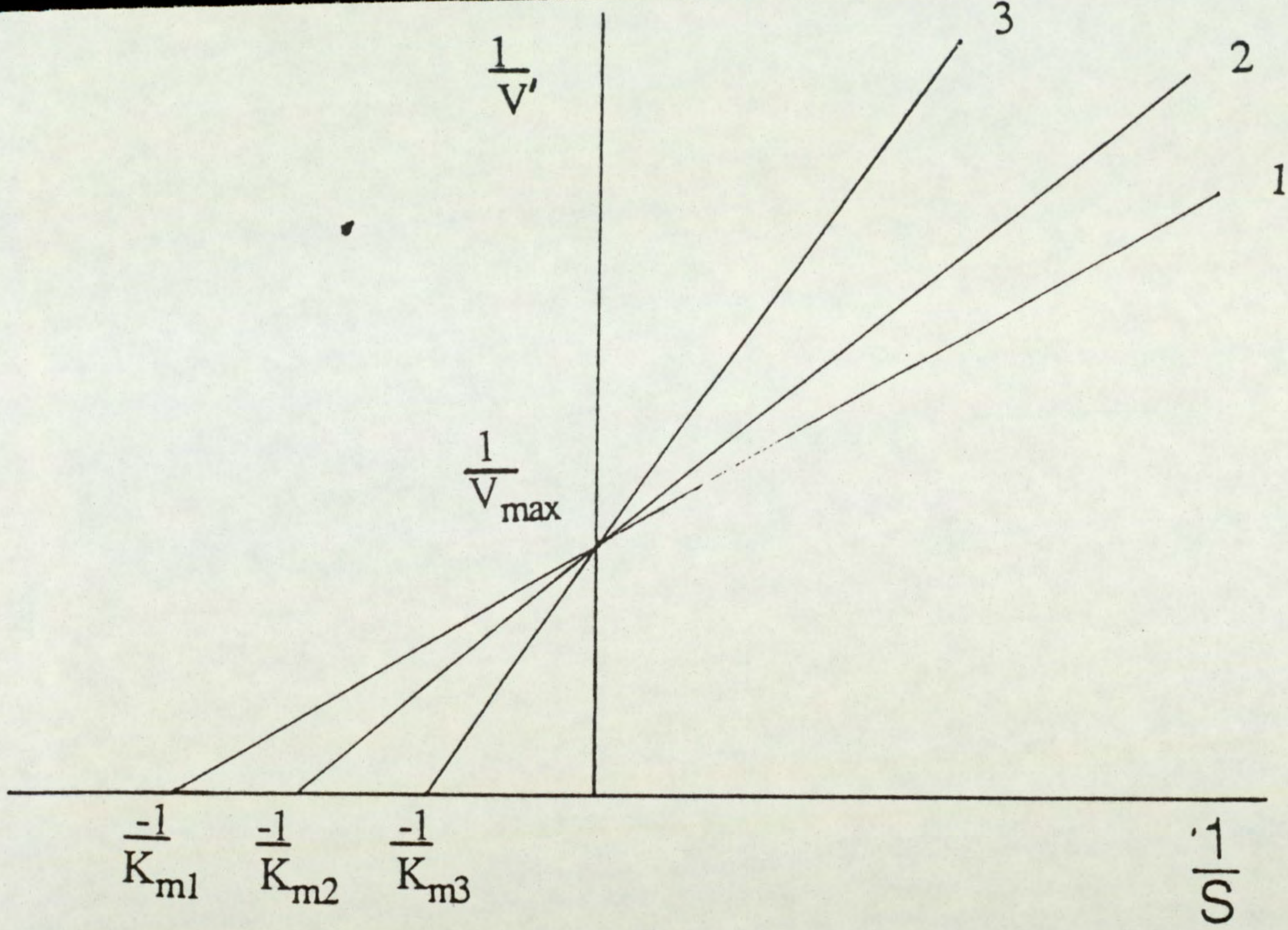



**Figure 2.4: Competitive inhibition**
**Inhibitor concentration 1<2<3**

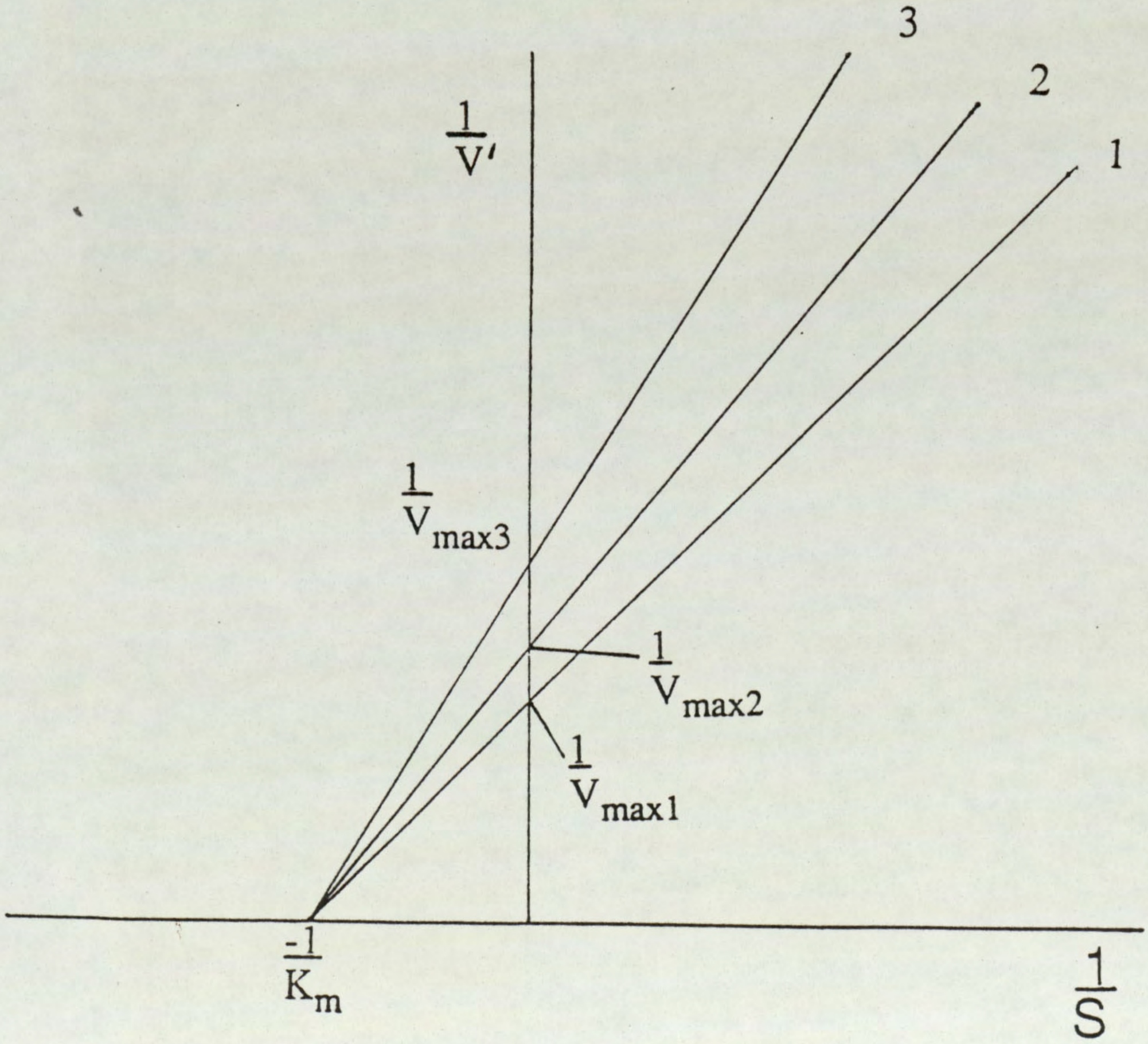



**Figure 2.5: Non-competitive inhibition**
**Inhibitor concentration 1<2<3**

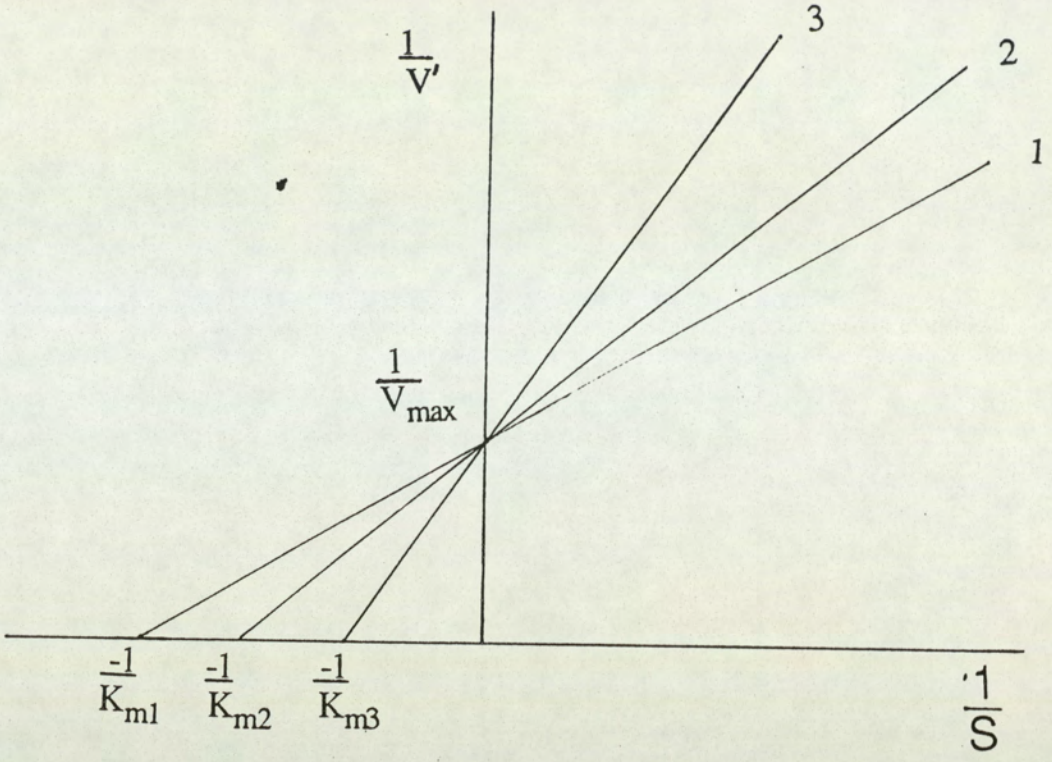



**Figure 2.4: Competitive inhibition**
**Inhibitor concentration 1<2<3**

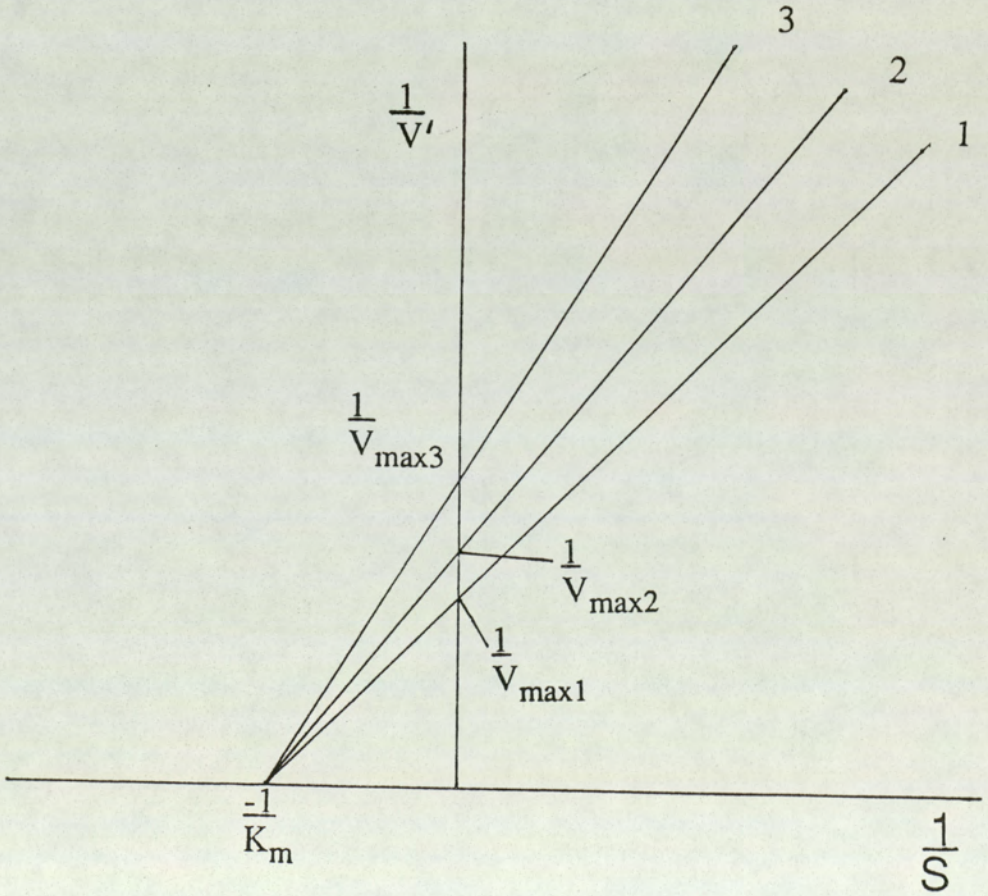



**Figure 2.5: Non-competitive inhibition**
**Inhibitor concentration 1<2<3**

medium. Non-competitive inhibition occurs when the inhibitor binds to some other part of the enzyme, e.g. the control or allosteric sites and alters the binding characteristics of the enzyme. This is not altered by changing the level of substrate.

Another method of obtaining the values of $K_m$ and $V_{max}$ is by the use of the Eadie-Hofstee plot (Figure 2.6) This uses the Lineweaver-Burke equation as the starting point, and multiplies both sides by $V_{max}V'$ to give a relationship for V' as shown in the equation below:

$$V' = \frac{K_m V'}{V_{max}} + V_{max} \qquad \text{..... 2.6}$$

The values of $V_{max}$ and $K_m$ are obtained as shown in Figure 2.6. Less accurate values of $K_m$ and $V_{max}$ can also be calculated by integrating the Michealis-Menten equation. This gives:

$$V_{max}t = (so-s) + K_m \ln\left(\frac{so}{s}\right) \qquad \text{..... 2.7}$$

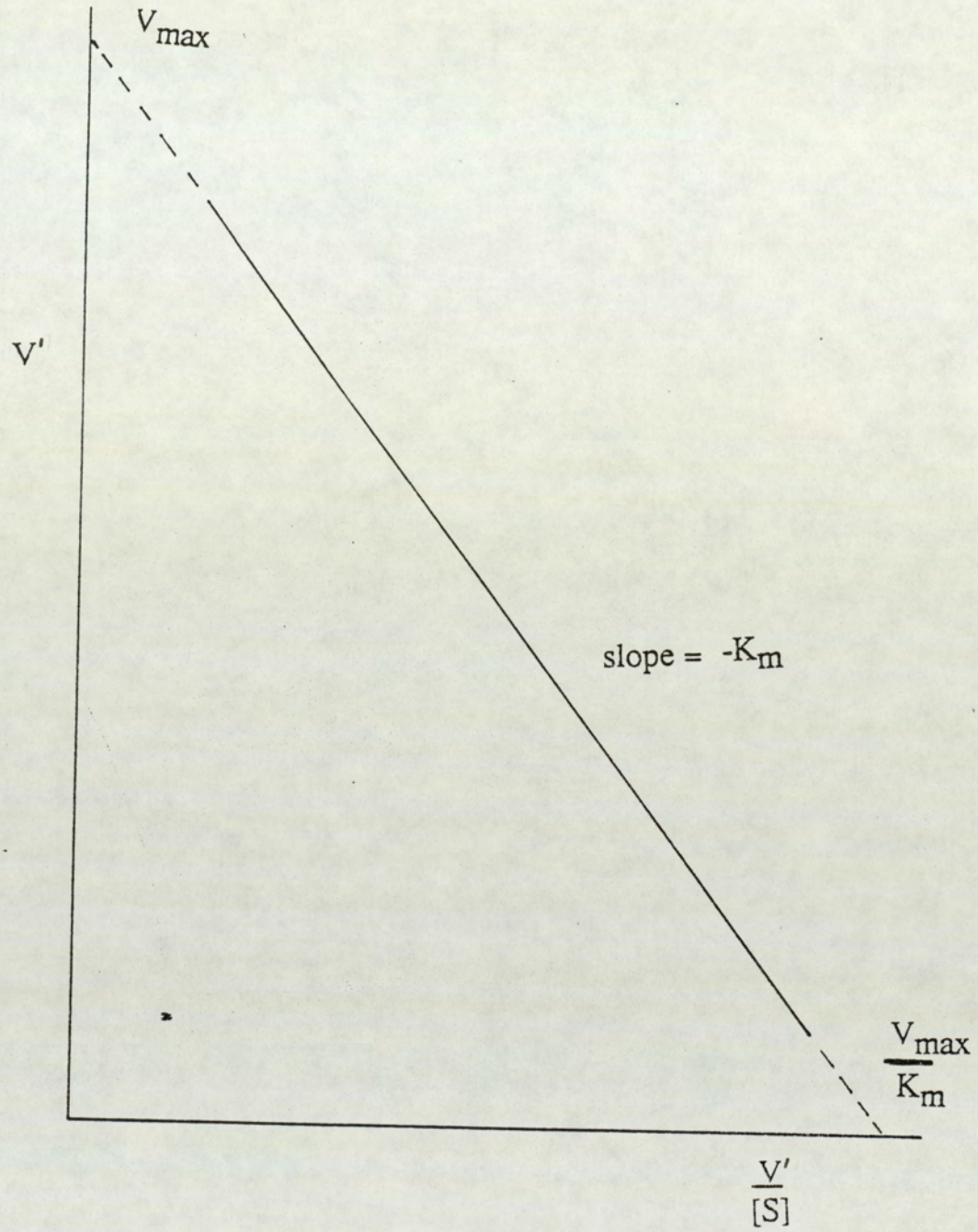



**Figure 2.6: Eadie- Hofstee plot**

## 2.7 METHODS OF FOLLOWING ENZYME REACTIONS

There are a number of methods for monitoring and assaying enzyme catalysed reactions. They usually involve a direct or indirect method of measuring either the rate of product formation, rate of substrate consumption or both. The methods should be both fairly quick to carry out and accurate. The most widely used are spectrophotometric, fluorescence, electrode, polarimetric, and sampling methods[6].

1) **Spectrophotometric method:** This method relies on the fact that many substrates and products of enzyme reactions absorb light either in the visible or ultraviolet regions of the spectrum. Usually, the products and substrates do not give identical spectra at the same wavelength. A suitable wavelength where conversion of substrate to product is accompanied by a considerable change in absorbance is used for monitoring the progress of such a reaction. The absorbance (or optical density) of the sample can then be related to the activity of the enzyme.

**2) Electrode method:** This method involves the use of a glass electrode to follow a reaction which produces an acid. The pH profile of such a reaction is an indication of the activity of the enzyme. Its main drawback when used in this way is the fact that the pH affects the activity of the enzyme (see Section 2.8). Also, the presence of proteins can increase the buffering ability of the solution and thus show a reduced pH change. As a result, this method is not suitable for use in crude enzyme systems. The technique is generally used by linking the reaction to a titration system where the pH is kept constant by the addition of alkali and the reaction rate related to the alkali addition. However, the initial amount of buffer still affects the results.

**3) Polarimetric method:** This method is quite useful for enzyme systems where the enzyme acts only on one optical isomer of a substrate. If either the substrate or product is optically inactive, a change in rotation may be used as a means of following the course of the reaction. Application of this method is also possible when both the substrate and product are active, but their specific rotations differ.

**4) Sampling method:** With this method, samples are withdrawn at intervals of time and the substrate or product analysed. The analytical methods include colourimetric and HPLC techniques. HPLC techniques are widely used because they have the advantage of simultaneously analysing both the substrate and the product, as well as showing other byproducts present. However, the type of HPLC column and eluent used, and the conditions of operation should be carefully chosen so as not to affect the results. This method sometimes suffers from the disadvantage of long analysis time.

**5) Other methods :** Other commonly used methods include fluorescence, manometric, and polarographic methods.

Fluorescence methods are used when one of the reaction components is a fluorescent substance.

Manometric methods are useful especially when the reaction involves either gas uptake, e.g. oxidase reactions ($O_2$ uptake) or gas production (e.g. $CO_2$ formation).

Polarographic electrodes such as dissolved oxygen electrodes could be used to study the rate of oxygen release or uptake and the rate related to that of the reaction.

When using the above methods, the activity of the enzyme is expressed as the initial rate of the reaction. Progress curves for most enzyme reactions are generally as shown in Figure 2.7. As the figure shows, the reaction rate falls with time because:

(1) Products of the reaction may inhibit the enzyme.

(2) The degree of saturation of the enzyme with the substrate may fall because of a reduction in the substrate concentration with time.

(3) The reverse reaction may become more important as the concentration of the product increases (see Equation 2.1).

(4) The enzyme may undergo some inactivation at the temperature or pH of the reaction owing to instability.

The enzyme activity should be measured by the initial velocity.

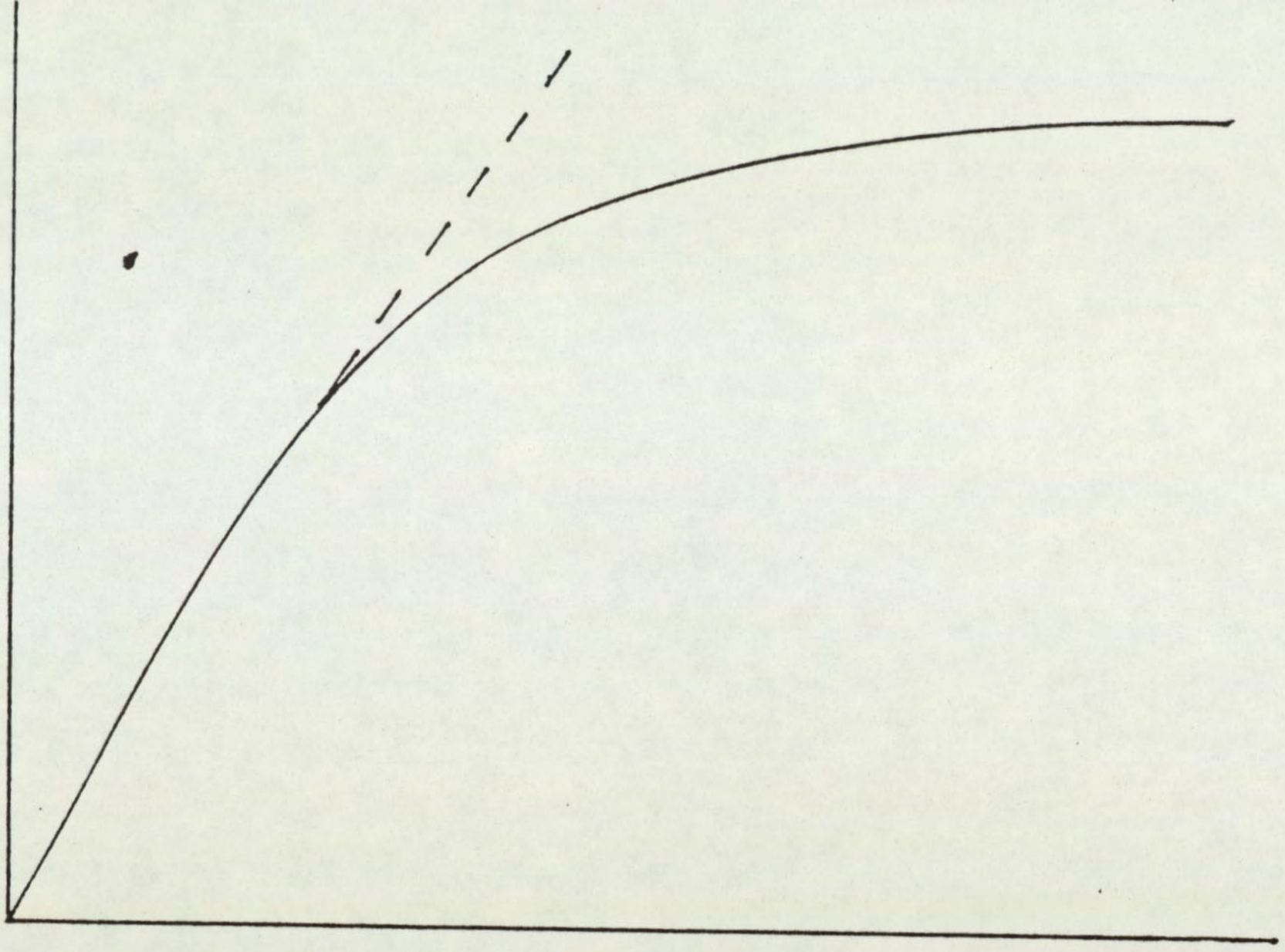



**Figure 2.7: Progress curve for an enzyme catalysed reaction**

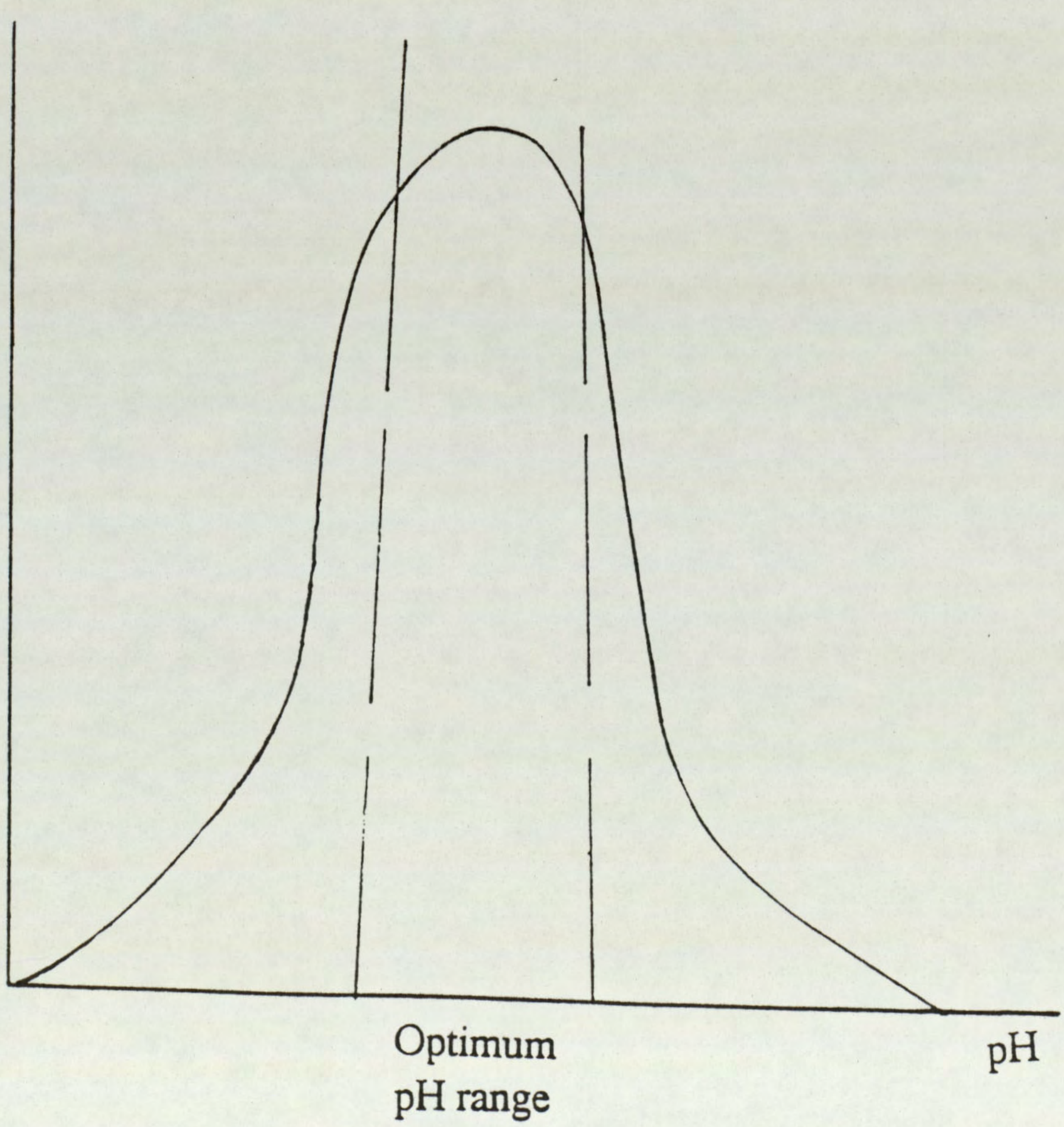



**Figure 2.8: Effects of pH on enzyme activity**

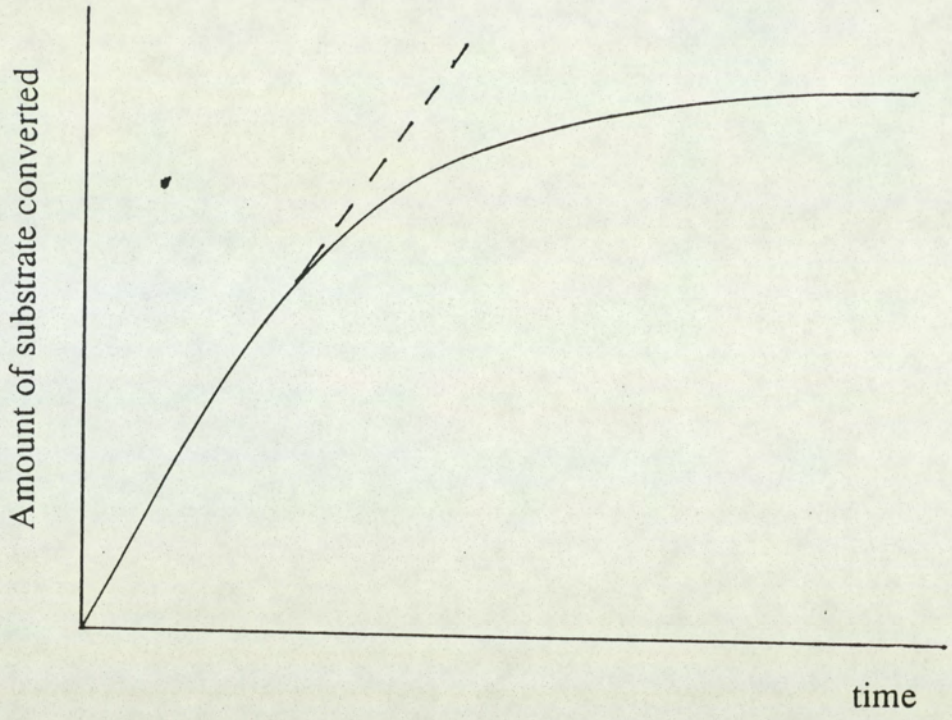



**Figure 2.7: Progress curve for an enzyme catalysed reaction**

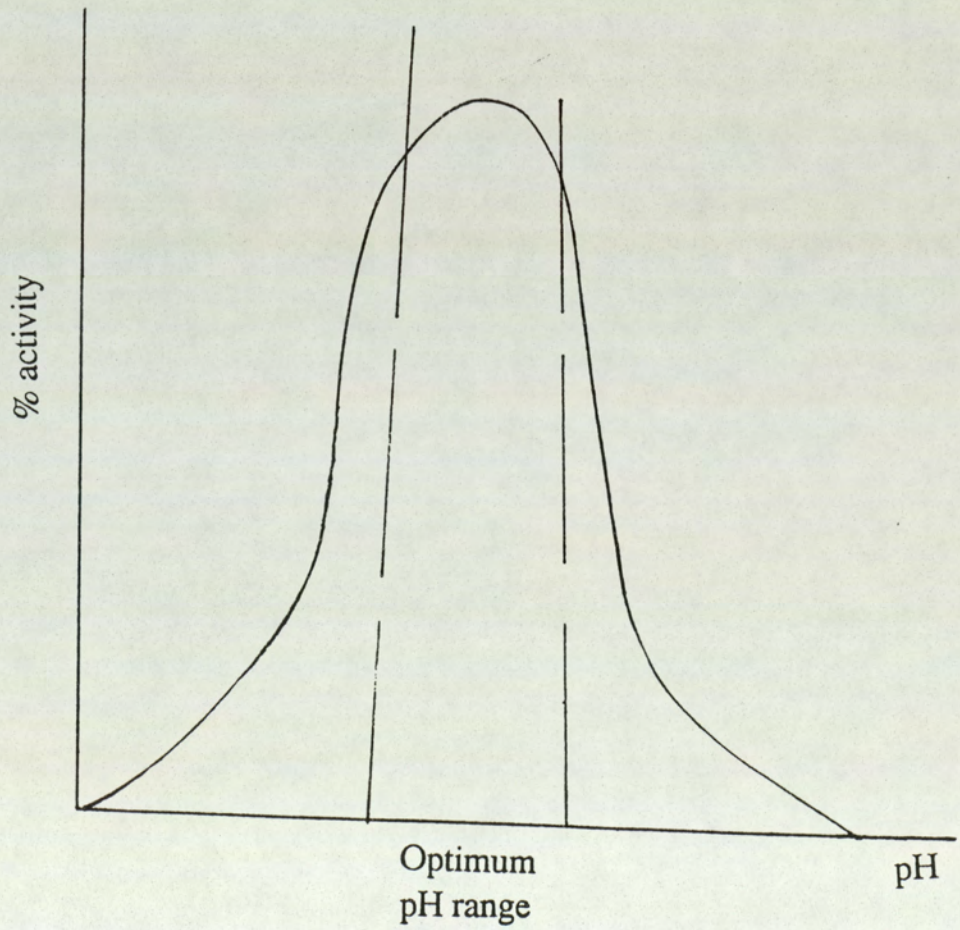



**Figure 2.8: Effects of pH on enzyme activity**

For non-continuous methods of analysis, at least three points are required in order to check the linearity of the curve. Due to high specificity, care should be taken to avoid contaminants. Finally, appropriate amounts of enzyme should be used so that the readings are properly taken. If necessary, dilution of the enzyme solution should be carried out.

## 2.8 FACTORS AFFECTING ENZYME ACTIVITY

Enzymes are relatively fragile substances with a tendency to undergo denaturation and inactivation under unsuitable conditions. The main physical factors that affect the activity and stability of enzymes are pH and temperature. The presence of certain chemicals, like alcohols may also affect enzyme stabilty.

Generally, pH values less than 5.0 or greater than 9.0 inactivate most enzymes[6,9]. All enzymes have an optimum pH range over which their activity is maximum (Figure 2.8). Small changes from optimum pH temporarily inactivate the enzyme. The activity is restored if the pH is moved towards the optimum. Change of pH above or below points of temporary inactivity generally result in gradual permenent inactivity. The optimum pH for some enzymes may also vary with the substrate type and composition.

High temperature (generally above human body temperature) completely inactivate a majority of enzymes. Lower temperatures slow the rate of enzyme activity but this is fully restored as temperature is increased to its optimum[1]. Enzyme reaction rate increases with temperature according to the Arrhenius relationship. Stability of the enzyme is affected by increased temperature. Optimum pH is also affected by temperature. It is thus necessry to make a compromise between these two effects.

## 2.9 ENZYME IMMOBILIZATION

Traditional enzyme reactions are carried out in solution (usually in water). When applied in such systems, the enzyme cannot be re-used. Techniques of immobilization like encapsulation, binding the enzyme to an inert support or making the enzyme insoluble have led to the enzyme being recovered or re-used. This has also enabled enzymes to be used in a variety of configurations of bioreactors which could be operated continuously, e.g.

immobilised enzymes used in the production of High Fructose Syrups (HFS) and semi-synthetic penicillins[11].

# CHAPTER THREE

# LITERATURE SURVEY 2

# CHAPTER THREE
# LITERATURE SURVEY 2

# FERMENTATION TECHNOLOGY

## 3.1 INTRODUCTION

Fermentation originally referred to the anaerobic metabolism of organic compounds by microorganisms or their enzymes to produce simpler products than the starting materials. This process provided energy to the organism in the form of adenosine triphosphate (ATP) molecules. The modern definition describes the processes in which cells, or other microorganisms, are cultured in a fabricated vessel (fermenter or bioreactor) in liquid or solid media for experimental or commercial purposes[11,16]. Included in this definition are anaerobic processes like ethanol and lactic acid production, and products containing them like beer, wine, vinegar, pickles etc. Aerobic metabolic processes such as antibiotics, organic acids, enzymes and vitamin production are also included in the modern definition.

A complete understanding of fermentation technology requires integral knowledge of the principles of biology, microbiology, biochemistry, genetic engineering, biochemical and chemical engineering to name but a few[17]. When this integration is achieved, fermentation technology enjoys the advantage over most chemical processes in that due to the rather simple and uniform series of techniques involved, the same facilities can be used to make chemically unrelated substances. Other advantages include less expensive raw materials, and lower energy costs. Since bio-processes are carried out at lower temperatures, higher efficiency than in multistep chemical synthesis, higher degrees of specificity and quality of final product can be obtained than by chemical synthesis. The fermentation times are relatively short. As a result, large scale extraction of enzymes and antibiotics from plant and animal tissues that require several years to grow are not widely used.

Problems facing a fermentation process arise because of its requirements. Some of them are the supply of a pure and viable culture as well as the maintenance of optimum growth conditions for the culture like pH, temperature and oxygen supply. The products of fermentations are

usually in very dilute concentrations in large volumes of medium. Expensive multistage purification processes are usually required in order to produce a particular product (see Figure 2.1). Economic considerations have therefore limited the use of fermentations in those processes where cost instead of the technology becomes the dominant factor. Environmental problems such as water pollution and the risk of the release of bioactive substances are also hindrances to the large scale development of certain fermentation processes.

The fermenter (bioreactor) is at the heart of any fermentation process. Fermentation technology therefore involves the design, control and scale-up of these reactors, together with their ancillaries[18,19]. The main components of a fermentation process are:

(1) Biological materials.
(2) Formulation of medium and sterilization.
(3) Production of pure and active inoculum for the fermenter.
(4) Growth kinetics.
(5) Optimization of growth to obtain required product-agitation and mass transfer, control.
(6) Design and scale-up.
(7) Product recovery and waste treatment/disposal.

## 3.2 BIOLOGICAL MATERIALS

The biological materials needed for the performance of fermentations are cells. Cells can be classified into two classes: Prokaryotes and eukaryotes[4,19-23].

**(1) Prokaryotes:** These are the simplest and smallest cells. They consist of different families of unicellular microorganisms (e.g. bacteria and blue-green algae). They can be classified in a number of ways: according to shape (rods, cocci, etc), their capacity for motion, their staining characteristics (Gram positive or negative), the nutrients they favour or the product they make. They do not contain a nucleus and generally reproduce at a fast rate by asexual cell division. Some prokaryotic cells are pathogenic while some are highly beneficial.

(2) **Eukaryotes:** This group contains the cells of all other living organisms. Included in this group are microbial cells such as fungi and protozoa. Eukaryotes are much larger in size, 20-30 μm in diameter compared to 1-5 μm for prokaryotic cells. They have a well formed nucleus, are surrounded by a paired membrane and have complex internal structures. They can reproduce asexually, but by a more complex process of mitosis and also undergo complex sexual conjugation in which there is exchange of genes.

(3) **Viruses:** A class of biological materials which has more destructive than useful effects to fermentation technology. These are non-living but biologically formed molecular assemblies that duplicate themselves in the appropriate host cells. They consist of nucleic acid molecules surrounded by a protective shell made of protein molecules. Once a virus gains entry into its specific host, it acts as an intracellular parasite. The viral nucleic acid carries genetic messages which take over and alter the host cells' normal activities. This diverts the cells' enzymes from performing their normal cellular roles to that of manufacturing new viral material.

## 3.3 FORMULATION OF MEDIUM AND STERILIZATION

### 3.3.1 Formulation of medium

All living cells require certain nutrients for their growth and development. The nutrients must contain the chemical constituents of cellular materials and structures as well as those required for membrane transport, enzyme activity and energy generation for biosynthetic processes[22,25]. Table 3.1 shows the major elements required by microbial cells.

**Table 3.1: The major elements required by microbial cells (ref 22)**

| Element | Source | Metabolic function |
|---|---|---|
| C | Organic compounds, $CO_2$ | Major components of cell material |
| O | $O_2$, $H_2O$, organic compounds, $CO_2$ | |
| H | $H_2$, $H_2O$, organic compounds | |
| N | $NH_4^+$, $NO_3^-$, $N_2$, organic compounds | |
| S | $SO_4^{2-}$, $HS^-$, S, $S_2O_3$, organic sulphur compounds | Component of cysteine, methianine thiamin pyrophosphate Coenzyme A, biotin and α-lipoic acid |
| P | $HPO_4^{2-}$ | Component of nucleic acids<br>ATP and other nucleotides, phospholipids |
| K | $K^+$ | Main inorganic cation within cell, cofactor of some enzymes |
| Mg | $Mg^{2+}$ | Cofactor of many enzymes (particularly kinases) present in cell walls and membranes |
| Ca | $Ca^{2+}$ | Cofactor of enzymes; present in important exoenzymes, e.g. amylases, proteases; an important component of endospores (Ca-dipicolinate) |
| Fe | $Fe^{2+}$, $Fe^{3+}$ | Component of cytochromes; iron-sulphur proteins, e.g. ferredoxins cofactors of many (e.g. hydrolases). |

There are two basic means by which nutrients are provided to cells. The cells can either make their own food (autotrophy) or obtain it from other organisms (heterotrophy)[20].

Autotrophs can convert relatively oxidised substances into reduced ones. This process requires energy which can either be supplied by light (photoautotrophy) or by chemical reactions (chemoautotrophy). When the carbon source is inorganic, the growth of the cells is referred to as photolithotrophy for the phototrophs and chemolithotrophy in the case of chemotrophs. If the carbon source is organic, the growth becomes known as photoorganotrophy and chemorganotrophy respectively.

Most industrial microorganisms are chemoorganotrophs, therefore the commonest source of energy will be carbon in the form of carbohydrates, lipids, proteins, alkanes and sometimes alcohols[19]. The choice of a carbon source to be used for any particular fermentation is dependent on a number of factors such as the availability of the source, its purity, the type of product, the rate at which the source is metabolized and its effect on product yields, government legislation, etc.

Nitrogen is also one of the most important components of cell material. It is usually supplied either as organic or as inorganic nitrogen. Inorganic nitrogen is supplied as ammonia gas, ammonium salts or nitrates. Organic nitrogen may be supplied as amino acids, proteins or urea. Due to the high cost of pure amino acids, they are normally supplied by the addition of cheaper complex organic nitrogen sources. These are non-homogeneous and usually contain varying amounts of inorganic compounds, proteins and vitamins. Examples of such sources include yeast extracts, corn steep liquors, soya meal, soy beans, pea-nut meal, and fish meal. Due to the undefined nature of these complex media, batch variations sometimes affect results of fermentations.

Minerals like magnesium, potassium, sulphur, calcium, etc (Table 3.1) are also essential. Their concentrations are usually such that they will be added separately as salts containing these compounds. Certain trace elements as Zn, Mn, Na, Cl, Mo, Se, Co, Cu and Ni are also required by most microorganisms. They are usually present as impurities in other major ingredients like the organic nitrogen sources.

Vitamins are required in extremely small amounts as cofactors for certain enzymes. Many of the natural carbon and nitrogen sources contain some or all of the required vitamins. Certain organisms have a more stringent demand for vitamins than others and it is sometimes necessary to supplement the amounts present in the medium. A list of some of the vitamins and related compounds which are frequently required for growth are shown in Table 3.2.

Other components of a fermentation medium are oxygen, buffers, precursors, inhibitors and inducers[19]. Oxygen is usually supplied by aerating the fermenter with air or pure oxygen. Sometimes the aeration conditions for biomass production are different from those of product formation. Therefore, this component is usually optimised when the other medium requirements have been satisfied. However, knowledge of the rate of metabolism of the microorganism, the rheology of the medium and the presence of antifoams and their effects on oxygen supply is needed during medium formulation.

Precursors are chemicals which, when added to certain fermentations, are directly incorporated into the desired product.

Inhibitors could be added either to enhance the production of a certain product, or accumulate an intermediate product which is normally metabolized.

Inducers are normally added in systems where inducible enzymes are produced and could be added either at the beginning of the fermentation or when enzyme production is required.

When control of pH is critical, buffers may be added into the fermentation medium. These could be nutrient sources like calcium carbonate, proteins or ammonia. The pH may also be controlled externally by using acids or alkalis.

Most fermentation media have the tendency of producing foam. Antifoaming agents such as silicones, fatty acids, esters etc, are added to the media. However, most tend to reduce oxygen transfer in aerated systems. Their use is thus restricted to the amount that will just prevent excessive foaming.

**Table 3.2: Vitamins and related compounds required for growth ( ref 22)**

| Compound | Metabolic function |
|---|---|
| P-aminobenzoic acid | Tetrahydrofolate precursor, involved in transfer of one-carbon units |
| Biotin | Coenzyme involved in carboxylation reactions |
| Folic acid | Tetrahydrofolate involved in transfer of one-carbon units |
| Nicotinic acid | Precursor and NAD and NADP |
| Pantothenic acid | Precursor of Coenzyme A (carrier of acyl groups) |
| Pyridoxine (vit $B_6$) | Pyridoxal phosphate involved in transmission and decarboxylation reactions |
| Riboflavin (vit $B_6$) | Component of FMN and FAD-prosthetic groups of flavoproteins |
| Thiamin (vit $B_1$) | Component of thiamine pyrophosphate in decarboxylases, transaminases and transketolases |
| Cyanocobalamine (vit $B_{12}$) | A coenzyme involved in re-arrangement reactions (glutamate mutase) |
| Vitamin K | Precursor of menaquinone - an electron carrier (e.g. fumarate reductase) |
| Coenzyme M | Involved in methanogenesis |

## 3.3.2 Sterilization

### 3.3.2.1 Objective

Most fermentations require pure cultures to be used for their inoculation. The media used for these processes are capable of supporting more than one species of organisms. The cost of sterilization and the maintenance of aseptic conditions throughout the process is a major component of the overall process cost. The main reason, therefore, for sterility throughout a fermentation process is to prevent invasion of the system by foreign organisms[19,24]. Some of the consequences of this invasion are:

(1) Loss in productivity due to competition for growth medium.

(2) Contaminant may outgrow product organism in the case of a continuous fermentation or where the organism grows at a slower rate.

(3) Foreign organism may contaminate or degrade the required product.

(4) Contaminant may produce a byproduct which represses yield of desired product.

The aim of sterilization is thus to eliminate these effects. A number of strategies that are applied to achieve sterility include using a pure culture to inoculate the fermenter and;

(a) sterilizing the medium, vessels, additional materials, and maintaining aseptic conditions throughout the process;

(b) operating under semi-aseptic or non-aseptic conditions where only those components of the medium which are more easily contaminated will be sterilized.

### 3.3.2.2 Medium Sterilization

Steam has generally been used for the sterilization of the medium. The medium can be sterilized in the fermenter or as separate batches. In some cases, the medium components are mixed and sterilized at once, while in others, some components (usually the sugars) are separately sterilized so that they do not react with other media components. Sterilization may be carried out either in a batch or continuous mode. The choice of the type of sterilization depends largely on the nutrient demand and on the stability of the nutrient components (especially proteins and vitamins) to heat. Typical sterilization conditions are 115 - 121 $^{o}$C, at 1 atmosphere pressure for 20 - 30 minutes. For larger volumes of material, the sterilization times are usually doubled.

Batch sterilization is carried out either by indirect heating using a cooker (autoclaves) or a heat exchanger (coil or jacket), or by direct heating. Direct heating involves the injection of live steam into the medium. This leads to volume increase and this should normally be determined in advance of the sterilization.

Continuous sterilization employs the same stages of heating, holding at the sterilization conditions and cooling as in the batch sterilization conditions but these are carried out continuously. The two types generally used are the continuous plate exchanger and the continuous injector-flash cooler. In the plate exchanger, the medium is heated, held at the sterilization temperature and then cooled in another exchanger. The continuous injector-flash cooler utilises live steam to heat the medium, holds it at the required temperature and then it is passed into a chamber where it is flash cooled. This is usually referred to as the High Temperature Short Time (HTST) sterilization and is less destructive to labile nutrient components.

Aerobic systems require large amounts of air. Sterilization of air is very costly. Air sterilization is achieved by either heat treatment or filtration. Heat treatment is not generally used because of the cost involved. Filtration is normally used to remove microorganisms. Two types of filters generally used are absolute and non-absolute filters. Absolute filters are generally sterilizable and have pore sizes much smaller than the microorganisms they remove

(typically 0.2 μm). The other type of filter includes those made from fibrous materials as cotton, glass, and steel wool. The gaps between them are usually in the range 0.5 - 15 μm. They are able to filter smaller particles because of the depth of packaging. These are cheaper, more robust and have a lower pressure drop. As a result, they are more widely used.

#### 3.3.2.3 Fermenter and ancillary equipment sterilization

Sterilization of the fermenter, together with all its feed lines is carried out by heating with jackets or coils, or by sparging steam into the system through all entries. Holding the vessel for about 30 minutes under 1 atmosphere of steam pressure has been found to be enough to sterilize the system. Exit and sample lines are also sterilized by steam. All other additional pieces of equipment like the tubings and reservoirs are sterilized in autoclaves.

## 3.4 PREPARATION OF INOCULUM

Cell cultures used for fermentations are normally preserved under conditions where viability is maintained but cell multiplication suspended[16]. Some of the methods used include, storing as cell suspensions in liquid nitrogen at -196 $^{o}$C, storing as freeze dried vials at -16 $^{o}$C or on agar slants at 4 $^{o}$C in the fridge. Fermentations require sufficient quantities of active and pure cultures for their inoculation. These are normally produced from the pure preserved cultures by a number of stages usually referred to as the inoculum development. The number of stages depend on the size of the production vessel. The procedure involves starting with the stock culture and building up the inoculum in stages using sterile media in shake flasks and seed vessels. This is done so that the inoculum going into the production vessel is about 3 -10 % of the medium volume[19]. The composition of the medium used in the inoculum development stage is generally the same as that of the final production vessel. However, specially formulated media which favour profuse growth of certain organisms are commonly used for this stage. In some cases, the difference in media composition has been deliberately allowed in order to eliminate the presence of certain byproducts.

## 3.5 GROWTH KINETICS

### 3.5.1Introduction

The whole essence of fermentation technology is to culture a specific organism. The desired product is either the biomass or a certain metabolite of the organism. In order to optimise the yield, detailed knowledge of the way in which the organism behaves in the growth medium is required. This is achieved by studying the kinetics of growth of the organism under defined conditions.

### 3.5.2 Measurement of cell growth

Growth of an organism can be considered at two levels; the growth of individual cells as described by their life cycles and the growth of cell populations. In fermentation technology, the component considered when measuring growth is that of population. Several methods are used for the measurement of cell population growth (henceforth referred to as cell growth or simply as growth). The most commonly used methods are cell dry weight, total cell number, absorbance, substrate consumption or product formation, heat evolution and fluorescence spectroscopy(25).

1 **Cell dry weight:** This is one of the most commonly used techniques. It involves either centrifuging or filtering a known volume of cell suspension, drying to constant weight and then determining the cell dry weight. Cell dry weight is important because kinetic parameters can be related to it to give their specific values. The problem with using this technique is the time factor involved which makes it difficult to use for quick estimation of cell growth. The method also does not differentiate between viable and non-viable cells.

2 **Total cell numbers:** This involves counting the number of cells in a known volume of broth. The technique is relatively rapid, but is tedious, relatively inaccurate and only applicable to unicellular organisms that do not form cell aggregates. It does not distinguish between dead and viable cells.

3 **Absorbance:** This is an extremely fast and accurate method for

determining growth of unicellular organisms growing in non-turbid media. The absorbance (also turbidity or optical density) of a culture is measured using a photospectrometer at a chosen wavelength with the sample placed in a glass, plastic or quartz cuvette. The absorbance is directly proportional to the cell concentration. However, this is usually true only up to a certain absorbance, after which the relationship is no longer linear. So it is necessary to determine the linear scale for the photospectrometer.

4 **Substrate consumption or product formation:** If biomass is the main fermentation product, then measurement of substrate depletion can be related to growth. This is only true if the yield coefficient ($Y_{X/S}$) is constant (see Section 3.5.3). Also, if the products are growth associated, their concentration can be related to the growth.

5 **Heat evolution:** All metabolic growth processes are accompanied by the liberation of heat. This is directly proportional to the number of viable cells in the culture. Heat generation can be measured by various calorimetric techniques and related to growth. This technique has the advantage that it can be applied for continuously monitoring cell growth.

6 **Other techniques:** Several other methods are used for cell growth determination. They include the measurement of cellular components, packed cell volumes, wet cell weight, fluorescence spectroscopy and viable cell number.

Cellular components such as protein or DNA (deoxyrhibonucleic acid) content of the culture can be measured. Provided the concentration per cell is constant, this can be related to the number of cells in the medium.

Viable count involves the dilution of a known volume of culture, incubating on agar plate and counting the number of colonies. This number can then be multiplied by the dilution factor to give the viable cell count. It is very inaccurate and takes a number of days to carry out.

Fluorescence spectroscopy relies on the principle that certain cellular components exhibit fluorescence (i.e. re-emit absorbed light with a spectral shift towards a longer wavelength). The intensity of the fluorescence light can

be measured and related to the composition of the cellular component. If this composition is constant to all cells, the cell growth can be determined.

### 3.5.3 Growth parameters

Information obtained when growth is quantitatively measured is usually expressed in terms of various growth parameters. The most commonly used growth parameters are specific growth rate ($\mu$), yield coefficients, metabolic quotients, productivity, substrate utilization, and maximum biomass[21,27].

#### 3.5.3.1 Growth rate

When a culture of biomass concentration $x_o$ is inoculated into a medium containing all the necessary growth requirements of the organisms, they will grow. The increase in biomass concentration (dx) after a time interval (dt) can be represented as follows:

$$dx = \mu x.\, dt \qquad \text{..... 3.1}$$

The parameter, $\mu$, represents the growth per unit amount of biomass and is known as specific growth rate. It is expressed by rearranging Equation 3.1 above:

i.e.

$$\mu = \frac{1}{x}\,\frac{dx}{dt} \qquad \text{..... 3.2}$$

$\mu$ is constant for a particular organism growing under a specific set of conditions and has dimensions of reciprocal time. Integrating Equation 3.2 gives an expression for biomass (x) in terms of time:

$$x = x_o\, e^{\mu t} \qquad \text{..... 3.3}$$

Cells growing according to this equation are described as growing at an exponential or logarithmic rate.

Two useful parameters normally derived from the relationships of specific growth rate are doubling time of the biomass and degree of multiplication.

Doubling time is obtained by taking the natural logarithm of both sides of the Equation 3.3 and replacing x by $2x_o$.

$$\ln (x/x_o) = \mu t \quad \text{..... 3.4}$$

hence,

$$t_d = \ln 2/\mu \quad \text{..... 3.5}$$

The degree of multiplication is given by $x/x_o$ which is equal to $e^{\mu t}$ (from Equation 3.3). The number of doublings (or generations), n, undergone by the growing organism is obtained from the following expression:

$$2^n = x/x_o \quad \text{..... 3.6}$$

### 3.5.3.2 Yield Coefficients

A number of yields of one parameter with respect to the other can be estimated from the measured parameters[21]. Yield coefficients are normally represented as $Y_{a/b}$ and this expresses the yield coefficient of parameter (a) with respect to (b). Examples of yield coefficients applied to growth kinetics are yield coefficient of biomass (x) with respect to substrate (s), ($Y_{x/s}$), coefficient of biomass with respect to oxygen ($Y_{x/O_2}$), of product (P) with respect to biomass ($Y_{p/x}$)[27], etc.

### 3.5.3.3 Metabolic Quotient

This is the rate consumption of a substrate in a culture at a particular moment and is given as

$$\frac{ds}{dt} = q_i x \quad \text{..... 3.7}$$

q is the metabolic quotient and, i, the substrate concerned, e.g. $q_{O_2}$ and $q_{sucrose}$ refer to oxygen and sucrose quotients respectively. For a short interval of time, the yield of cells with respect to substrate is given by:

$$Y_{x/s} = \frac{dx}{ds} \qquad \text{..... 3.8}$$

Substituting dx as defined by Equation 3.1 and rearranging,

$$\frac{ds}{dt} = \frac{\mu x}{Y_{x/s}} \qquad \text{..... 3.9}$$

Comparing Equations 3.7 and 3.9,

$$q = \mu / Y_{x/s} \qquad \text{..... 3.10}$$

### 3.5.3.4 Substrate Utilization

The relationship between cell growth rate and substrate utilization is represented by the Monod equation:

$$\mu = \frac{\mu_{max}\, s}{s + K_s} \qquad \text{..... 3.11}$$

where $K_s$ is the saturation constant and is related to the affinity of the organism to the substrate. If this value is high, the affinity of the organism for the substrate is low and vice versa. It is the substrate concentration when $\mu$ equals half of $\mu_{max}$ ( Figure 3.1), where $\mu_{max}$ is the maximum growth rate when substrate concentration is in excess and there is no inhibition.

More accurate values of $K_s$ and $\mu_{max}$ can be obtained by rearranging Equation 3.11.

$$\frac{1}{\mu} = \frac{K_s}{\mu_{max} s} + \frac{1}{\mu_{max}} \qquad \text{..... 3.12}$$

A plot of $\frac{1}{\mu}$ against $\frac{1}{s}$ will be linear with an intercept on the ordinate axis of $\frac{1}{\mu_{max}}$ and slope of $\frac{K_s}{\mu_{max}}$ (Figure 3.2).

The substrate used is generally the limiting component like the carbon source, nitrogen, phosphate or even a vitamin.

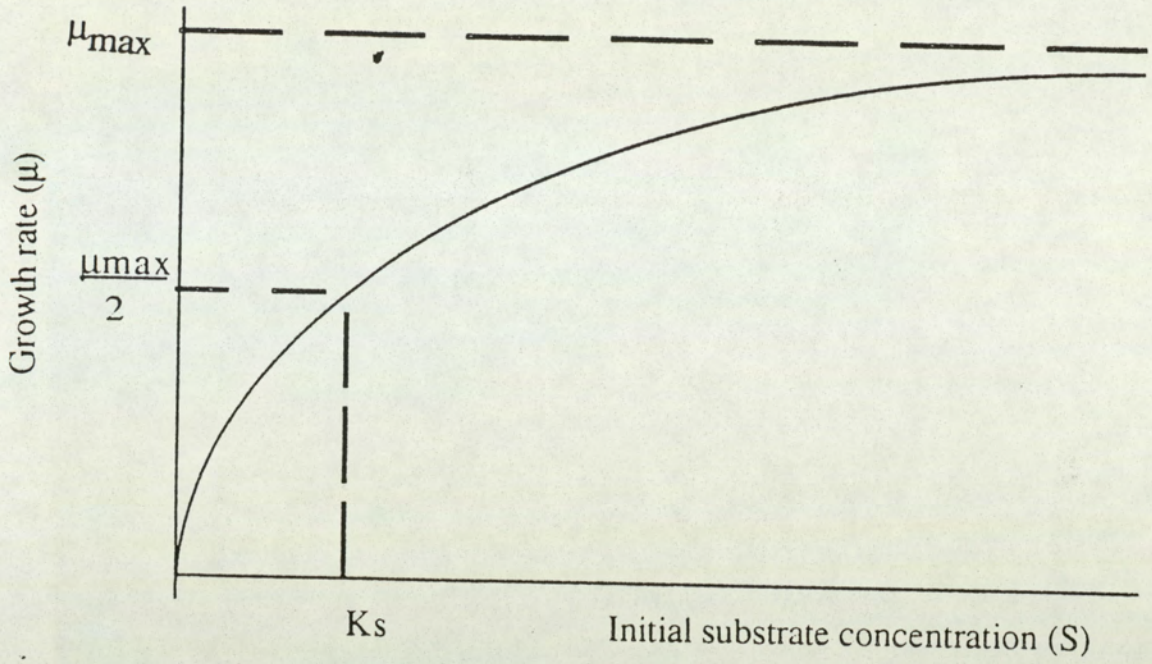



**Figure 3.1: Variation of growth rate with initial substrate composition**

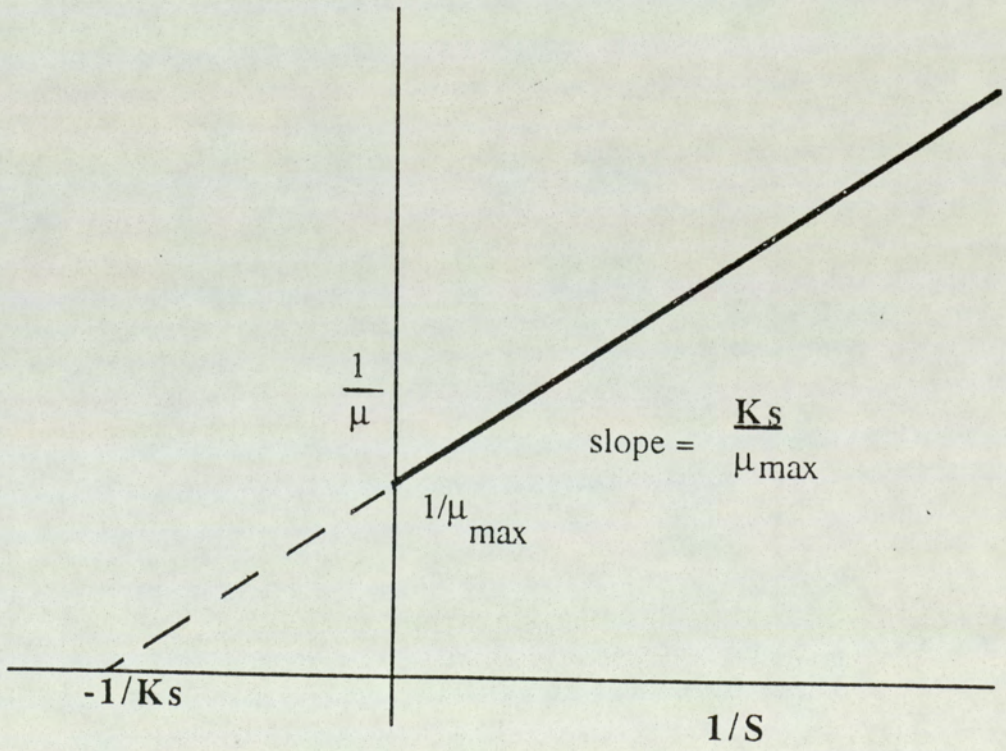



**Figure 3.2: Lineweaver- Burke plot for the determination of $K_s$ and $\mu_{max}$**

In systems where the substrate is utilized both for growth and product formation, the Monod kinetics tend not to fit growth exactly. The effect of product formation is considered for the different types of fermentations (batch, fed-batch and continuous) below.

### 3.5.4 Batch Fermentation Kinetics

Cell growth in a batch culture generally follows the pattern shown in Figure 3.3. The growth occurs in a number of phases. These can ideally be divided into six phases. Under batch conditions, the bioreactor is inoculated with a pure culture and operated under optimum conditions without addition of any fresh medium. Growth normally terminates when either some nutrient(s) have been exhausted or when there are changes in the environmental conditions (e.g. accumulation of toxic materials, pH changes).

Phase 1 is the lag phase. This is the period after inoculation when no growth is observed. During this period, the culture adapts itself to growth in the new medium. The length of this phase is dependent on the difference between the conditions of the inoculum and the fermenter medium, and sometimes on inoculum cell concentration (size).

Phase 2 is the acceleration phase. It represents the period after the lag phase when the growth rate of the organism increases until it becomes constant at the exponential phase (Phase 3). This phase is usually quite short and not clearly noticeable.

Phase 3 is the exponential or constant growth rate phase It is the region where the growth equation (i.e. Equation 3.1) is satisfied, i.e. where $\mu$, the growth rate is constant.

Phase 4 is known as the deceleration phase. It occurs at the end of the exponential phase and usually arises when there is a depletion of nutrients, or accumulation of toxic products.

Phase 5, the stationary phase, occurs when there is no net cell growth. Cells in this stage may either be growing, but at a rate equal to the death rate, or just remain as viable cells.

Phase 6, the decline phase, occurs in some cases when the death rate of cells becomes greater than the growth rate.

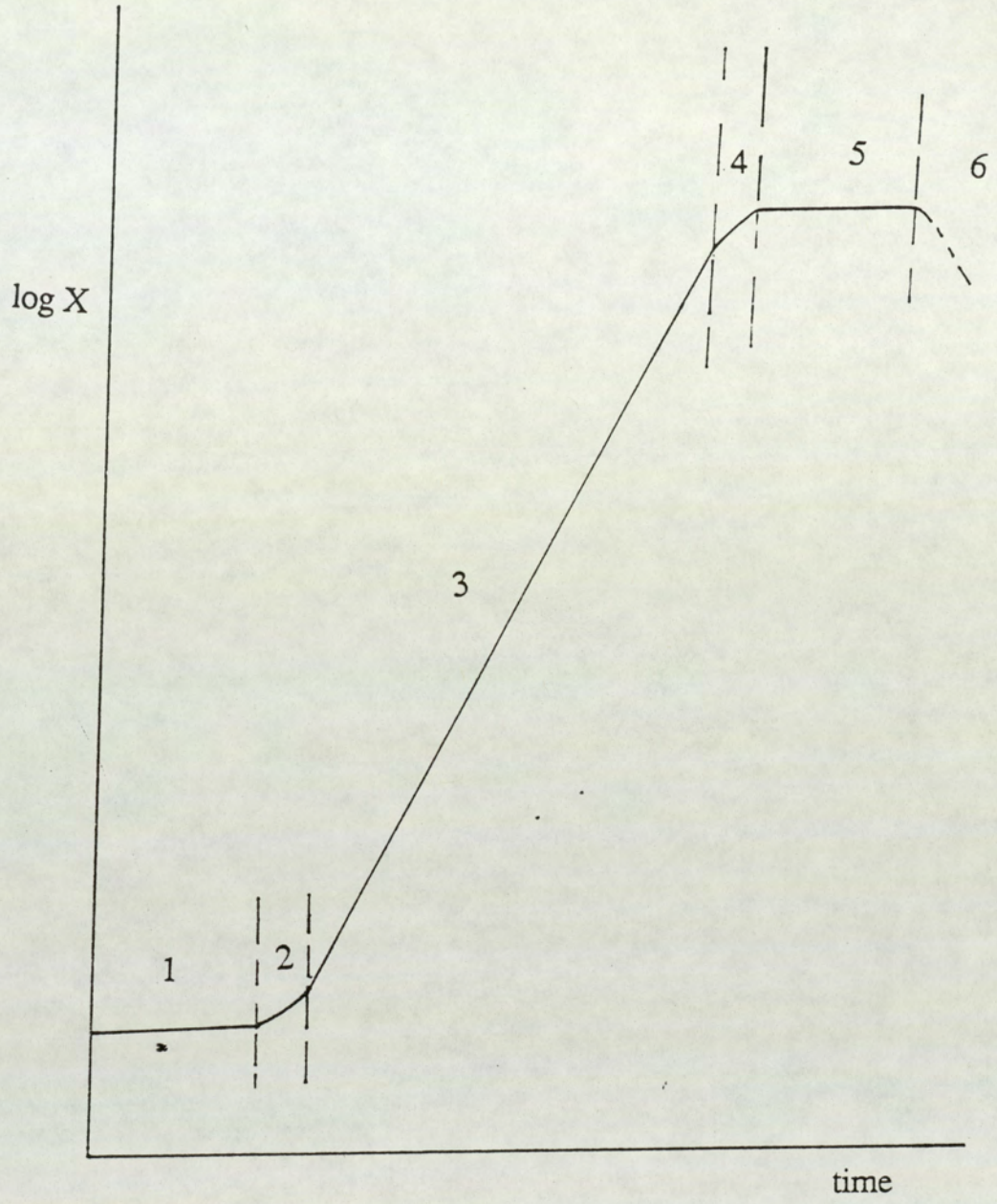



Figure 3.3: Batch growth cycle
Growth phases: 1, lag; 2, acceleration;
3, exponential; 4, deceleration;
5, stationary; 6, decline or death

### 3.5.4.1 Determination of specific growth rate ($\mu$)

At any time during a batch growth a cell material balance can be carried out as follows:

$$\frac{dx}{dt} = \mu x - \frac{F}{V} x - \alpha x \qquad \text{..... 3.13}$$

Cell dry weight accumulation | Growth | Cell removal | Cell death

Since no cell removal takes place in the batch fermentation,

$$\frac{dx}{dt} = x(\mu - \alpha) \qquad \text{..... 3.14}$$

At the exponential phase, $\mu >> \alpha$, and this equation becomes the form shown in Equation 3.1. From this, $\mu$ can be determined as the slope of the plot of natural logarithm of x against time ( Figure 3.3).

### 3.5.4.2 Substrate utilization

When cells grow in a batch system, the substrate is utilized until it runs out. The limiting substrate determines how far the growth will go. There are a number of ways that the substrates are used. These include, provision of energy for growth and maintenance of culture and synthesis of product in the cases where the substrate is required for this purpose. A balance on a substrate component gives the following equation:

$$\frac{ds}{dt} = \frac{\mu x}{Y_{x/s}} + \frac{q_p x}{Y_{p/s}} + mx \qquad \text{..... 3.15}$$

substrate consumed | Cell growth | Product formation | Cell maintenance

For systems where the substrate is not involved in product formation, this expression reduces to Equation 3.9. The maintenance term is usually negligible compared with the growth term. Substrate and cell growth profiles

in a batch culture normally follow the pattern shown in Figure 3.4.
The above relationships are true only for single substrate systems. Various models have been proposed for multi-substrate systems[28-30].

For a single limiting substrate system, $Y_{x/s}$ can be determined by growing the organism under different substrate concentrations and determining the final biomass concentration at the stationary phase. The relationship between biomass concentration $(x-x_o)$ and substrate concentration $(s_o-s)$ is shown in Figure 3.5. $Y_{x/s}$ is the slope of the linear section[19]. Over region A—>B of Figure 3.5, substrate concentration is zero at point of cessation of growth.

### 3.5.4.3 Product formation

Formation of products by organisms is either growth related, partially growth related or non-growth related (Figures 3.6 to 3.8).

For growth related product formation, the relationship between cell concentration and product is as follows:

$$\frac{dp}{dt} = q_p x - \frac{F}{V} p - \beta p \quad \text{..... 3.16}$$

| Product accumulation | Product synthesis | Product removal | Product denaturing |
|---|---|---|---|

In batch processes, there is no removal by product. If the product is being formed at a faster rate than is being denatured, ß will be negligible.
Equation 3.16 thus becomes:

$$\frac{dp}{dt} = q_p x \quad \text{..... 3.17}$$

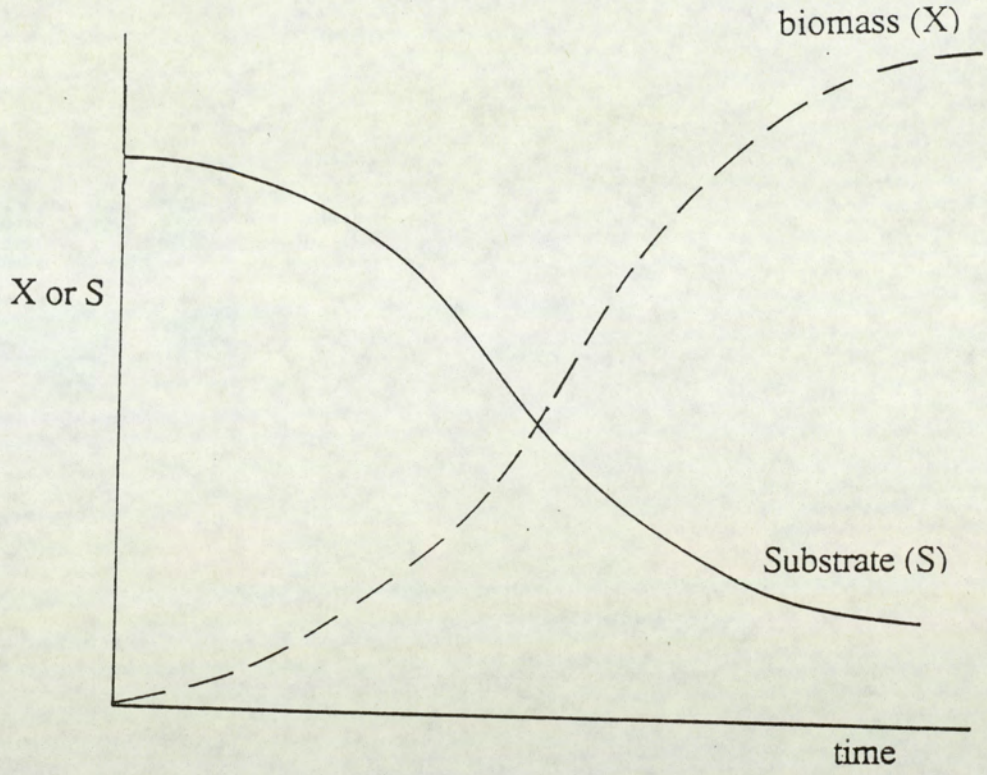



**Figure 3.4: Substrate utilization and growth in a batch culture**

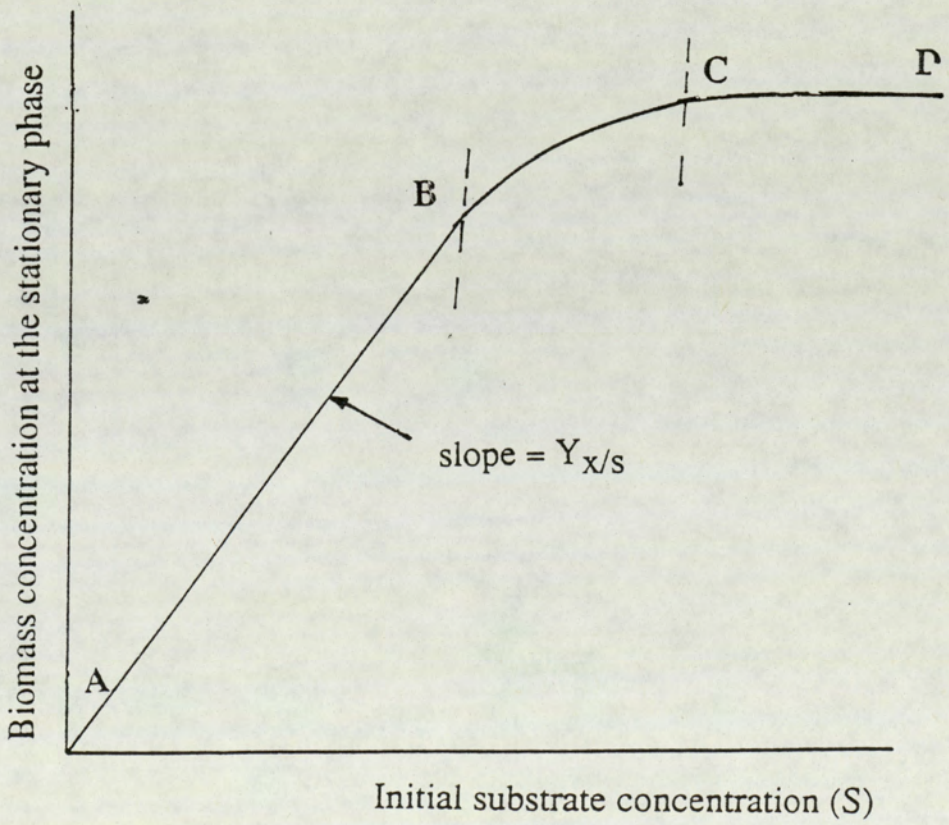



**Figure 3.5: Variation of final biomass concentration with initial substrate concentration under batch fermentation conditions**

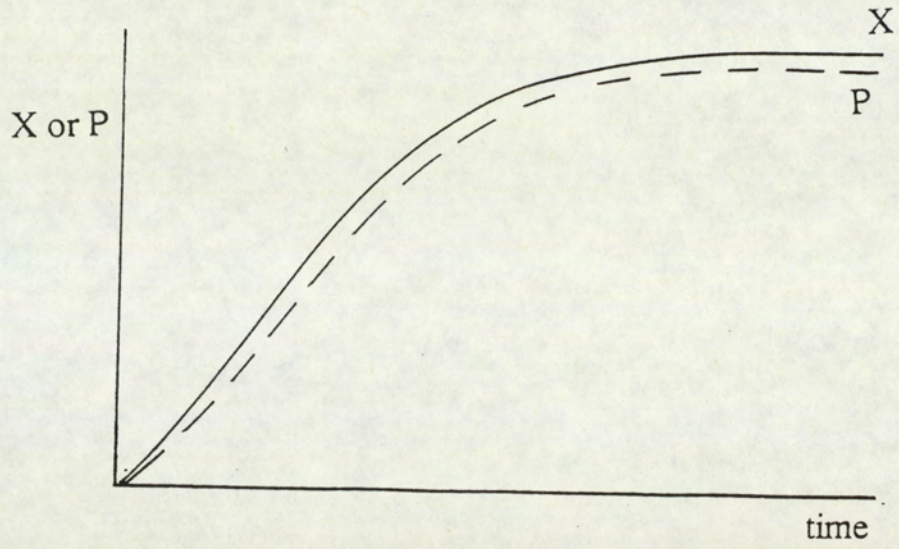



**Figure 3.6: Growth-related product formation**

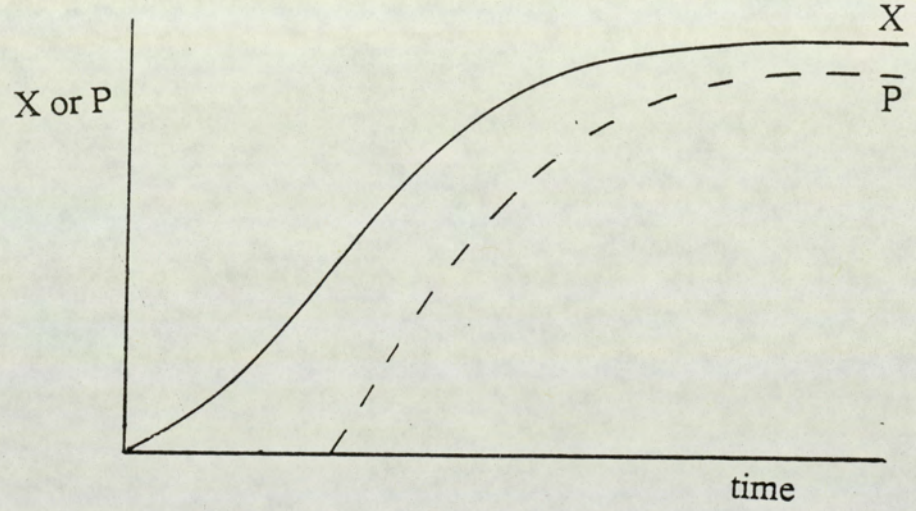



**Figure 3.7: Partial growth-related product formation**

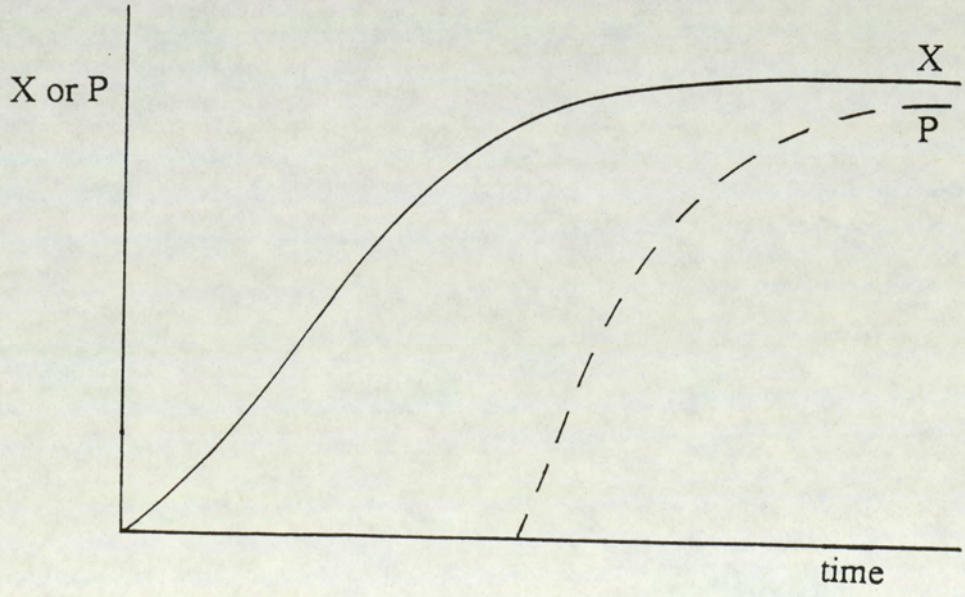



**Figure 3.8: Non growth-related product formation**

Comparing Equations 3.17 and 3.9 (substrate utilization),

$$\frac{dp}{dt} = \frac{\mu x}{Y_{p/x}} \quad \text{..... 3.18}$$

Hence, $q_p = \mu / Y_{p/x}$ ..... 3.19

Luedeking and Piret[31] have described an equation for partial growth-related product formation.

$$\frac{dp}{dt} = A \frac{dx}{dt} + Bx \quad \text{..... 3.20}$$

where A and B are coefficients for growth and non growth-related terms respectively. Dividing both sides by x, and comparing with Equation 3.17:

$$\frac{1}{x} \frac{dp}{dt} = A\mu + B = q_p \quad \text{... 3.21}$$

Since $\mu = \frac{1}{x} \frac{dx}{dt}$ ( from Equation 3.2).

### 3.5.4.4 Productivity

This is the measure of the amount of product formed per unit volume per hour. It is expressed as the overall batch cycle time productivity (taking time to prepare, sterilize, run and empty vessel into consideration). It can be measured either as maximum productivity ($P_m$) or overall productivity ($P_o$) (Figure 3.9).

For growth related product formation systems or those systems where cells are the desired product, maximum productivities are generally employed. For partial or non-growth related processes (e.g. antibiotic fermentations) overall productivities apply.

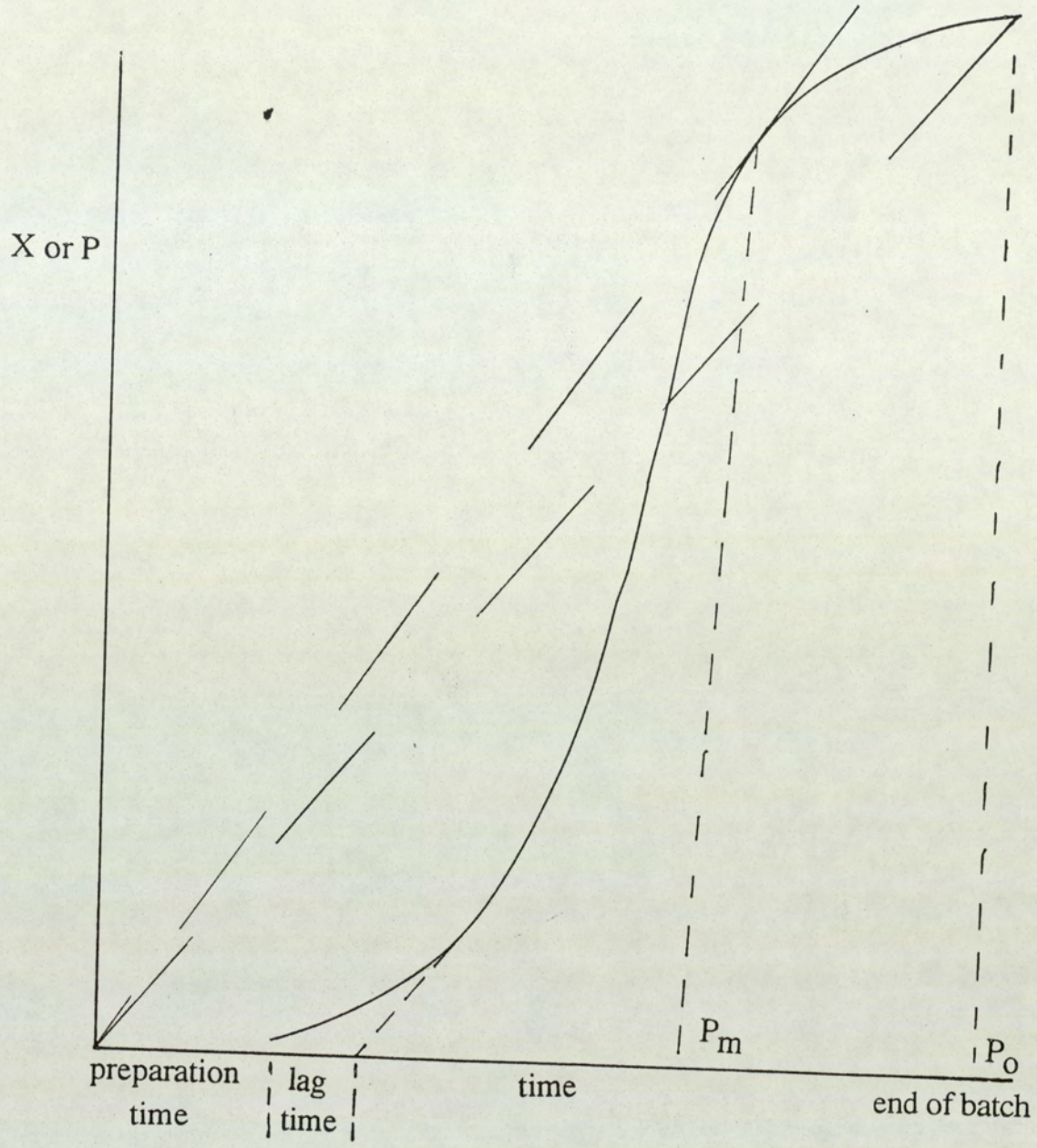



**Figure 3.9:** **Productivity in a batch culture**
**$P_o$ - maximum productivity time**
**$P_m$ - overall productivity time**

Overall productivity can be calculated from the following equation:

$$P_o = \frac{x_f}{\frac{1}{\mu_{max}} \ln \frac{x_f}{x_o} + t_T + t_L + t_D} \quad \text{..... 3.22}$$

Where

$x_f$ final cell concentration

$x_o$ initial cell concentration

$t_T$ turn around time (washing, sterilizing, filling etc)

$t_D$ delay time until inoculation

$t_L$ lag time after inoculation

### 3.5.5 Fed-batch Kinetics

In a fed-batch process, there is a continuous input of certain nutrients (usually the carbon source) during the fermentation. There is no removal of material from the system. As a result, the volume of the medium will continuously increase. The addition of the nutrients can be continuous or discontinuous. Moser[30] has proposed a number of equations for modelling fed-batch systems. In general, the kinetic terms are determined for the batch process.

### 3.5.6 Continuous Fermentation Kinetics

Continuous fermentation is carried out either by operating the system as a turbidostat or a chemostat.

A turbidostat is operated by maintaining a constant cell density and then supplying nutrients as required.

A chemostat operates by supplying essential growth limiting nutrients at a constant rate with the result that the cell density and growth rate adjust themselves to the supply.

Both systems require a method of controlling a constant reactor volume. This is normally achieved by using overflow tubes. Turbidostats, in addition, require photocells to monitor cell density. Chemostats are generally employed and continuous fermentation kinetics studies have mainly been carried out on them [32-34]. However, both systems have the same basic kinetic theory.

#### 3.5.6.1 Growth rate

Cell mass balance in a chemostat is given by:

$$\frac{dx}{dt} = \frac{Fx_o}{V} - \frac{Fx}{V} + \mu x - \alpha x \qquad \text{..... 3.23}$$

Cell accumulation = Cells in - Cells out + Growth - Death

The feed is usually pure and contains no cells (ie. $x_o = 0$), and $\alpha$ is usually negligible compared to $\mu$, thus the equation becomes:

$$\frac{dx}{dt} = \mu x - \frac{Fx}{V} \qquad \text{..... 3.24}$$

The term F/V is the dilution rate D ($h^{-1}$), therefore:

$$\frac{dx}{dt} = x(\mu - D) \qquad \text{..... 3.25}$$

Under optimum growth conditions, a steady state will be established where $dx/dt = 0$, thus $\mu = D$. Therefore, altering the value of D will lead to a change in $\mu$. At a certain value of $D = Dc$ (critical dilution rate), the growth rate becomes a maximum. Beyond this point, cell concentration starts decreasing as the cells are being washed out ( Figure 3.10).

### 3.5.6.2 Substrate Utilization

The relationship between dilution rate and substrate concentration can be examined by a material balance on the limiting substrate.

$$\frac{ds}{dt} = \frac{FS_R}{V} - \frac{Fs}{V} - \frac{\mu x}{Y_{x/s}} - mx - \frac{q_p x}{Y_{p/s}} \qquad \text{..3.26}$$

| Nutrient accumulation | Nutrient in | Nutrient out | Consum-ption | Main-tenance | Product formation |
|---|---|---|---|---|---|

In general, the maintenance term is much smaller than the amount consumed. At steady state where there is no product formation, Equation 3.26 simplifies to

$$x = Y_{x/s}(S_R - s) \qquad \text{..... 3.27}$$

At steady state, the Monod Equation 3.11 can be re-written as:

$$D = \frac{U_{max}S}{K_S + s} \qquad \text{....3.28}$$

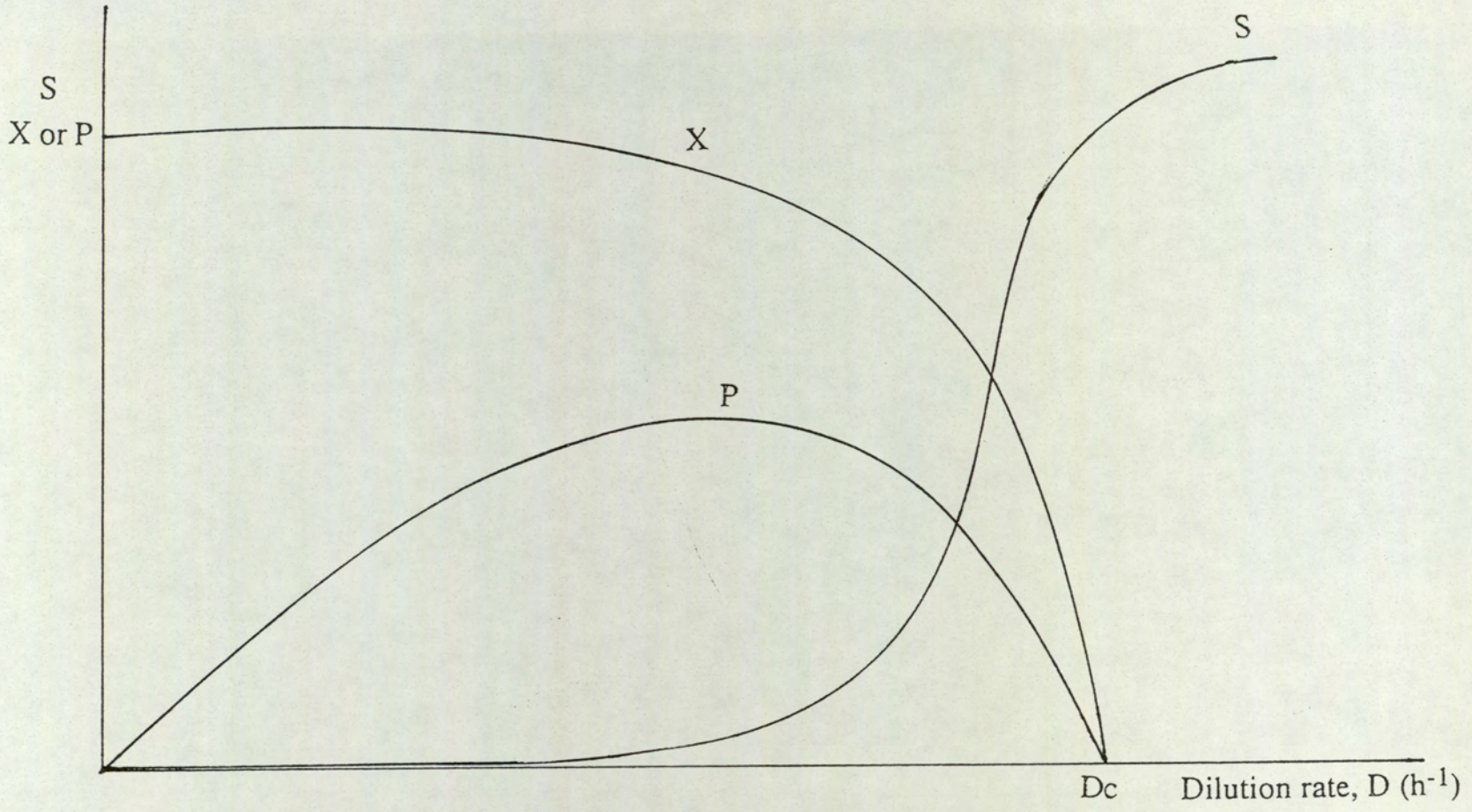



**Figure 3.10: Relationship between dilution rate during continuous fermentation and steady state biomass, substrate and product concentrations.**

Solving for s and replacing in Equation 3.27,

$$x = Y\,x/s \left( S_R - \frac{K_S D}{\mu_{max} - D} \right) \quad \text{..... 3.29}$$

### 3.5.6.3 Productivity

Productivity in a continuous culture is defined as:

$$P = Dx \quad \text{... 3.30}$$

Substituting for x as in Equation 3.29,

$$P = D\,Y\,x/s \left( S_R - \frac{K_S D}{\mu_{max} - D} \right) \quad \text{..... 3.31}$$

where $S_R$ is the substrate concentration in the reservoir. The relationship between biomass, substrate, productivity and dilution rate is shown in Equation 3.10.

## 3.6 MONITORING AND CONTROL OF FERMENTATIONS

The most widely monitored parameters of a growing culture are the cell density, nutrient levels, temperature, pH and product formed[50]. In order to maintain the culture under optimum growth conditions, control of these parameters is necessary. Cell growth and product formation are measured using different assaying techniques discussed earlier.

Nutrient levels are assayed by taking samples and analysing for the particular component. For aerobic systems, oxygen is an important component. It is measured by a variety of ways including sterilizable dissolved oxygen (DO) probes and mass spectrometry. Control of the level of oxygen or air required can be achieved by different design strategies[19,28-29] which facilitate oxygen transfer. Such strategies include ways of increasing aeration and agitation rates. Different types of fermenters have been used to improve mass transfer[35].

Temperature is usually monitored and controlled automatically using

various proprietary temperature controllers which incorporate heaters and coolers. Growth and death of organisms follow the Arrhenius relationship. The optimum growth temperature usually depends on whether the organism is a psychrophile (grows below 20 $^{o}$C), mesophile (grows at 20 - 37 $^{o}$C) or thermophile (grows above 38 $^{o}$C).

The pH is critical because microorganisms tend to grow under limited pH ranges. This characteristic could be advantageous in that certain contaminants would not be able to grow at the organisms' optimum pH range. Sterilizable probes are widely used for pH monitoring. Control is achieved by automatic addition of acids and/or alkali. Buffers are sometimes used, but they are quite expensive, and they also have the disadvantage of increasing the salt content of the medium. This is undesirable especially if the products are to be used as feedstocks.

## 3.7 SCALE-UP

Development of fermentation processes is always carried out in the laboratory mainly because of cost considerations. To transfer these processes to production, they would have to be scaled-up, usually through a number of stages. Three types of bioreactors currently used in industrial productions are non-stirred, non-aerated bioreactors; non-stirred aerated; and stirred (aerated and non-aerated) bioreactors.

Non-stirred, non-aerated fermenters are generally used in traditional processes like beer, wine and cheese fermentations. Most new product processes require growth of organisms in aerated, agitated vessels.

The main components of a scale-up strategy are mass transfer, shear, and heat transfer. These parameters affect mixing and growth of microorganisms in a bioreactor. Scaling-up by merely altering the dimensions of the fermenter (while maintaining geometric similarity) has been found to be unsuitable. In practice, therefore, scale-up is based on one or more of the following criteria:

1 **Mass transfer:** Growth of aerobic organisms in submerged culture is controlled by the availability of substrates and energy. Due to the

heterogeneous nature of culture media, growth can be limited by the rate of substrate or product transfer. Agitation is one of the ways by which uniform mixing is achieved. Oxygen transfer rate (OTR) in aerated systems is one of the main parameters for scaling-up. OTR is dependent on the mass transfer coefficient ($K_la$). Various correlations are available for scaling-up different bioreactors based on OTR[29]. The normal criteria chosen for scale-up to ensure proper mass transfer are constant agitation power per unit volume of culture, constant gas flowrate per unit volume and geometric similarity of vessel.

2 **Shear:** Shear rate is a function of agitator speed and diameter. It is given by the relationship; shear rate = NDi ( Di - agitator diameter, N - agitator speed). To maintain constant shear, NDi should be kept constant during scale-up.

3 **Heat transfer:** During cell growth, heat is produced by the organisms as well as by agitation. Removal of this heat is normally achieved by using cooling coils or jackets. Scale-up based on heat removal includes techniques of maximizing cooling area like passing of water through baffles as well as cooling coils and jackets. Various correlations have been proposed for removing heat from bioreactors containing both Newtonian and non-Newtonian cultures[36].

Different fermenter types are currently used for large scale aerobic fermentations. Some of them include the stirred tank, air-lift, loop and immobilised bioreactor systems.

Stirred tank systems are the most commonly used. Air-lift (pressure cycle) systems are aerated by circulating pressurised air. These produce very low shear and can be used for growing plant and animal cells. They can be very tall (e.g. the ICI single cell protein plant) and the pressure at the bottom increases oxygen solubility. Heat removal is much easier. However, they are expensive to construct, require high energy inputs for the large amount of air needed, and are much more difficult to maintain at uniform nutrient compositions.

Loop reactors are modified air-lifts in which a pump transfers both air

and liquid through the vessels.

Immobilised systems are those where air is circulated over a film of microorganisms growing on a solid surface (e.g. sewage treatment).

## 3.8 PRODUCT RECOVERY AND WASTE TREATMENT

Products of a fermentation process are usually in very dilute concentrations. The desired product is either cells, or some metabolic product of cells which could be inside the cells (intracellular) or outside the cells (extracellular). The first stage usually involves cell separation after which the product can be concentrated and purified. This is normally referred to as downstream processing and the various processes have been described in Chapter 2.

The undesired components of the culture broth require some sort of treatment before being disposed of. Various techniques have been widely reported in the literature. Some of them could actually be an additional fermentation stage.

## 3.9 FERMENTATION ECONOMICS

Most fermentation products are also obtainable from other natural sources like plants and animals. In order for the product of a fermentation process to be competitive in price, the cost of producing it must be kept to a minimum. The yield of the product should be predictable and the quantities lost during extraction and purification should be small.

The chosen organism should be capable of producing large amounts of product. In some cases genetic engineering techniques are used to improve the strain of the organism.

The raw materials used should be cheap. Raw material costs can be up to 73 % of the total cost. Storage, handling and mixing should be carried out with care. Components that are required continuously such as air for aerobic systems should be sterilized using the most economic method.

Aeration, agitation, heating, cooling and control should just be sufficient for the system.

The degree of purification should not be more than necessary for the use of the desired product, and the purification technique should not lead to significant loss of product. If the product is more stable in the unpurified form, purification should be employed just before use.

# CHAPTER FOUR

# LITERATURE SURVEY 3

# CHAPTER FOUR
# LITERATURE SURVEY 3

# DEXTRANSUCRASE

## 4.1 INTRODUCTION

Dextransucrase (sucrose: 1, 6-α-D glucan 6-α- 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:-

$$\underset{\text{sucrose}}{nC_{12}H_{22}O_{11}} \xrightarrow{\textit{dextransucrase}} \underset{\text{dextran}}{(C_6H_{10}O_5)n} + \underset{\text{fructose}}{nC_6H_{12}O_6} \qquad \text{---4.1}$$

Bacteria of the family *Streptococceae* produce dextransucrase (henceforth also referred to as the enzyme ). The genera belonging to this family that have generally been used for dextransucrase production are *Streptococcus* and *Leuconostoc*[21]. Different species of these bacteria have been used. However, the enzyme (and thus the dextran produced ) has been found to be species dependent[37-38]. Dextransucrase is an inducible enzyme that requires only sucrose for its induction.

Numerous studies have been undertaken on the production and purification of dextransucrase, its reaction mechanisms, its immobilization, and use, i.e. for the production of dextran. 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* (LM) NRRL strain B512(F). The enzyme produced by these organisms synthesizes dextran which is non-pathogenic and highly soluble in water. This dextran is used in the pharmaceutical industry as clinical dextran.

## 4.2 DEXTRAN

### 4.2.1 Introduction

Dextran is the name given to a class of extracellular polysaccharides synthesized from sucrose by bacterial enzymes (glucansucrases or glucosyltransferases ). 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[38-39]. The degree of branching differs with the species of bacteria used, and affects the solubility and other rheological properties of the dextran[40]. The main properties of dextrans are their viscous and near Newtonian behaviour, and the fact that they contain no charged groups[41]. Jeanes[38] has published a list of different strains of bacteria and the dextrans produced by their enzymes. Neely [42] has reviewed the different methods of studying the structural characteristics of dextrans.

### 4.2.2 Production

Traditional production of dextran is carried out in sucrose rich culture media. A suitable medium is inoculated with a specific bacterial culture. The growing cells secrete dextransucrase into the medium where it converts the excess sucrose to dextran. The optimum conditions for dextran synthesis are a pH of 5.0 - 5.3 (Figure 4.1) and a temperature range of 25 - 30 °C. Alsop[40] reported that the optimum sucrose concentration required is 17.9% w/v.

Most modern methods of dextran synthesis employ cell-free enzyme preparations. Enzyme production is normally carried out at pH values greater than 6.0 in order to avoid dextran formation. After synthesis, the dextran is separated from the reaction mixture and purified by a series of alcohol precipitation, acid hydrolysis and spray drying techniques. The product of these purification stages is often referred to as crude or native dextran. This dextran has a wide range of molecular weight distributions (MWD). In order to be useful for any particular purpose, the molecular weight should be within a narrow range of MWD. Several methods have been used to produce dextrans of the required MWD.

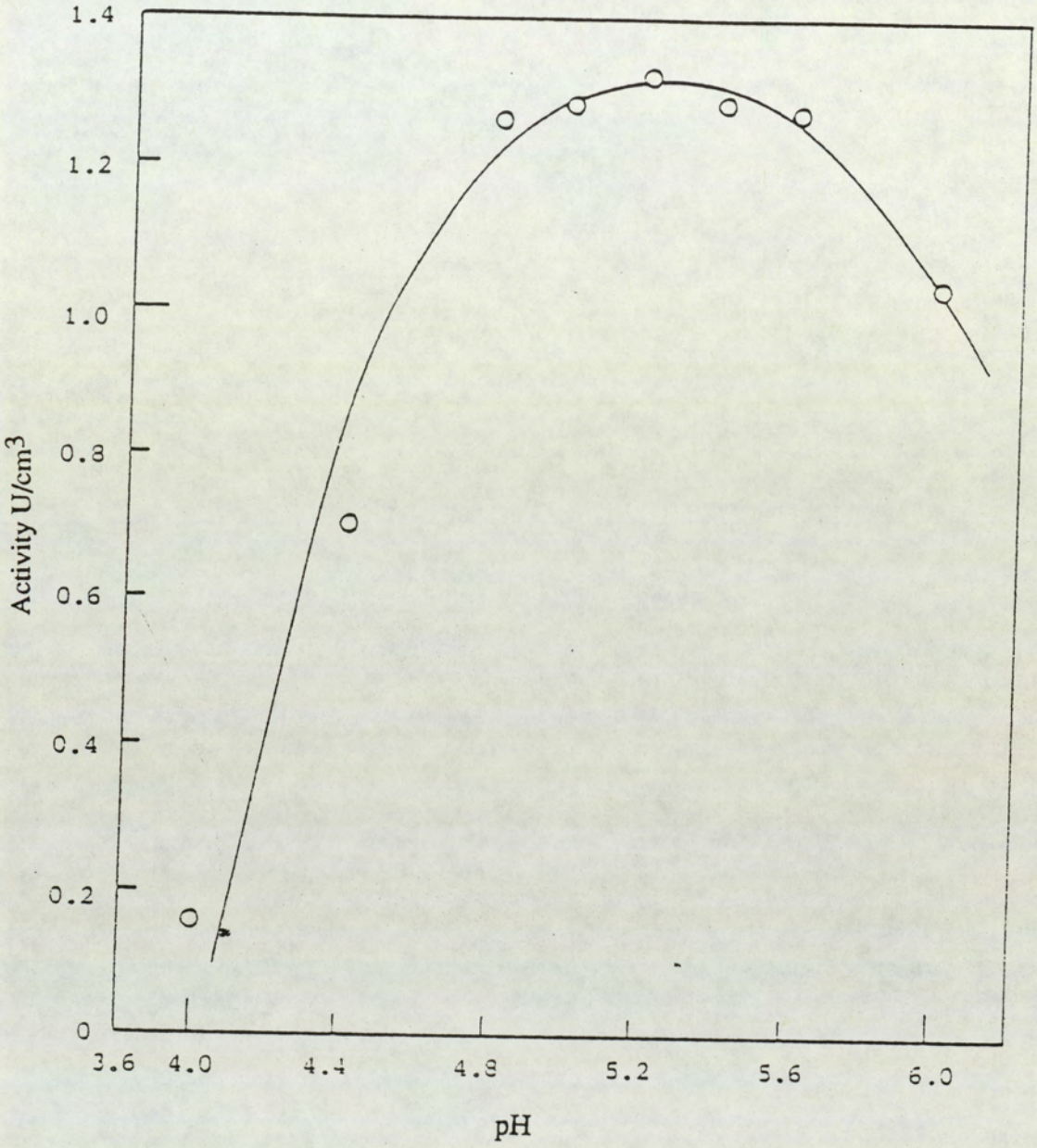



**Figure 4.1: Effect of pH on dextransucrase activity at 30 $^{o}$C (ref 60)**

Some of these processes have been used to produce dextran standards of given molecular weight fractions termed T fractions, e.g. dextran T70, denoting dextran of 70,000 daltons molecular weight.

Alsop *et al.*[40,43-44], Barker *et al.*[91-92] and Poland[93] have reported methods of characterising the MWD of dextran. They described processes for producing clinical dextran as well as dextrans of different MWDs. These involved the combined use of gel chromatography, ultrafiltration, and ion exchange chromatography. These systems have been operated batchwise [70], semicontinuously and as continuous cascades.

### 4.2.3 Uses of dextran

Dextran has had its widest use in the pharmaceutical industry, either as a blood volume expander (molecular weight of 70,000 daltons) or as a blood flow improver (molecular weight of 40,000 daltons). It has also been used as an iron complex to produce iron-dextran which is used for the treatment of iron deficiency anaemia in humans and animals.

Alsop[40] has compiled a list of uses of dextran. Included are its uses in the food industry as a gelling agent, in the petroleum industry for secondary oil recovery and as oil drilling muds.

Dextran is also increasingly being used for preparing separation membranes which are generally marketed under different proprietary names e.g. Sephadexes[38-41].

## 4.3 PRODUCTION OF DEXTRANSUCRASE

Early workers[38,45-47] produced dextransucrase and used it simultaneously for dextran production. The medium they used was basically similar to that by Jeanes[68]. Hehre[47] was the first to produce dextran from cell-free extracts from *Leuconostoc mesenteroides* (LM). Since then, there have been numerous studies aimed at optimising the yield of dextransucrase. Most of the work has been carried out using dextransucrase produced by LM NRRL strain B512(F). This enzyme produces dextran which is relatively stable, highly soluble in water, non-pathogenic has 95% of its bonds being $\alpha$-1,6 glucosidic bonds and 5% being $\alpha$-1,3 branched linkages. This section will mainly be involved with the enzyme produce by LM NRRL B512(F)

(henceforth also referred to simply as LM). LM B512(F) are heterofermentative Gram positive, non-pathogenic microorganisms, present as spheres or cocci (0.5 - 1.2 μm), and exist in pairs or short chains[48]. They require rich media containing such growth factors as vitamins (folic acid, biotin, thiamine) and amino acids[49]. These bacteria are capable of utilizing a number of carbon sources such as fructose, glucose, maltose, and sucrose.

Temperatures of 20 - 30 °C and pH of 6.0 - 7.0 have generally been used to produce dextransucrase from LM[51]. However, pH 6.7 has been found to be the optimum for maximum enzyme production[52]. Schneider and co-workers[53] and Alsop[40] have also reported 23 °C as the optimum temperature. The degree of aeration required for maximum enzyme production has not had as much attention given to it as that give to additives, or modifications of the basic medium of earlier workers. Neely and Nott[66] used different carbohydrates and proved that only sucrose was needed for the elaboration of dextransucrase by LM.

Many of the early researchers on dextransucrase produced the enzyme so as to use it for kinetic studies. This was mainly carried out in shake flasks or batch bioreactors with very low yields being obtained. Lawford *et al.*[37] carried out aerated batch fermentations at 25 °C using a 2 litre fermenter and obtained about 80 units of dextransucrase activity. The activity was defined as the micro grams of reducing sugar produced/min/$cm^3$. They reported that the enzyme production was discontinuous during the exponential cell growth and that media containing in excess of 1% phosphate were detrimental to the enzyme activity.

Lopez and Monsan[65] obtained 180 DSU/$cm^3$ of enzyme activity by continuously feeding the fermenter with sucrose at a rate of 20 g/l/h. One DSU (Dextransucrase Unit) is defined as the amount of enzyme that will convert 1 mg of sucrose to dextran in 1 hour at 25 °C and at a pH of 5.2. Their process conditions included mild aeration of 0.06 VVM (i.e. volumetric air flow rate per unit volume of medium) and an agitation rate of 280 rpm. Their process was carried out in a 3 litre fermenter.

Schneider and co-workers[53] reported yields much higher than those of Lopez and Monsan. They obtained enzyme activities of up to 400

DSU/$cm^3$ by using highly aerated conditions and reported that it is absolutely necessary to maintain the level dissolved oxygen (DO) in the fermenter at between 40% and 70% of the initial saturated value all through the fermentation process. Their processes were carried out at scales of up to 300 litres at which they obtained up to 300 DSU/$cm^3$ of enzyme activity. The process conditions also differed from those of Lopez and Monsan(65) in that sucrose was not added at a constant rate but at an increasing rate so as to maintain the sucrose level in the fermenter between 5 - 10 g/l. The amount of yeast extract and phosphate they used was also much higher than that used by previous workers. Their medium contained 4% w/v yeast extract and 2% phosphate. In comparative studies, they reported yields of 105 DSU/$cm^3$ when the DO level was allowed to drop to 0%, up to 180 DSU/$cm^3$ under 100% DO saturation, and 150 DSU/$cm^3$ when the system was not aerated.

Other studies have been carried out on the conditions for improving dextransucrase production. Brown and McAvoy(67) observed that fermentations with different yeast extracts suffered from some form of limitation late in the exponential growth phase.They used $CO_2$ and $O_2$ analysis to locate the occurrence of this limitation.By aseptically adding known components to the fermentation medium, they found out that the component in the yeast extract that caused this limitation was folic acid. Lee *et al.* (68) studied the effects of aeration and agitation rate on the yield of dextransucrase. Using a 3 litre vessel, they recommended an aeration rate of 0.5 VVM and agitation of 750 rpm. They used only two aeration rates; 0.5VVM and 1.0 VVM. Their results showed no difference in the activity when the two aeration rates were applied with the same agitation speed. For example, they reported enzyme activities of 30.84 DSU/$cm^3$ and 30.06 DSU/$cm^3$ for 1VVM and 0.5 VVM aeration rates respectively. The agitation speed was 500 rpm for both cases.

Very little work has been done on continuous dextransucrase fermentation. Lawford *et al.*(37) used a dilution rate of 0.53 $h^{-1}$ and obtained 9 DSU/$cm^3$/h of enzyme activity. Kurt and Curt(69) used a dilution rate of 0.095 $h^{-1}$ to obtain 25 DSU- Units/ $cm^3$/h. One DSU- Unit is defined as the amount of enzyme that will produce 1mg of fructose in 1 hour at 23 °C and pH 5.2. This is roughly about 48 DSU/$cm^3$, however, they determined

their enzyme activity at 23 $^{o}$C instead of 25 $^{o}$C that is generally used. Their enzyme was then used for continuous synthesis of clinical dextran with 80 - 90% conversion of sucrose being achieved. More recently, Paul and co-workers[64] reported 70 DSU/cm$^3$/h at a dilution rate of 0.4 h$^{-1}$. The fermentation medium they used contained 2% w/v sucrose and 1% yeast extract.

Dunsap and Gros[48] produced a model for estimating the various parameters for dextransucrase production under batch and continuous conditions. They fitted results from work on dextransucrase carried out by different researchers into their models and estimated various yield coefficients.

Veljkovic *et al.*[85] used dynamic methods for the determination of $K_l a$ values of dextransucrase fermentations. By employing an oxygen electrode placed in a bypass cell, they estimated $K_l a$ values of LM strain ZDRAVLJE S-P growing under different aeration and agitation conditions.

## 4.4 PURIFICATION OF DEXTRANSUCRASE

Fermenter broths containing dextransucrase also contain many impurities. Dead cells, unused nutrients, salts from pH buffers, proteins, and other byproducts such as fructose, glucose, mannitol, leucrose, oligosaccharides, and dextran are some of the impurities. Removal of these impurities is always accompanied by loss of enzyme activity. The higher the degree of purification applied, the greater the process time and the loss of enzyme activity.

The first stage in the purification of the enzyme is cell separation by centrifugation. This is normally carried out below 8 $^{o}$C, either using batch or continuous centrifuges. Zafar[70] reported that high centrifugal forces led to loss of activity at 4 $^{o}$C and 15 minutes centrifuging time ( Figure 4.2).

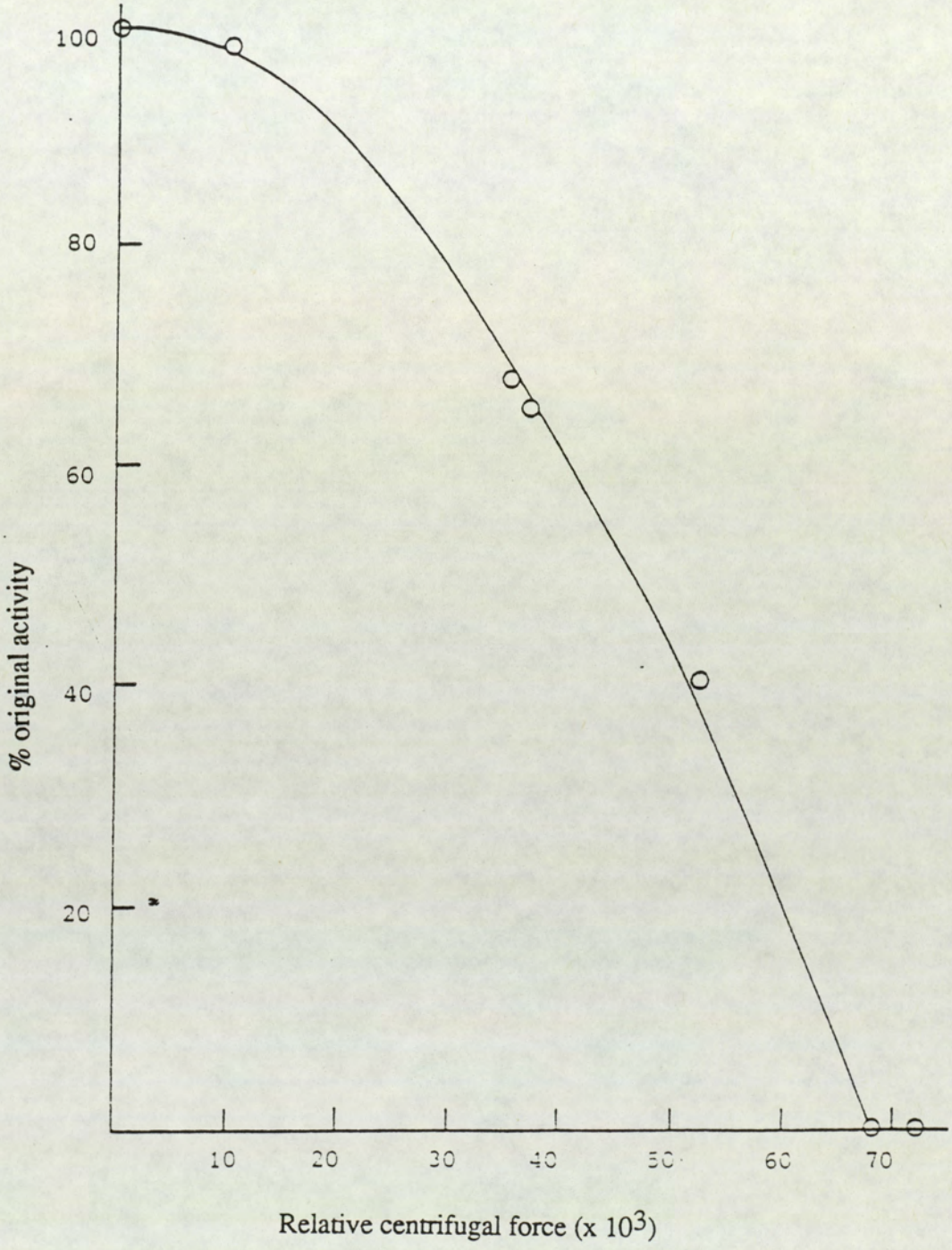



**Figure 4.2: Effect of centrifugal force on dextransucrase activity (ref 70)**

Numerous purification methods have been used. These have either involved precipitation, ultrafiltration, gel chromatography, and ion exchange chromatography or a combination of these[45-48,52-65,70-72].

Hehre[46-47] purified the enzyme by adding ammonium sulphate and then washing the precipitate three times with half saturated ammonium sulphate in 0.1% acetic acid. He extracted the enzyme at pH 6.3 with citrate buffer and then precipitated it in alcohol.

Lawford *et al.*[37] also isolated the enzyme from cell-free cultures by ammonium sulphate precipitation. The crude enzyme was desalted by gel filtration with Sephadex G-25 and applied it to a column of DEAE-(diethylamino ethyl) cellulose, equilibrated with acetate buffer at pH 5.5, and containing 0.2 mM $CaCl_2$. Under these conditions, over 90% of the enzyme activity bound onto the ion exchanger and was eluted with buffer containing 250 mM NaCl.

Kobayashi and co-workers[63,73] used a lengthy method which involved precipitation of the enzyme with hydroxyapatite, dextranase treatment and separation by ion-exchange chromatography.

Reilly[57] used an Amicom 402 ultrafilter containing a XM- 100A membrane to concentrate the enzyme supernatant. He then purified it by gel permeation chromatography using a column packed with LXB Ultrogel AcA 34 and obtained 56% yield of enzyme activity. Later, Lopez and Monsan [65] used this method and had a yield of 96.4 %.

Steward and Jackson[58] modified the process by Schneider *et al.*[53] to obtain pure enzyme by a single stage anion-exchange chromatographic process. The crude enzyme was applied to a column of DEAE Sephadex A-25 and eluted with buffer solutions to remove contaminants before changing the buffer to elute the enzyme.

Phase partition techniques have also been used for dextransucrase purification[55-56]. Paul *et al.*[64] used repeated phase partition and obtained specific activities of 3500 DSU/mg of proteins. They further developed a process for simultaneous purification and concentration of the enzyme by phase partitioning[54]. This involved the addition of polyethylene glycol ( PEG) and dextran to a culture supernatant, mixing and allowing to settle. The mixture separated into two phases: a dextran rich phase containing

the enzyme and the PEG phase containing the impurities. The advantage of this process is that it could be carried out continuously.

More recently, Miller and co-workers[72] used a sequence of dextranase treatment, DEAE-cellulose chromatography, affinity chromatography on Sephadex G-200, and chromatography on DEAE- Tris-acryl M to obtain about 1890 -3600 DSU/mg of enzyme activity with yields of 30 - 50%. Although lengthy and requiring narrow ranges of conditions they claimed it could easily be scaled-up.

## 4.5 DEXTRANSUCRASE REACTIONS

Dextransucrase has its optimum activity at pH 5.0 - 5.3 ( Figure 4.1 ) and temperature in the range of 25 - 30 $^{o}$C (Figure 4.3). However, above 27.5 $^{o}$C the enzyme tends to rapidly decay[57]. A lot has been reported on the effects of different conditions on the reactions of the enzyme. It is inhibited by compounds like EDTA (ethylenediamine tetraacetic acid )[37] and divalent ions like copper, lead, and mercury[61]. Activation of dextransucrase can be caused by $Ca^{2+}$ and exogenous dextran[37,64,73] . Kobayashi *et al.*[74] studied the effects of various chemicals and substrate analogs on the activity of the enzyme. They reported that compounds such as D-glucose, and D-fructose are capable of inhibiting dextransucrase activity by as much as 60 - 80%.

Robyt and co-workers[59,61-62,77-80] have carried out a lot of work on the reaction mechanisms of dextransucrase. They made extensive use of pulse and chase techniques using labelled [ $^{14}$C] sucrose, fructose, maltose and oligosaccharides to study the mechanisms of dextran synthesis and the acceptor reactions that take place when the enzyme reacts. Robyt *et al.*[77] insolubilized dextransucrase in Bio-Gel P2 beads. The insolubilized enzyme was pulsed with labelled sucrose. Labelled products (glucose and dextran) were obtained by heating the reaction mixture at 90 $^{o}$C for 10 minutes.

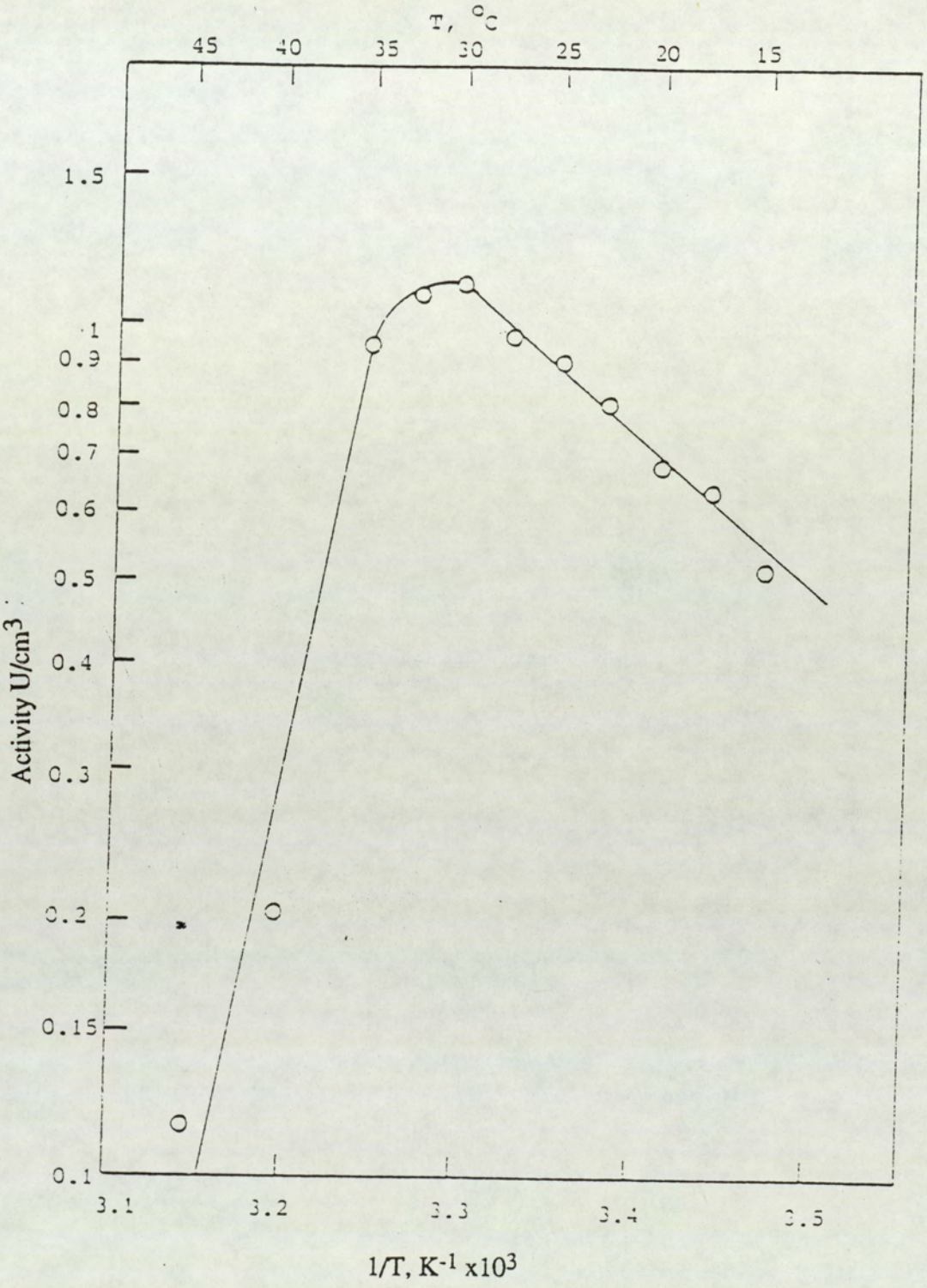



**Figure 4.3: Effect of temperature on dextransucrase activity at pH 5.2 (ref 60)**

A similar experiment was carried out with non-labelled sucrose, and the dextran produced in either case was hydrolysed. By comparing the results they concluded that dextran is synthesized from sucrose by the transfer of glucose from the sucrose to the reducing end of the growing chain. Also, that during the synthesis, there is a glucosyl and a dextranosyl group covalently attached to the enzyme active site. From these studies, they proposed a mechanism for the formation of dextran by the enzyme.

This mechanism assumes that there are two similar active groups in the active site of the enzyme (Figure 4.4). Two nuclophiles at the active site termed $X_1$ and $X_2$ attack two bound sucrose molecules to give two glucosyl groups covalently linked to the nuclophiles through the $C_1$ carbon[78]. In subsequent steps, the -OH on the $C_6$ carbon of one glucose unit makes a nuclophilic attack onto the $C_1$ of the other forming an $\alpha$-1,6 linkage, and releasing the active site nuclophile which attacks another sucrose molecule to give a new glucosyl group. This group then attacks then $C_1$ of the isomaltosyl group to form another $\alpha$-1,6 linkage. The process continues with the two active sites alternately forming covalent complexes with glucose and the growing chain until stopped by an acceptor. Acceptors do not initiate polymerization, but cause termination of the polymer chain at the reducing end by displacing glucose and the growing dextran from the active sites of the enzyme[59]. Larm and co-workers[81] carried out studies on the site chains of dextran produced by the enzyme and reported that at least 85% of them are made up of one or two D-glucose units.

Low molecular weight sugars have been found to have acceptor effects on the enzyme reactions. D-glucose, D-fructose, maltose, and isomaltose are some of the acceptors commonly present in dextransucrase reactions. Their presence in a sucrose-enzyme mixture leads to the formation of low molecular weight dextran and a homologous series of oligosaccharides[59]. Prat and co-workers[76] have successfully used borate ions to reduce the acceptor effects of fructose in dextransucrase reactions. This is done by the borate ions complexing with the fructose, thus preventing it from binding with the enzyme active sites.

Some kinetic parameters for dextransucrase reactions have been estimated.

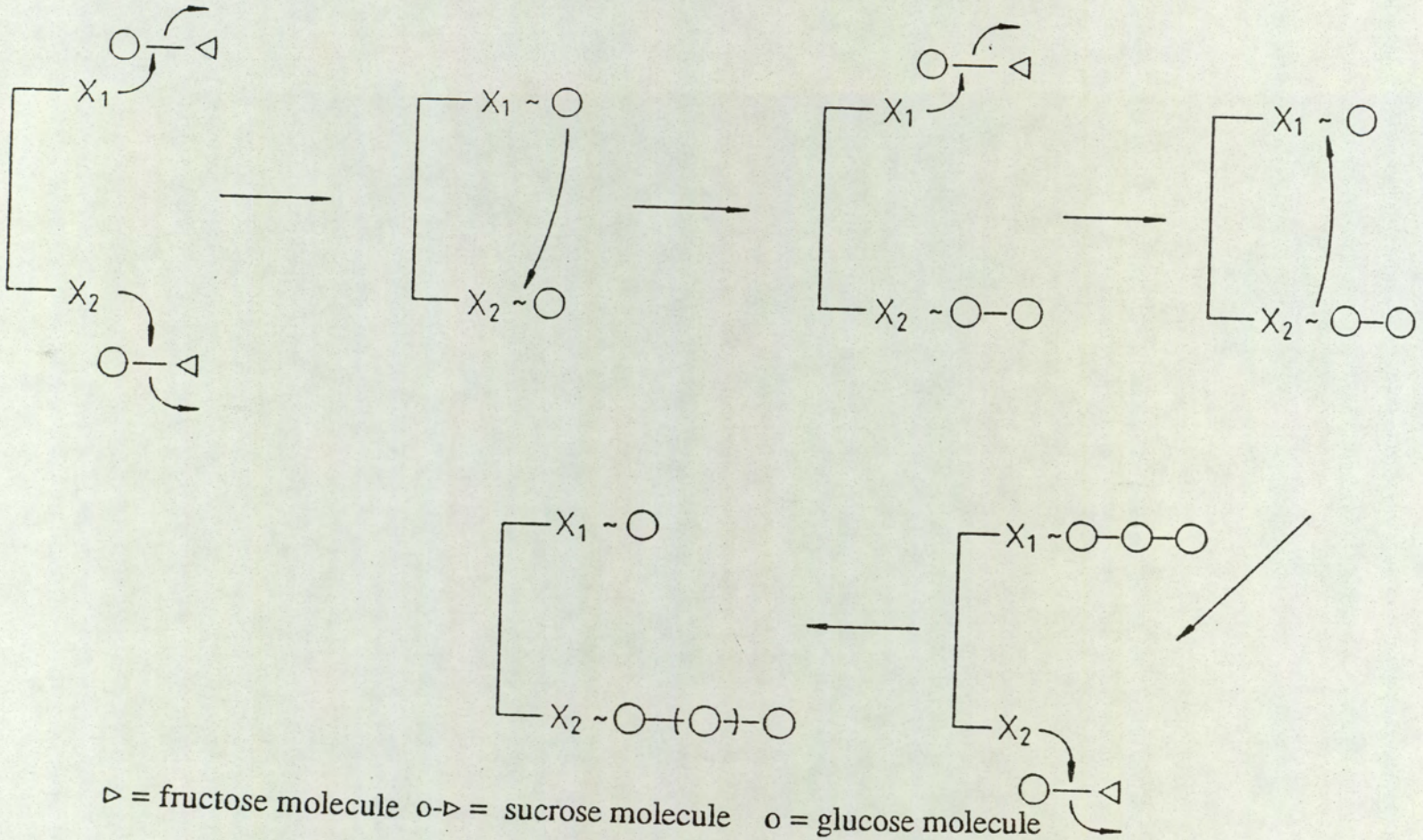



**Figure 4.4: Schematic representation of the mechanism proposed for the action of dextransucrase (ref 78)**

Lawford *et al.*[37] reported a $K_m$ value for the enzyme acting on sucrose to be 0.02M at 30 °C. Later, Lopez and Monsan[65] used the Eadie - Hofstee method (see Chapter 2) and obtained a value of 0.018 M. Kobayashi and co-workers [74] evaluated kinetic constants for different modes of action (competitive, non-competitive and mixed inhibition ) using various substrate analogs. Miller and Robyt[79], while studying the effect of calcium on the enzyme action found that at 0.1mM, $Ca^{2+}$ had activating effects. When the concentration became greater than 1.0 mM, they found that the $Ca^{2+}$ produced inhibitory effects. A model was proposed according to which dextransucrase has two $Ca^{2+}$ sites, one activating and the other inhibitory. The $Ca^{2+}$ at the inhibitory site prevents binding of sucrose. They also reported that LM B512 dextransucrase had two molecular weight forms - 159,000 daltons and 176,000 daltons. The 176,000 dalton form predominated in fresh preparations, while on storage, the 159,000 daltons form increased at the expense of the former.

## 4.6 DEXTRANSUCRASE IMMOBILIZATION

Not many attempts have been made at immobilizing dextransucrase. However, developments of novel types of bioreactor/separators, and the need for re-use of the enzyme is making the need for immobilization more important.

Kaboli *et al.*[60,71] obtained low yields upon immobilization using alkylamine silica and porous Spheron beads. However, they reported that immobilization did not change the optimum pH or temperature of the enzyme. Monsan and Lopez[65,83] achieved up to 830 DSU/g support by covalently coupling the enzyme onto an amino porous silica support (Spherosil), activated with a 2% gluteraldehyde solution in 0.05 M pyrophosphate buffer at pH 8.6. They found that in order to achieve this, the pore sizes of the support had to be wide enough to allow not only enzyme fixation but also the diffusion of dextran and sucrose. Maltose enhanced immobilization by eliminating dextran from the enzyme-dextranosyl complex thereby increasing the enzyme-support contact.

# CHAPTER FIVE

# CHAPTER FIVE

# EXPERIMENTAL AND ANALYTICAL EQUIPMENT AND TECHNIQUES

## 5.1 INTRODUCTION

Three sizes of fermenters were used for the batch studies. These were a 16 litre, an 80 litre and a 1000 litre fermenter. Shake flasks were used for simple screening experiments. Continuous fermentations were carried out using a 500 $cm^3$ chemostat.

The analytical techniques employed throughout the course of the work were cell growth determination by turbidity (optical density) measurements at 590 nm, i.e. $OD_{590}$ measurements, and enzyme activity determinations. Tests for contamination, gas analysis, cell dry weight determination and fermenter broth analysis using HPLC were also carried out from time to time. The Hostettler's method[87] was used to measure the fructose released in the enzyme activity assay. The HPLC technique was used as a confirmatory test.

## 5.2 EXPERIMENTAL EQUIPMENT AND TECHNIQUES

### 5.2.1 Batch experiments

Except during scale-up processes, all batch and fed-batch experiments were carried out in a 16 litre New Brunswick "microgen" SF116 fermenter (see Plate 1). It has a working volume of 3 - 12 litres, but the actual volume that was used for all the experiments was 6 litres. It is steam sterilizable and is fitted with an Ingold pH electrode, a dissolved oxygen (DO) probe and an antifoam probe which are all sterilizable. These probes and electrodes are linked to their respective controllers which have interfaces for outputting their respective readings to a chart recorder or computer.

DO was controlled by automatically varying the agitation speed between the range of 100 and 1200 rpm.

## KEY TO PICTURE

1 Fermenter vessel

2 pH probe

3 DO probe

4 pH, DO and antifoam pump

5 pH, DO and antifoam controller

6 Feed pump

Plate 1: Picture of New Brunswick Microgen fermenter

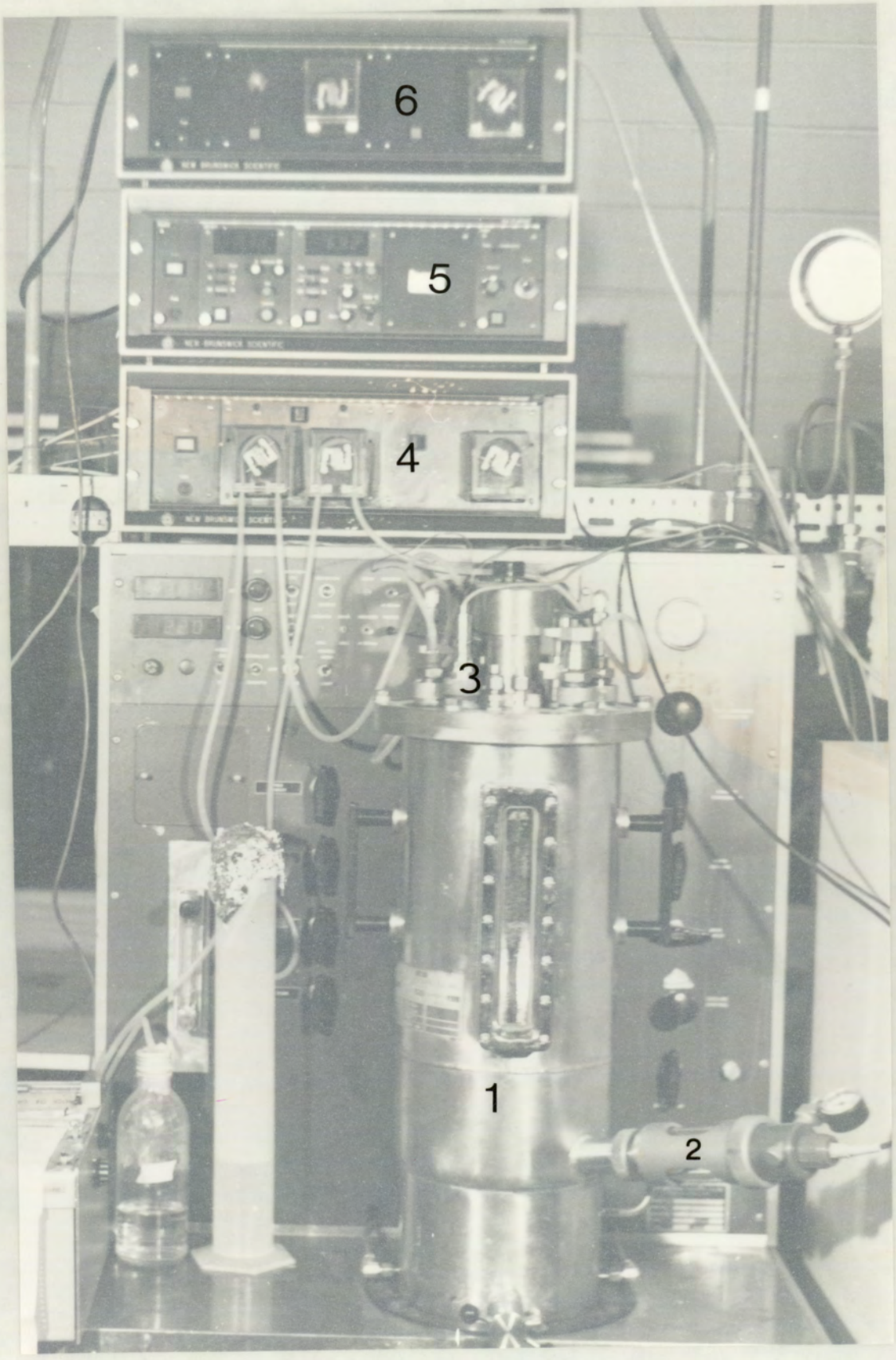
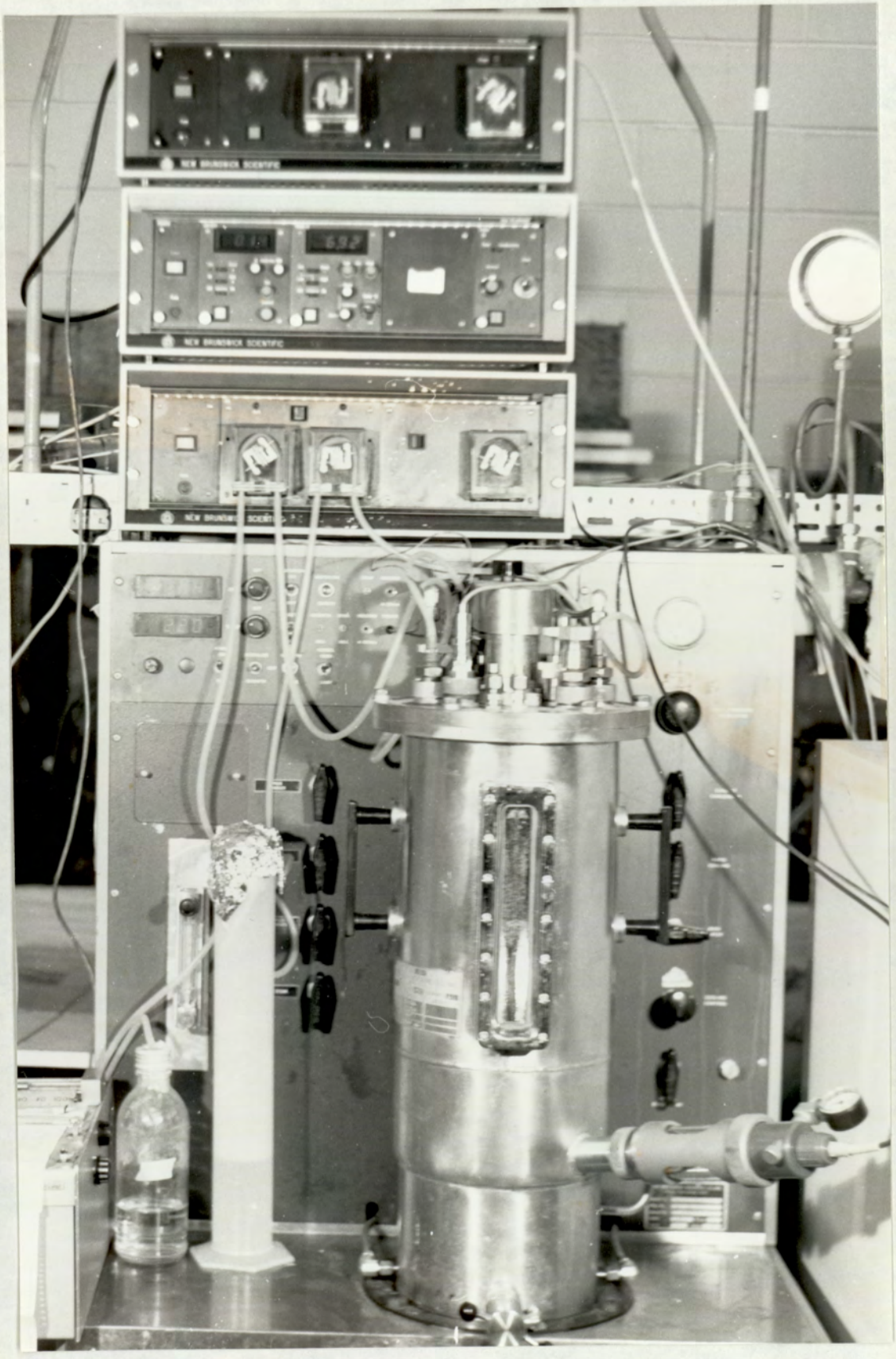


Plate 1: Picture of New Brunswick Microgen fermenter



Air was supplied through an air flow meter of 0-15 l/min throughput. This was later replaced by a bigger flow meter capable of delivering up to 50 l/min of air. Temperature was also controlled automatically by altering the temperature of water flowing through a heat exchanger in the fermenter. The controller was capable of controlling temperatures from 5 $^{o}$C above the cooling water temperature up to 70 $^{o}$C. During sterilization the cooling water was turned off and steam allowed to pass through the heat exchanger, giving sterilization temperatures of up to 125 $^{o}$C. Air inlet lines and filters were sterilized by purging steam through them.

The 80 litre fermenter was a pilot plant fermenter located at Teeside Polytechnic, Middlesborough, England and was equipped with facilities for measuring and controlling temperature, DO, and pH. DO control was not required since the scale-up processes were anaerobic. At the time this fermenter was used, a computer which was being linked to it was found to interfere with the controllers and was thus disconnected. No other recording facilities were available. Agitation was by means of a single bladed disc turbine stirring a fixed speed of 100 rpm.

The 1000 litre fermenter was a modified industrial seed fermenter with a fixed speed agitator of 120 rpm. This fermenter system was located at Fisons Pharmaceutical Plc., Holmes Chapel, Cheshire. Temperature was controlled by manually opening valves to allow the right amounts of cooling water and steam to flow into a jacket on the vessel. The pH was monitored with a pH probe inserted in a recirculating loop and controlled automatically by the addition of the sucrose/alkali mixture. This loop, the pH monitoring and control system, and the alkali feed line were the only modifications that were made to the existing fermenter. DO control was not required.

### 5.2.2 Continuous fermentation

A New Brunswick Bio-Flo model C30 chemostat was used for the continuous fermentations. The working volume was 320 $cm^3$. It was equipped with a temperature and pH monitor and the relevant control facilities. Agitation was by means of a magnetic stirrer (200 - 1000 rpm ). It also had facilities for aeration, but these were not required. The vessel was

not sterilizable, so sterilization was achieved by autoclaving after the medium had been put in it and all the ancillary connections made. The pH probe used was an Ingold autoclavable probe.

### 5.2.3 Sterilization equipment

All the fermentation media were prepared and put into the fermenters and then sterilized. Media for inocula and additional media were sterilized using a small bench top autoclave capable of sterilizing at temperatures of up to 125 $^{o}$C. The sterilization conditions for the various media components are shown in Section 5.4.

## 5.3 MICROORGANISM

The microorganism used for all experiments was *Leuconostoc mesenteroides* (LM) NRRL B512(F). This was supplied by Fisons Pharmaceuticals Plc., Holmes Chapel, Cheshire, England. The bacteria were supplied as freeze dried vials ( Fisons C31 cultures ). These vials were stored frozen at -10 $^{o}$C until required.

## 5.4 PROCESS MEDIA

The media were used for the preservation and propagation of the culture, sterility checks, the main fermenter and as additional media.

### 5.4.1 Culture preservation

The freeze dried vials were reconstituted in sterile broth, the cultures grown on agar slopes and stored in the refrigerator at about 4 $^{o}$C. These provided cultures for up to a year. The vials were reconstituted by adding about 2 $cm^3$ of sterile MRS (de-Man Rogosa Sharpe) broth into each vial to dissolve its contents. The preparation of the MRS broth is detailed in Section 5.4.2. The cultures in the suspension were used to inoculate sterile MRS agar slants. These were incubated for at least 48 hours at 25 $^{o}$C and then stored in the refrigerator until they were needed for the inoculum preparation (Section 5.4.2).

The MRS agar was supplied by Oxoid, Basingstoke, England. It was prepared according to the manufacturer's instructions, i.e. 62g of agar were dissolved in a small amount of boiling water and the volume made to 1 litre with distilled water. Small quantities were immediately transferred to sterilizable bottles and sterilized at 121 $^{o}$C for 15 minutes. After sterilizing, the bottles were slanted and cooled to 25 $^{o}$C. These sterile agar bottles were incubated for at least 3 days at 25 $^{o}$C. Any one that showed any signs of growth were discarded. The remaining sterile slopes were then inoculated with LM cultures.

Some of the sterile agar that was not inoculated with bacteria was heated to above 45 $^{o}$C to melt and was poured into sterile petri dishes. These were allowed to cool to 25 $^{o}$C and immediately used for a sterility check (Section 5.5.4 ).

### 5.4.2 Culture propagation

This stage is generally referred to as the inoculum preparation stage. The medium used for reconstituting the freeze dried cultures and for the inoculum preparation was the MRS broth, also supplied by Oxiod. This was prepared by dissolving 52g of MRS broth in a small amount of warm distilled water and making the volume up to 1 litre. The pH was adjusted to 7.0 - 7.2 using 5M NaOH, sterilized at 119 - 121 $^{o}$C for 15 minutes and cooled to around 25 $^{o}$C. The sterile broth was incubated at this temperature for at least 3 days. After this period, any broth which did not remain clear was thrown away.If they remained sterile, they were then used for the inoculum preparation when required. The sterile broths were found to be still useful after several months in the incubator.

The inoculum was prepared by aseptically transferring LM from one agar slope into 10 - 15 $cm^3$ of sterile broth. This was incubated at 23 - 25 $^{o}$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. Unless specified otherwise, all inoculum preparations were carried out in two stages as described here.

### 5.4.3 Fermenter medium

The composition of the medium used was identical to that of Schneider *et al.*[53] and is shown in Table 5.1.

**Table 5.1: Composition of fermenter medium**

| | | |
|---|---|---|
| Sucrose | 10.0 g/l | |
| Yeast extract | 40.0 g/l | |
| $K_2HPO_4$ | 20.0 g/l | |
| R* salts | 0.5 % v/v | |
| Antifoam | 0.1 % v/v | |
| **R* salts:** | | |
| | $MgSO_4.7H_2O$ | 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 115 $^oC$ for 20 - 25 minutes and cooled to 23 $^oC$.

### 5.4.4 Additional media

For batch processes caustic used for pH was the only component added. Sterile 5M NaOH was used for this purpose. For fed-batch experiments, sucrose and NaOH were added. Sucrose addition was coupled to the alkali demand (86). The composition of the sucrose/alkali mixture used was as shown in Table 5.2.

**Table 5.2: Composition of sucrose/alkali mixture.**

| | |
|---|---|
| Sucrose | 900 g |
| NaOH | 200 g |
| Water | to 1500 $cm^3$ |

The procedure for producing this mixture is as follows:-

1) Dissolve the NaOH in a small amount of cold water to make, say, a 15M solution.
2) Dissolve the sucrose in hot water so that the total volume of the mixture will be 1500 $cm^3$ (or the total required ).
3) Sterilize the two solutions separately at 121 $^oC$ for 30 minutes and cool.
4) Mix the solutions by slowly adding the caustic to the sucrose solution while stirring. This mixing is highly exothermic and causes a high temperature rise. The mixture should be allowed cool to around 25 $^oC$ before more caustic is added.

The sucrose/alkali mixture has a golden yellow colour. In some cases the solution turned dark red, especially when there was a large increase in temperature. This also happened when low grade NaOH was used.

However, this was not found to affect the results of the fermentations.

For continuous fermentation, the additional medium was of the same composition as the fermenter medium (Table 5.1), with the exception that it contained 20 g/l of sucrose instead of 10 g/l. 5M NaOH was used for pH control.

## 5.5 ANALYTICAL EQUIPMENT AND TECHNIQUES

### 5.5.1 Cell growth determination

Cell growth was measured half hourly or hourly throughout the course of the fermentation once the lag phase was exceeded. Growth was determined by measuring the turbidity or OD of the sample using a Pye Unicam SP1800 UV/visible photospectrometer. Other types were used during the scale-up experiments carried out away from the University, i.e. at Teeside Polytechnic and Fisons Pharmaceuticals Plc., Holmes Chapel.

To measure the OD of a sample, it was diluted 25 times with saline (9 % w/v NaCl solution). The OD value was read at 590 nm wavelength ($OD_{590}$) using 10 mm plastic, glass or quartz cuvettes. Before taking the readings, the photospectrometer was zeroed using saline. $OD_{590}$ values greater than 0.6 were found to be at the edge of, or outside the linear range of the photospectrometer. The 25 fold diluted sample was further diluted, the $OD_{590}$ value read and the result multiplied by the dilution factor to give a more accurate value of the 25 times diluted sample. To correct for the effects of the growth medium, the $OD_{590}$ of the sterile uninoculated medium was subtracted from each 25 fold diluted sample.

Cell dry weight determination was also carried out under standard fed-batch fermentation conditions (see Chapter 7). A calibration curve for $OD_{590}$ against cell dry weight (g/l) has been produced (Figure 5.1). The procedure for the determination of the cell dry weight is as follows:-

Cleaned centrifuge tubes were marked and dried in an oven at 90 $^{o}$C for at least 48 hours.

After drying, the tubes were taken out of the oven, immediately placed in a desiccator and allowed to cool down to room temperature,

and then carefully weighed.

Broth samples were taken from the fermenter, their $OD_{590}$ values determined and a known volume put in a dry centrifuge tube. At least three tubes were used for each sample.

The broth containing tubes were then placed in a centrifuge (Damon/IEC CU-5000 model) and centrifuged at 3000 rpm for 30 minutes. The supernatants were decanted and the cell sediments resuspended in saline and centrifuged. This rinsing was done twice and the resulting precipitate dried to constant weight at 90 $^{o}C$ for at least 48 hours.

The dried tubes and cells were placed in a desiccator to cool to room temperature and weighed. The weight of the dry cells was determined by subtracting the weight of the dry tubes from that of the dry cells and tubes.

To take account of solids present in the medium, their weight was determined using sterile medium that had not been inoculated. The true dry cell weight was then obtained by subtracting that due to solids in medium.

The cell dry weight was expressed in grams per litre by dividing by the volume of broth used for each tube. The average cell dry weight was calculated using the results of all the tubes containing any particular sample. This value was then plotted against the corresponding $OD_{590}$ of that sample to obtain Figure 5.1.

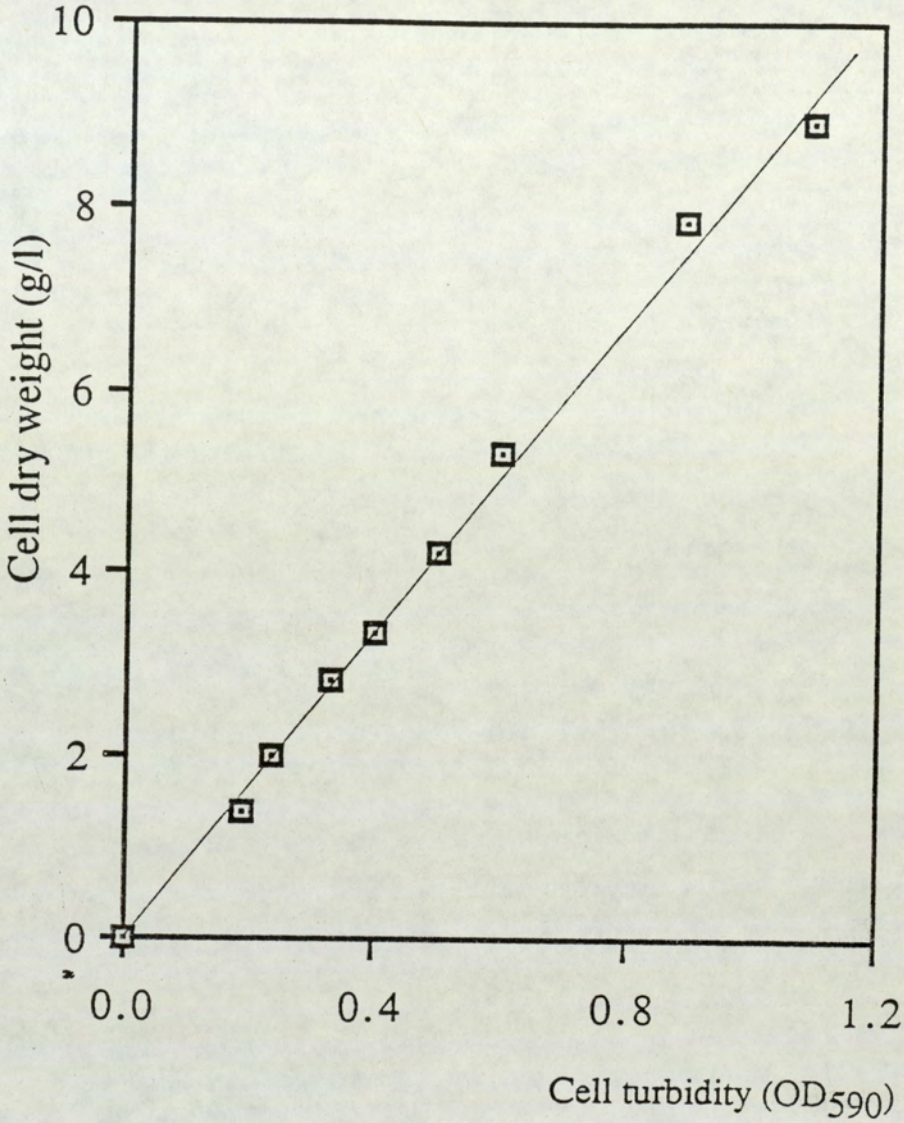



**Figure 5.1: Cell dry weight calibration curve**

### 5.5.2 Enzyme activity determination

The activity of the enzyme was determined using the Hostettler's method[87] to follow the fructose released during the action of the enzyme on sucrose. This was determined colorimetrically using a UV/visible spectrophotometer. The HPLC technique was used to monitor either the sucrose consumption or fructose and dextran released during the enzyme assay.

#### 5.5.2.1 Hostettler's method

The procedure for assaying the enzyme activity by the Hostettler's method is as follows:

1 $cm^3$ of enzyme containing 30 - 50 DSU/$cm^3$ (or an appropriate dilution of it) was added to 4 $cm^3$ of 6.25% w/v sucrose in 0.1M sodium acetate buffer at pH 5.2 and shaken. Immediately 0.5 $cm^3$ of this mixture were taken out and the remainder incubated in a water bath at 25 °C. The 0.5 $cm^3$ sample was added to 1.0 $cm^3$ of Sumner reagent (10g 3-5-dinitrosalicylic acid, 300g potassium-sodium-tartrate dissolved in 1 litre of 0.4M NaOH), placed in boiling water for about 5 minutes, cooled and the volume adjusted to 12.5 $cm^3$ with distilled water. This provided the unincubated sample. Every 5 to 10 minutes, a sample was taken from the reaction mixture in the water bath and treated in this way for the next 20 minutes. The samples prepared as described were stable for up to 24 hours and their optical densities were read at 530 nm ($OD_{530}$) against a blank, using the same photospectrometer employed for growth determination ($OD_{590}$). The blank was prepared by adding 0.5 $cm^3$ of the 6.25% sucrose solution in acetate buffer at pH 5.2 to 1.0 $cm^3$ of Sumner reagent, boiling for 5 minutes, cooling and adjusting to 12.5 $cm^3$ with distilled water. The enzyme activity was then calculated from the following formula :

$$DSU/cm^3 = \frac{(OD_i - OD_u) \times d \times 60 \times 2}{OD_s \times 0.52 \times 0.2 \times t} \qquad \ldots\ldots 5.1$$

$OD_i$ - Optical density at 530 nm of incubated sample .

$OD_u$ - Optical density at 530 nm of unincubated sample .

d - Dilution factor of enzyme solution .

t - Incubation time in minutes .

$OD_S$ - The optical density of a 2g/l fructose solution, prepared using 0.5 $cm^3$ fructose solution, 1.0 $cm^3$ Sumner reagent and treating as for all other samples .

#### 5.5.2.2 Practical aspects of the Hostettler's method

When properly analysed, the activities calculated for the different incubation times, up to at least 30 minutes, were the same, i.e. the relationship between $OD_{530}$ and incubation time $OD_{530}$ and incubation time, t, was linear (Figure 5.2).

It was found that in order to obtain $OD_{530}$ values within the linear region of the photospectrometer, it was necessary to have an appropriate dilution factor for the enzyme solution. As a rough guide, a dilution factor of 10 times the $OD_{590}$ (cell turbidity) value gave good results.

It was also observed, only after a number of experiments (up to 13) had been wrongly analysed, that the sucrose solution was easily contaminated by the enzyme. This was evident by the fact that the blank became darker, instead of being a bright yellow solution. Sometimes it was so heavily contaminated that the $OD_{530}$ value exceeded that of the fructose standard, i.e $OD_S$ of $0.80 \pm 0.05$ (see Figure 5.3). To overcome this, it was decided that the sucrose solution be freshly prepared daily. The 0.1M sodium acetate buffer at pH 5.2 kept longer without any contamination. So this was prepared substantial quantities (typically one litre) and small amounts (say 100 $cm^3$) used to prepare fresh sucrose solutions. To double check, the fresh sucrose was used to prepare a blank and another blank was prepared using distilled water (i.e. 0.5 $cm^3$ water added to 1.0 $cm^3$ Sumner reagent instead of the sucrose). The $OD_{530}$ values of these two solutions should be the same if sucrose was not contaminated.

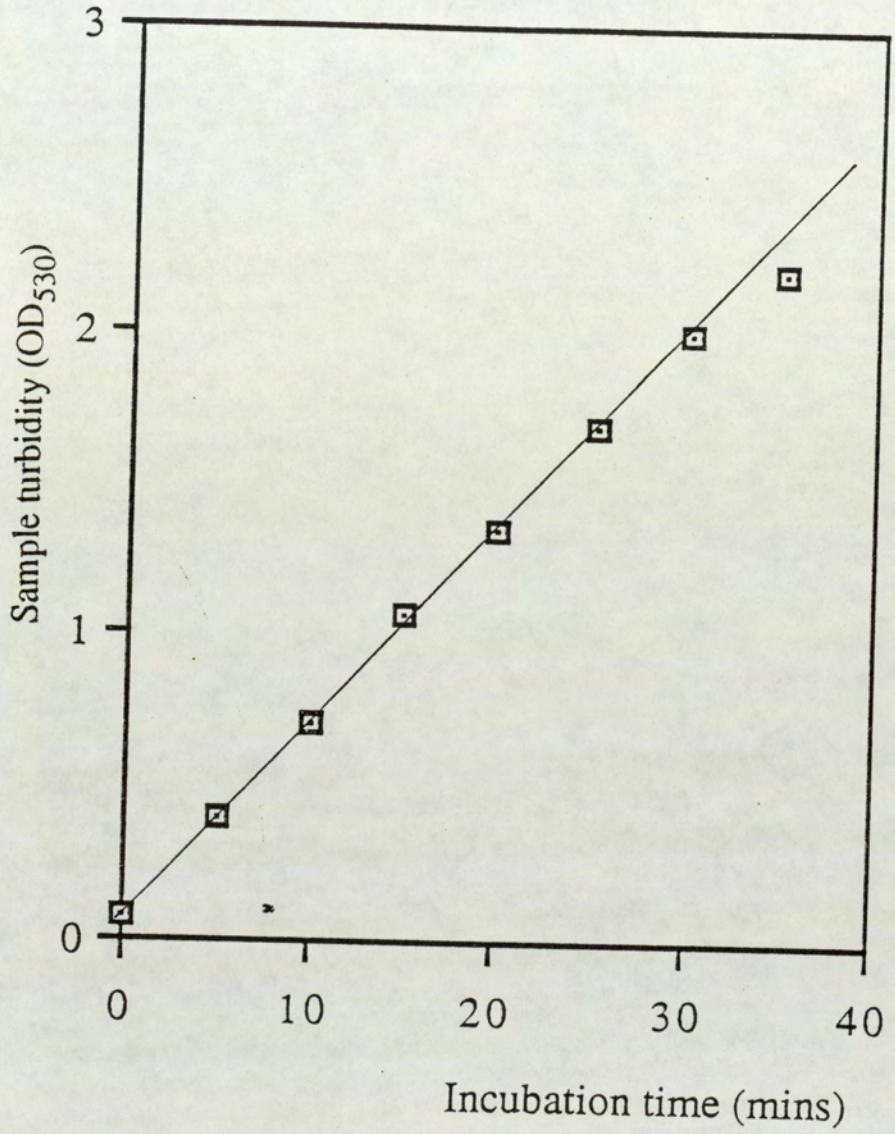



**Figure 5.2: $OD_{530}$ profile during enzyme activity determination**

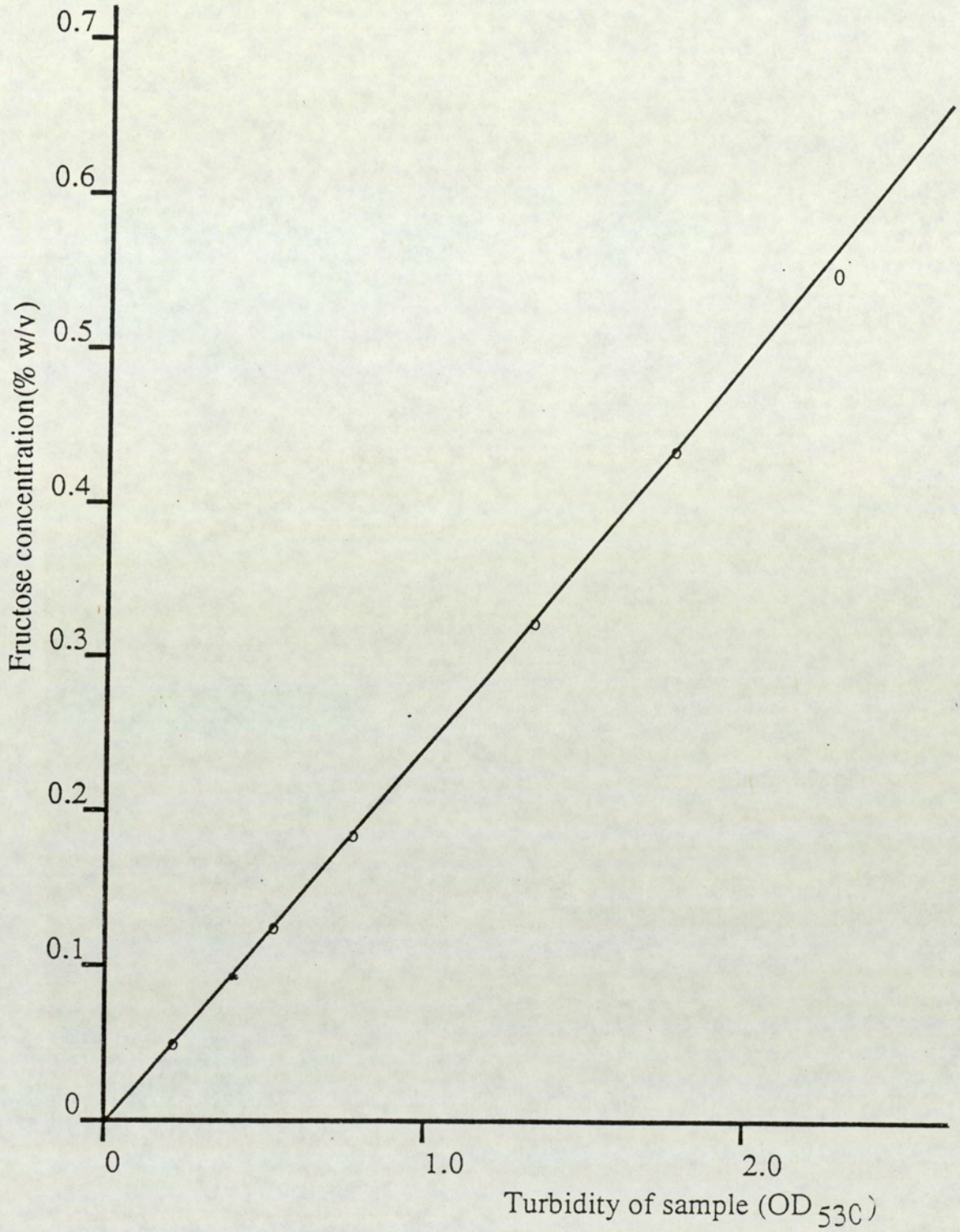



**Figure 5.3: Fructose calibration curve**

### 5.5.2.3 HPLC equipment

The HPLC system used for all the HPLC enzyme assays during the course of this research and for broth analyses had an Aminex carbohydrate HPX 87C column, supplied by Bio-Rad Laboratories, Watford, England. The system also had a pump, column heater and a refractometer, all supplied by Bio-Rad Laboratories. A schematic diagram of the equipment is shown in Figure 5.4. It consisted of an eluent reservoir, an isomantle, a pump, a sample injection valve, a guard column, a HPLC column, a column heater, a refractometer and an integrator/chart recorder.

The eluent used was de-ionised distilled water to which about 0.02% w/v calcium acetate was added. This was regularly degassed by raising the temperature of the reservoir to 80 $^{o}$C, with the aid of a thermal cut out device (Laboratory Thermal Equipment, Greenfield, England). The degassed eluent was pumped through a filter, a de-bubbler and the guard column (Bio-Rad Laboratories), using a dual piston Bio-Rad model 1330 pump.

Samples were filtered using 0.45 μm disposable Whatman filters and injected into the HPLC through a Rheodyne valve (Bio-Rad Laboratories). These samples passed through the guard column (which acted as a prefilter and thus increased the life time of the column) to the HPLC column. The column temperature was maintained at 85 $^{o}$C with the aid of the column heater.

Each sample was separated into its components in the column and these were then pumped through a differential refractometer (Bio-Rad Laboratories model 1750A). Due to the difference in the refractive index (RI) of the components and that of a reference filled with the eluent, a voltage peak was produced by the refractometer. The voltage was proportional to the concentration of the component.

These voltage signals were transmitted to an integrator (Hewlett Packard model 3390A) where the peak areas were calculated, and the actual concentrations obtained by comparing the area of each peak with that of a known concentration. The operating conditions of the HPLC column are as shown in Table 5.3.

**Table 5.3 Operating conditions of the HPLC system**

| | |
|---|---|
| Column dimensions (mm) | 300 x 7.8 |
| Operating pressure (bar) | up to 100 |
| Eluent flow rate ($cm^3$/min) | 0.5 |
| Retention times (mins) : | |
| dextran | 8.03 |
| sucrose | 10.02 |
| glucose | 12.00 |
| fructose | 15.32 |

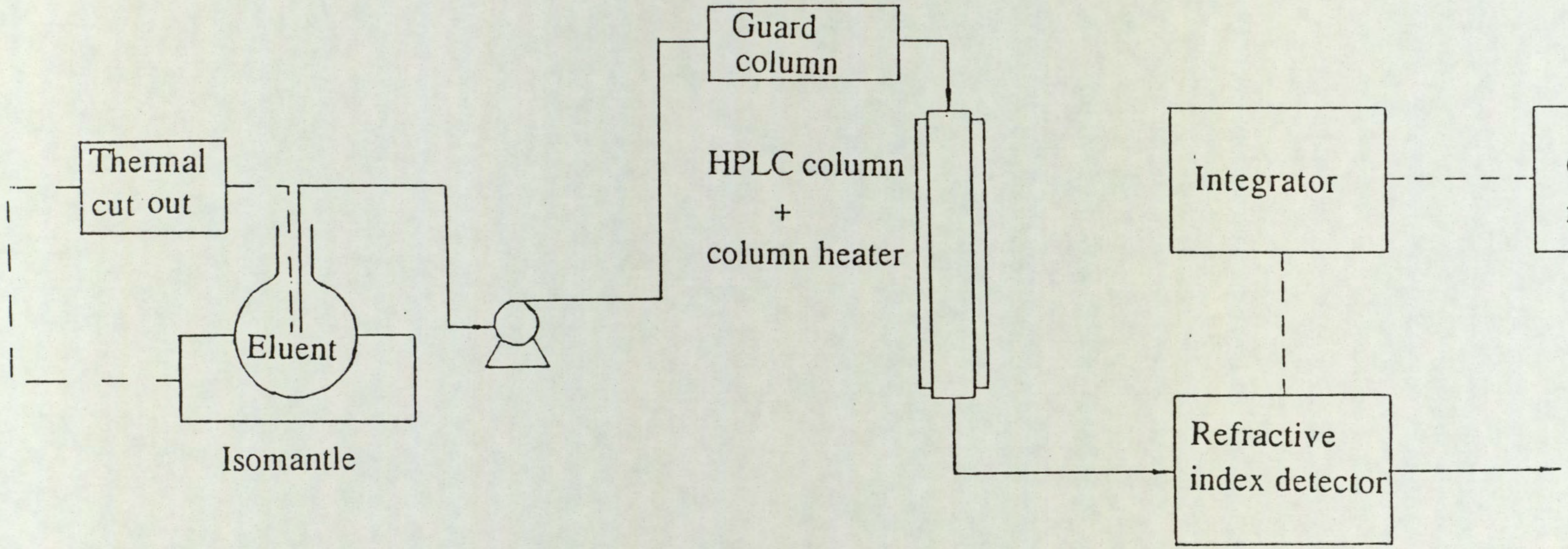



Figure 5.4: HPLC analytical system

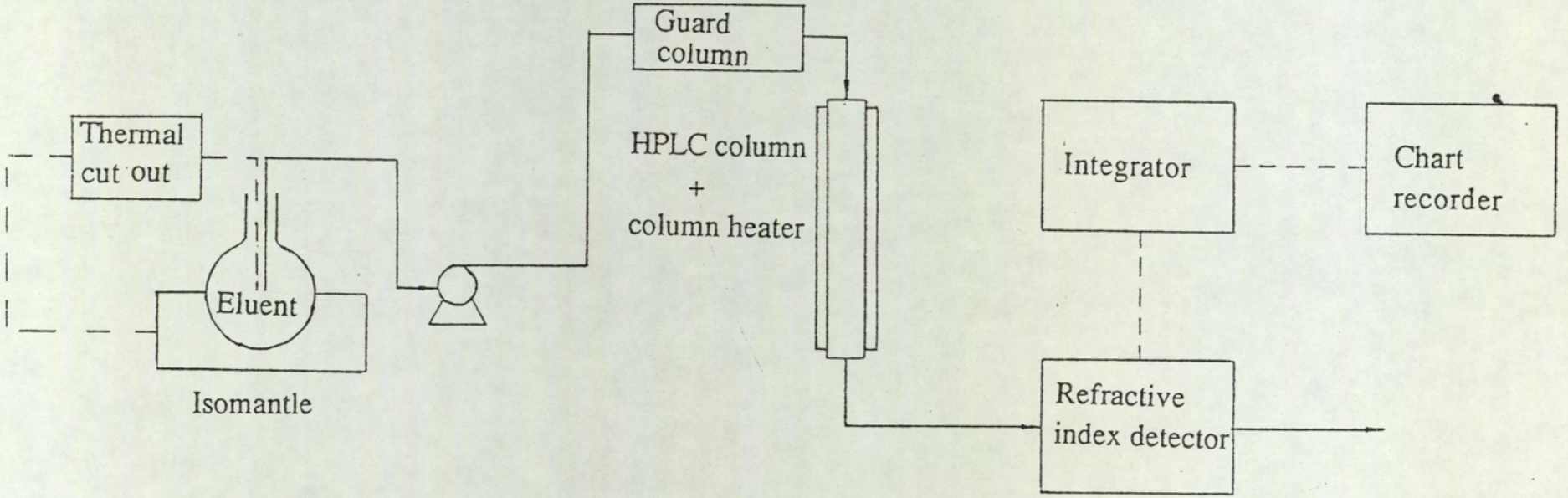



Figure 5.4: HPLC analytical system

**5.5.2.4 HPLC method of enzyme activity assay**

The use of the HPLC technique for assaying the enzyme activity depended on the determination of either the sucrose consumed, or the fructose and/or dextran produced during the enzyme reaction with sucrose. The procedure was as follows:

20 $cm^3$ of enzyme containing approximately 50 DSU/$cm^3$ of activity (or an appropriate dilution of the enzyme solution) were added to 80 $cm^3$ of a solution at pH 5.2, containing 2.0g of sucrose. This was shaken and a sample immediately taken, filtered and injected into the HPLC system. The remaining mixture was placed in a water bath maintained at 25 $^oC$. Every 10 or 20 minutes, for the next 2 hours, samples were withdrawn from the reaction mixture and injected into the system. If the sample was not to be assayed immediately, it was heated in boiling water for about 5 minutes to denature the enzyme and stop further reaction. The expected yield of fructose from 2.0g of sucrose after 2 hrs is 1.052g fructose (Equation 4.1).

The sucrose, fructose and dextran compositions present in the mixture at the times it was assayed were calculated by comparing their respective areas with those of standard 1% w/v solutions. Figure 5.5 shows a plot of the residual sucrose concentration in the reaction mixture during a typical assay. The activity was calculated from the following equations:

Activity based on sucrose consumption:

$$DSU/cm^3 = \frac{M_1}{1.0} \times 50 \times d \qquad \text{..... 5.2}$$

Activity based on fructose produced:

$$DSU/cm^3 = \frac{2 \times M_2}{1.052} \times 50 \times d \qquad \text{..... 5.3}$$

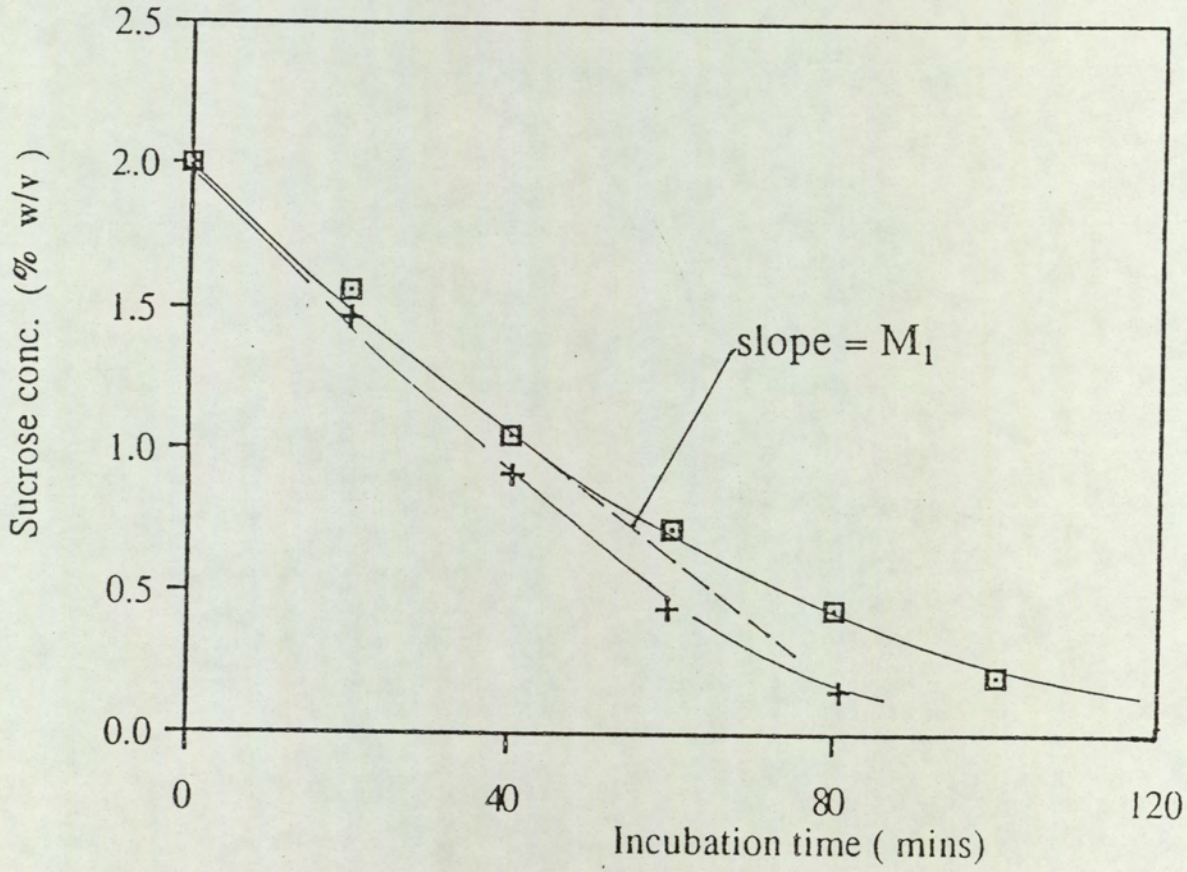



**Figure 5.5: HPLC enzyme activity determination based on sucrose conversion**

where $M_1$ and $M_2$ are the initial slopes for the sucrose and fructose curves respectively, expressed in % w/v/h, and d, the enzyme dilution factor.

The time involved in carrying out an assay using this method is considerable when compared to the Hostettler's method. However, it had the advantage that the compositions of the fructose and glucose could be monitored in order to investigate the presence of contaminating invertase and levansucrase. Invertase converts sucrose to glucose and fructose, while levansucrase converts it to levan and glucose[54]. An increase in glucose during the reaction is an indication of the presence of these contaminating enzymes. Figure 5.6 shows the profiles obtained during a typical assay, and it can be observed that the only peaks increasing are those of dextran and fructose. As a result, this technique was also used from time to time to check for these contaminating enzymes.

### 5.5.3 Fermenter broth analysis

During the fermentations, samples were taken and assayed using the HPLC to determine the sucrose level (especially in the batch processes where the sucrose/alkali mixture was not added). Broth analysis was also used during the continuous fermentation studies to determine the residual sucrose concentration in the fermenter.

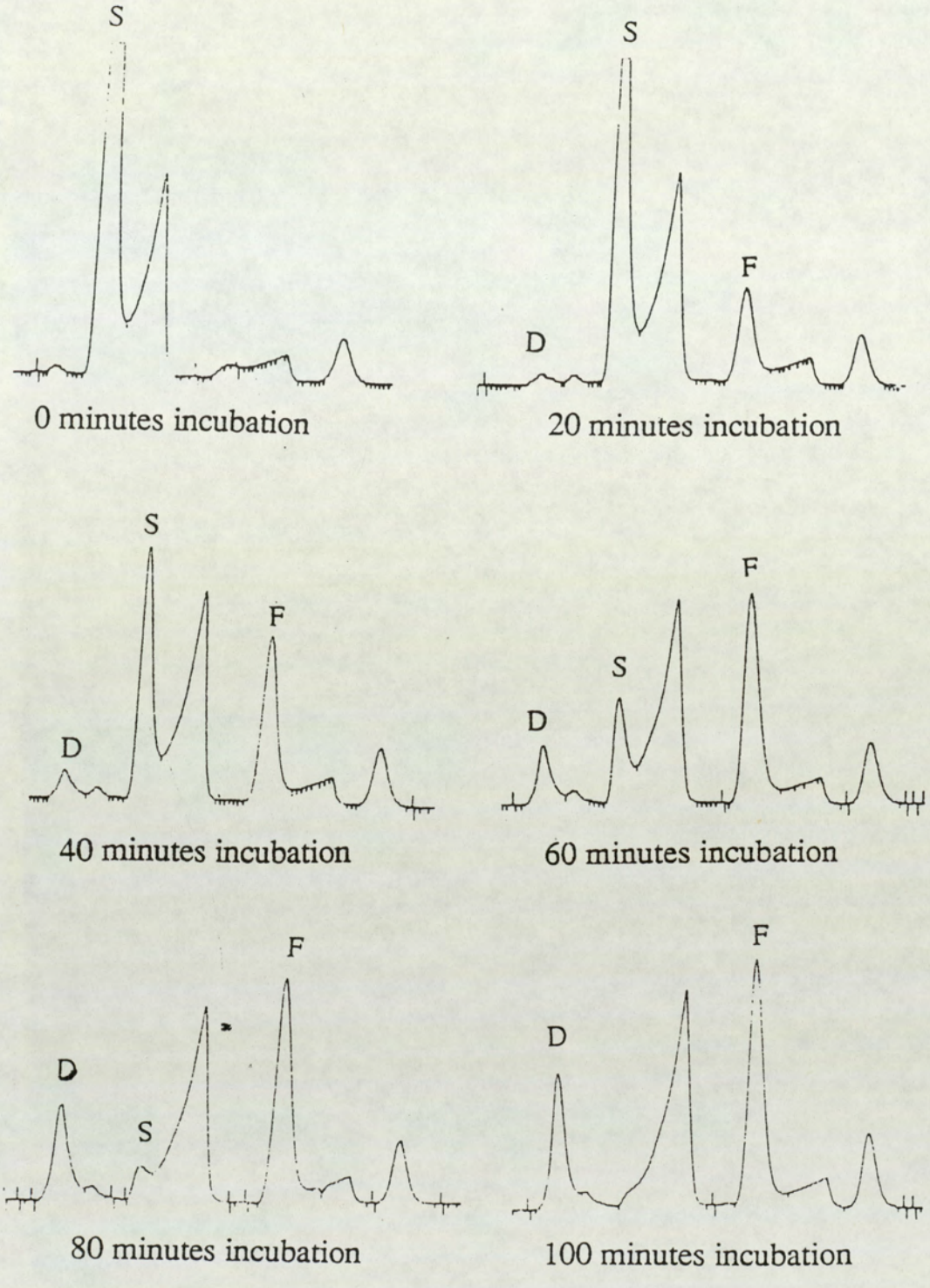



**Figure 5.6: HPLC profiles during enzyme activity assay**

### 5.5.4 Aseptic techniques

At the beginning of this project, certain basic microbiological studies were carried out at the microbiology department of Fisons Pharmaceuticals Plc. The aseptic techniques that were studied during this period were widely used throughout the course of the project. All transfers and mixing of sterile materials were carried out in a Gelaire VB85 laminar air flow ( LAF ) cabinet, supplied by Gelman Instruments, Herts., England. A 70% v/v alcohol solution was used to swab the working surface of the LAF cabinet before use. The fermenter addition ports and the ends of all sterile tubings were also swabbed with alcohol and flamed before use.

A positive pressure of 0.3 - 1.0 bar was maintained in the fermenter throughout the process so as to minimise the chances of any contaminants being sucked into the vessel. Contamination was checked either by microscopic examination or by plating out the sample on agar plates and observing visually. Microscopic observation was carried out by diluting a sample with saline, placing it on a slide and observing directly. Alternatively, a Gram stain was carried out on the sample before viewing under a microscope. The staining was carried out using Gram stain reagents supplied by Sigma Chemicals Co. Ltd., Dorset, England. When viewed through a microscope, the stained pure cultures contained crystal violet cocci shaped cells existing either singly or as short chains. The staining also had the advantage of showing that the organism is Gram positive.

The other method of checking the purity of the culture was to plate out a diluted sample on an agar plate containing sterile MRS agar in petri dishes. The plated cultures were incubated at 25 $^{o}$C for 48 hrs. The colonies were compared with those produced using a pure stock culture. Purity checks were carried out on the sterile broth and on the fermenter broth at the end of the fermentation. The former contained no organisms when examined, while the latter contained only LM cells.

### 5.5.5 Analysis of fermenter gases

The DO level in the fermenter was monitored continuously using the sterilizable DO probe in the vessel. The output from this probe was

continuously recorded with a chart recorder linked to the fermenter DO control module.

The composition of the fermenter off-gases was initially measured using a Centronic 200 MGA mass spectrometer. The moisture in the gases adversely affected the results from this instrument. As a result, the mass spectrometer was replaced with a Pye Unicam Series 204 gas-liquid chromatograph (glc). Samples were taken periodically from the air space above the fermenter medium by means of a sterile tube connecting the vessel to a stainless steel bicycle pump via a sterile filter. These air samples were either collected in plastic gas bags and analysed immediately or in stainless steel gas cylinders and analysed at a later time.

## 5.6 CONDUCT OF THE FERMENTATION

The inocula for all the laboratory batch and fed-batch fermentations were prepared in two stages using MRS broth as described in Section 5.4.2. At the end of the incubation period, the $OD_{590}$ values of the inocula were between 0.1 and 0.2.

The bioreactor containing sterile medium at 23 $^{o}C$ was inoculated by aseptically transferring the inoculum into it. Before inoculation, a sample of the broth was taken for a sterility check and for the determination of the $OD_{590}$ of the medium. The sucrose/alkali addition tubing was also fitted to one of the addition ports on the vessel.

Inoculation of the vessel normally took place immediately after sterilization and cooling. If the fermenter was not to be inoculated within 24 hours, the temperature was maintained at 50 - 60 $^{o}C$ until just before inoculation when it was cooled to 23 $^{o}C$. Under these conditions, no contamination was observed.

After inoculation, there occurred a lag phase of 4 - 7 hours. After this period, the pH started to drop from its initial value of around pH 7.0 - 7.1. When it reached 6.7, it was automatically controlled at this value by the addition of the sucrose/alkali mixture. The $OD_{590}$ value at this point was normally about 0.1. From this point onwards, samples were taken and assayed for the $OD_{590}$ and the enzyme activity ($DSU/cm^3$). Sampling was

continued at hourly or half-hourly intervals until the end of the fermentation. The DO and pH of the medium were continuously recorded on a chart recorder linked to their respective controllers. When required, air samples were taken at different intervals of times for analysis. If the sucrose level was required, samples were also taken and assayed as described above.

Termination of the fermentation occurred when either the enzyme activity had peaked, cell growth terminated or when it was observed that the rate of addition of sucrose/alkali had started decreasing. Batch fermentations were ended when the sucrose level in the vessel became zero.

If the final enzyme broth was required for downstream processes, it was collected and stored in the freezer. If not required, the contents of the vessel were sterilized before discarding. The vessel was then cleaned and the probes stored in their respective storage solutions. The DO probe was stored in a 1M KCl solution while the pH probe was stored in a 3M KCl solution.

# CHAPTER SIX

# CHAPTER SIX

# PRELIMINARY EXPERIMENTS AND AEROBIC FERMENTATIONS

## 6.1 INTRODUCTION

Before starting this research project, studies were carried out on the ways of handling the bacterial cultures and of producing inocula for the fermentations. These studies were undertaken in the microbiology department of Fisons Pharmaceuticals Plc., and included such microbiological techniques as asepsis, purity checks and analysis of broth media. These techniques have been described in the preceding chapter.

Preliminary experiments were based mainly on the conditions available in the literature, some of which involved the use of glucose and fructose as the carbon sources, and the selection of the best yeast extract type. More recently, tests were carried out in order to investigate the effect of the inoculum incubation period on the final cell concentration and enzyme yield. Due to the large number of experiments required, most of the preliminary screening experiments were carried out in shake flasks. From the results of these screening experiments, only those that required additional investigation were selected and then carrried out on a larger scale using the 16 litre fermenter.

This chapter also describes the different aerobic fed-batch fermentations that were carried out. The initial volume of the broth in the fermenter was 6 litres for all the experiments reported in this section, while the volume of the broth at the end of each fermentation was between 7.3 and 8.5 litres. The inoculum preparation was carried out in the normal two stages, and the pH was controlled by the addition of the sucrose/alkali mixture as described in Chapter 5. The first set of aerobic fermentations involved the use of different yeast extract types under the highly aerated conditions described in the literature[53]. In these fermentations, a high aeration rate was used and the agitation rate was varied to maintain the desired DO level. Results of the aerobic fermentations in which different aeration rates were applied but a fixed agitation rate of 100 rpm maintained are also presented. Antifoam was

added to all the media so as to prevent excessive foaming.

## 6.2 SHAKE FLASK EXPERIMENTS

These were carried out using 250 $cm^3$ flasks, with the actual working volumes used being 150 - 200 $cm^3$. The media were prepared, sterilized and left in the incubator for at least 48 hours. After this period, they were checked for contamination before inoculation.

All the sterile media flasks of each set of experiments were inoculated with the same amount of inoculum taken from a culture bottle containing inoculum in the second stage of preparation. The amount used was approximately 15 $cm^3$. Immediately before and after inoculation, each bottle was aseptically sampled using sterile syringes that were supplied by Sterilin Ltd., Feltham, England. The flasks were then incubated in a shaker bath maintained at 23 - 25 $^oC$ assayed periodically.

### 6.2.1 Experiments with sucrose, fructose and glucose

Two flasks were used for each sugar. The media compositions were the same as shown in Table 5.1, with the exception that in four of the six flasks used, two contained glucose and the other two fructose instead of sucrose. No antifoam was added to any of them.

Once the cells started growing, sterile sucrose/alkali was added to three of the flasks, i.e. one each of the sucrose, glucose and fructose flasks. About 2 $cm^3$ of the sucrose/alkali mixture were added to the flasks. Nothing was added to the other three flasks. The fermentations were terminated when no more cell growth was observed. Samples were then taken from each flask and analysed for turbidity ($OD_{590}$) and enzyme activity.

#### 6.2.1.1 Results and discussion

The results of the samples assayed from the flasks at the end of the fermentations are shown in Table 6.1.These results show higher cell growth and enzyme yield when sucrose was used as the carbon source than for glucose and fructose. Also, there was no enzyme production when only glucose or fructose were used. Addition of sucrose caused a slight increase in cell growth and a small amount of enzyme production.

**Table 6.1 Yields obtained using fructose, glucose and sucrose as substrates.**

| Carbon source | No sucrose/alkali added to medium | | Sucrose/alkali added to medium | |
|---|---|---|---|---|
| | Cell turbidity ($OD_{590}$) | Enzyme activity ($DSU/cm^3$) | Cell turbidity ($OD_{590}$) | Enzyme activity ($DSU/cm^3$) |
| Fructose | 0.073 | 0 | 0.089 | 4 |
| Glucose | 0.096 | 0 | 0.103 | 5 |
| Sucrose | 0.126 | 17 | 0.217 | 31 |

#### 6.2.1.2 Conclusion

The results showed that LM is capable of growing on these different substrates. Growth in the sucrose medium was much higher than in the glucose or fructose media. In the glucose and fructose media, no enzyme was produced. However, when sucrose was added some enzyme production occurred, showing that the enzyme is inducible, and requires sucrose for its induction.

### 6.2.2 Fermentations with different yeast extract types

The aim of the experiments in this section was to select the best type of yeast extract to use during the course of the research work. Yeast extracts supplied by Oxoid, England; the Distillers' Co. Ltd., England; Gist-Brocades, Holland and Orhly, W.Germany were used. The media were prepared, put in flasks, sterilized and checked for sterility as descibed earlier.

Each was inoculated, incubated in a shaker bath at 23 - 25 $^{o}$C and assayed until no more growth was observed. The yeast extracts that gave the highest enzyme activities were then used for larger scale (6 litre) fermentations.

### 6.2.2.1 Results and discussion

The results of trials using three types of the Gist-Brocades yeast extracts (Gistex standard, Gistex X-2 and Maxarome) and the Oxoid yeast extract are shown in Table 6.2. From the results, it was observed that the Maxarome yeast extract gave the lowest yields. No further work was done on this type of yeast extract. Fresh batches of the Gistex Standard and Gistex X-2 were purchased for the 6 litre scale experiments. The Oxoid yeast extract gave the highest $OD_{590}$ value but lower activity. No reason could be given for this since none of the suppliers was prepared to divulge any information regarding the compositions of their yeast extracts. However, it was noticed that the different yeast extracts had different colours after autoclaving. The Gist-Brocades samples had a clean golden colour while the Oxoid extract had a slightly darker shade. The DCL extract was the darkest of all the yeast extracts used. It even had solid particles which settled at the bottom of the flask.

After several trials with the Oxoid extract, it was decided not to use it any more since it did not give high enzyme activities. Its high cost was also a major determining factor. Yields of both cell growth and enzyme much lower with the DCL yeast extract. As a result, this extract was also eliminated from routine use. The Orhly extract gave results that were better than the Oxoid yeast extract.

**Table 6.2 Effects of yeast extract types on LM fermentation**

| Yeast extract type | Cell turbidity ($OD_{590}$) | Enzyme activity ($DSU/cm^3$) |
|---|---|---|
| Gistex Standard | 0.31 | 64 |
| Gistex X-2 | 0.31 | 65 |
| Maxarome | 0.12 | 13 |
| Oxoid | 0.38 | 47 |

### 6.2.2.2 Conclusions

The observations of these runs showed that the different yeast extracts affect the fermentation results. This fact has been thought for some time to be the cause of the difference in results obtained from LM fermentations by some dextran producers[89]. The Gistex Standard, Gistex X-2 and Orhly yeast extracts gave the best results. It was, therefore, decided to carry out larger scale fermentations with these yeast extracts.

## 6.3 AEROBIC FED-BATCH FERMENTATIONS

Monsan and Lopez[65] showed that the best results of LM fermentation are obtained under fed-batch conditions. This method has been adopted by several workers. Cell-free enzyme processes have generally been carried out under aerated conditions. Schneider *et al.*[53] reported that a DO level of 40 - 70 % of the initial saturation value, maintained throughout the fermentation was necessary in order to achieve high enzyme activities. With these in mind, the experiments described in this section were carried out. The aim was to optimise the aeration and agitation rates that produced the highest enzyme activities. In order to do this, it was necessary to first of all select the best yeast extract. Afterwards, an attempt was to be made to reproduce the results of Schneider *et al.* and optimise them further.

The inoculum preparation and sucrose/alkali compositions used for all these experiments was as described in Chapter 5. The pH was controlled at 6.7 ± 0.05, and temperature at 23 ± 0.5 °C. The initial volume of the medium in the fermenter before inoculation was 6 litres and the initial pH was 6.9 - 7.1.

Before inoculation, the broth media were saturated with air. This was achieved by aerating the medium with sterile air under the operating aeration and agitation rates for at least 1 hour. When the DO level remained at a steady value, this was taken as the 100% saturation. The DO controller was then adjusted to read 100% at this point.

To inoculate the vessel, the air flow rate was reduced and the back pressure, i.e. the pressure of the air space above the medium, allowed to drop to zero. Immediately after the inoculation, the aeration was restored and the back pressure regulator reset. The back pressure was maintained between 0.3 and 1.0 bar throughout the process.

As soon as the inoculation was completed and the aeration restored, the DO level started decreasing. This continued until the value reached the DO controller set point of 40 - 50% of the initial saturation. Once the DO level reached this set point, the controller then maintained it at or above this point by altering the agitation rate between the range of agitation speeds used.

### 6.3.1 Fermentation with different yeast extract types

Fermentations were carried out using the Gistex Standard, Gistex X-2, and Orhly yeast extracts. The air flow meter that was on the fermenter at the start of this project was replaced by another of larger capacity, capable of delivering up 50 l/min of air.

To achieve the 40 - 70 % DO saturation used by Schneider and co-workers[53], the agitation rate was set at 400 - 650 rpm. The aeration rate was set at 24 l/min, i.e. 4 VVM. Table 6.3 shows the results of fed-batch runs using these yeast extracts under the above conditions. Figure 6.1 also shows the cell growth and enzyme elaboration profiles during two such runs involving the Gist-Brocades yeast extracts.

The information available on the Gistex yeast extracts indicated that the X-2 type was of higher quality. It was also more expensive. The results obtained were, therefore, contrary to what would have been expected. Nevertheless, both gave identical cell growths and growth rates. Since the X-2 is mainly used as a food flavour, the higher quality may not necessarily have been that for enzyme production. Subsequent runs with the Orhly and Gistex Standard yeast extracts led to the choice of the latter as the best yeast extract for this research work.

**Table 6.3: Effects of yeast extract types on aerobic fedbatch LM fermentations.**

| Yeast extract type | $OD_{590}$ | enzyme activity* ($DSU/cm^3$) | sucrose used (g) | growth rate, $\mu$ ($h^{-1}$) | duration (h) |
|---|---|---|---|---|---|
| Orhly | 1.20 | 108 | 525 | 0.32 | 18 |
| Gistex Standard | 1.19 | 130 | 618 | 0.32 | 16 |
| Gistex X-2 | 1.20 | 93 | 602 | 0.31 | 17.5 |

* These activities were assayed at pH 7.3 instead of pH 5.2

### 6.3.2 Aerobic fermentations with Gistex Standard

A series of experiments were conducted using the Gistex Standard yeast extract paste. In one set, the DO level was maintained at 40 - 70 % of the saturated value throughout the fermentation period. This was achieved using an aeration rate of 4 VVM and agitation range of 400 - 650 rpm. Other parameters, i.e. temperature and pH were maintained as for all other fermentations.

#### 6.3.2.1 Results and discussions

Figure 6.2 shows the cell growth, enzyme production and DO level in the fermenter during the course of a typical run. The carbon dioxide composition in the exit gases was determined using a mass spectrometer. Figure 6.3 shows the $CO_2$ and cell growth profiles during a typical aerobic fed-batch. Table 6.4 also shows the results of a number of runs carried out under the above conditions using the Gistex Standard yeast extract paste.

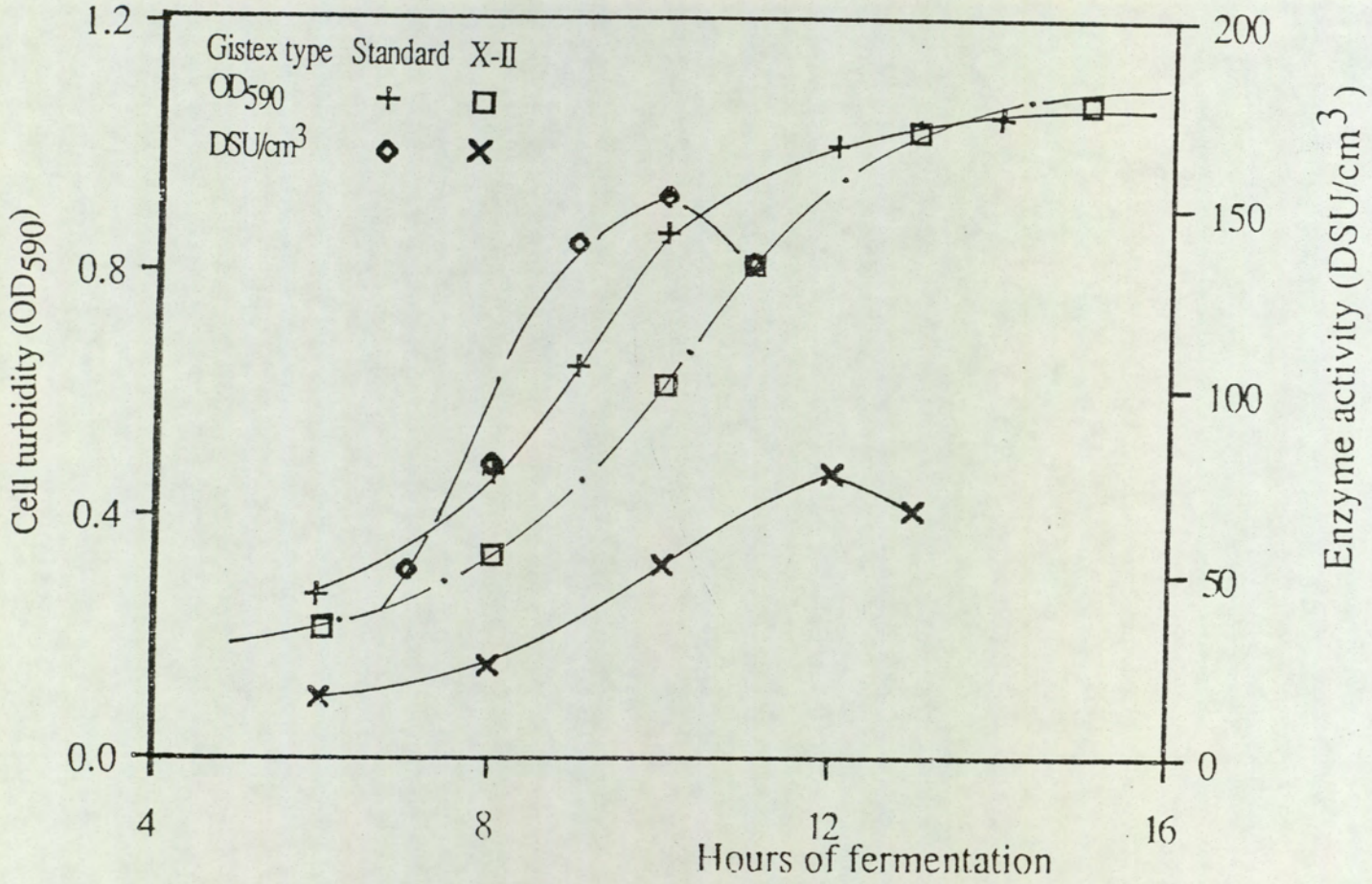



**Figure 6.1: Effects of yeast extract type on cell growth and enzyme production during aerobic fermentation**

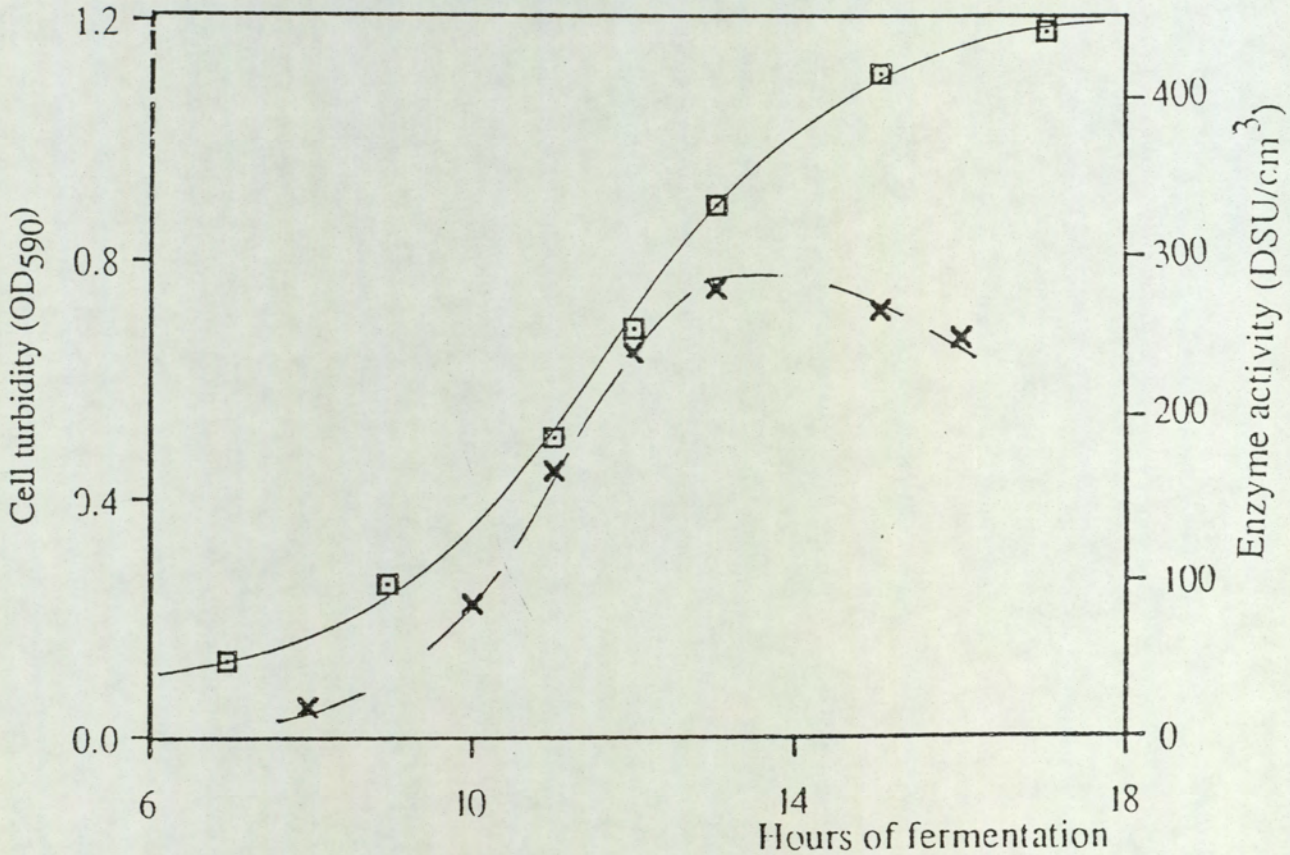



**Figure 6.2: Cell growth ( ⊡ ) and enzyme production ( ✕ ) profiles during aerobic fermentations containing Gistex Standard yeast extract - experiment 17-11-86**

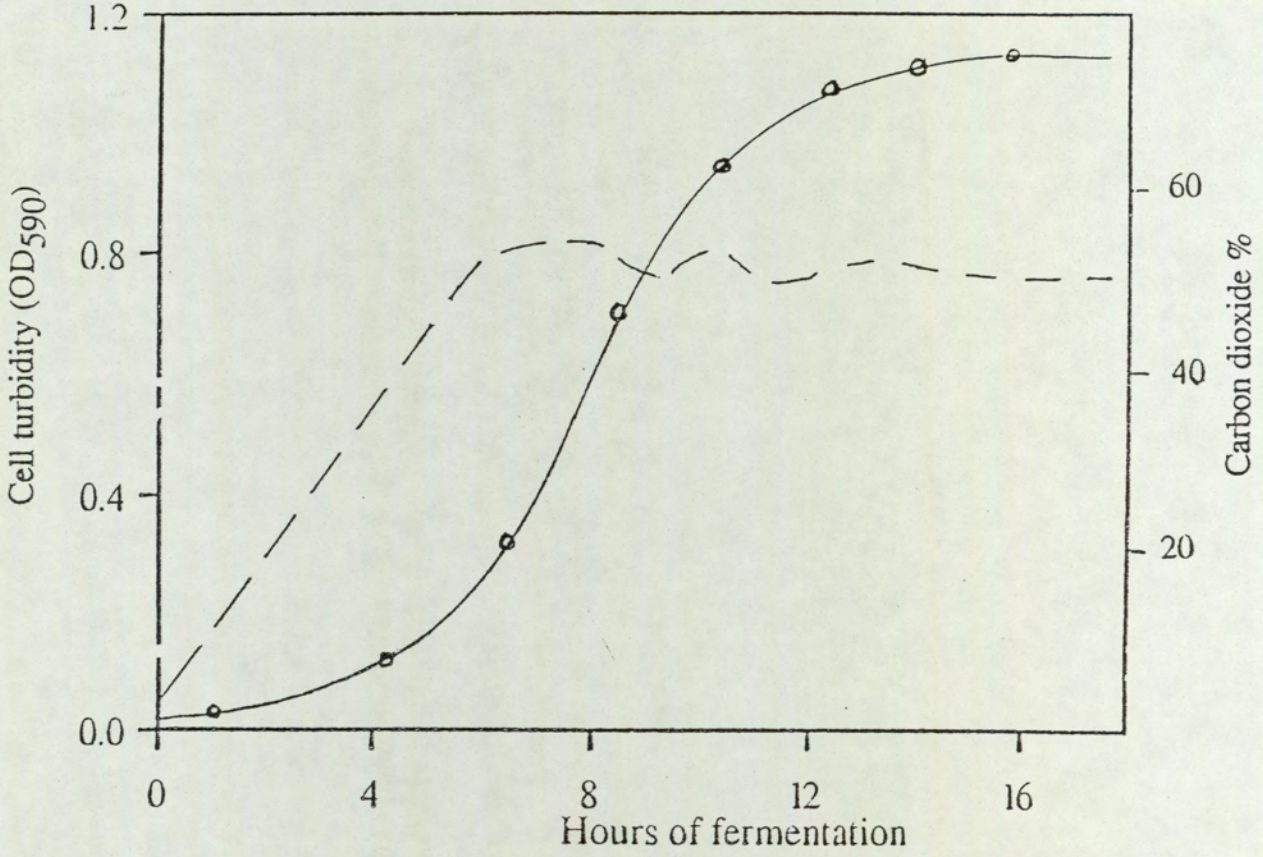



**Figure 6.3: Carbon dioxide content in exit gases (— –) and cell growth (—o—) during aerobic fermentations - experiment 34-04-87**

Table 6.4: Results of aerobic fed-batch fermentations

| Experiment number | $OD_{590}$ | enzyme activity (DSU/$cm^3$) | sucrose added (g) | minimum DO % | growth rate, $\mu$ ($h^{-1}$) | duration of fermentation (h) |
|---|---|---|---|---|---|---|
| 34-04-87 | 1.15 | 348 | 510 | 42 | 0.377 | 16 |
| 35-05-87 | 1.27 | 415 | 582 | 36 | 0.358 | 17.5 |
| 36-05-87 | 1.20 | 347 | 570 | 39 | 0.320 | 17 |
| 37-05-87 | 1.23 | 334 | 612 | 32 | 0.340 | 18 |

The enzyme activity tended to peak just before the termination of cell growth and started to drop as the cells entered the stationary growth phase (Figure 6.2). This suggested that the high aeration/agitation conditions used could have adversely been affecting the enzyme production. Oxygen is also known to affect some enzymes by oxidizing the sulphur-hydrogen bonds in the enzyme molecule[22,27]. These combined effects could, therefore, have been responsible for the drop in the enzyme activity at the end of the fermentations. It is possible that these effects were exhibiting themselves throughout the course of the fermentation, but became noticeable only towards the end of the process when the rate of enzyme elaboration levelled off at the end of the exponential growth phase.

#### 6.3.2.2 Conclusions

The activities of the enzyme produced under 40-70% DO saturation were typically around 350 DSU/cm$^3$. These results indicated that it was possible to produce activities in the order of 350 DSU/cm$^3$ as Schneider and co-workers claimed in their patent[53]. Only in one case was the activity more than 400 DSU/cm$^3$. All the other aerobic fermentations carried out (including those with other yeast extract types) did not produce such levels of activity under the aerobic conditions. Taking analytical errors into account, it was concluded that enzyme containing in excess of 400 DSU/cm$^3$ could not be obtained by using the yeast extract types that were available and the highly aerated conditions recommended by Schneider *et al.*[53].

### 6.3.3 Effects of aeration rate

Several experiments were undertaken using different aeration rates. The same agitation power input was maintained for all the experiments by applying the same agitation rate of 100 rpm. This speed was the lowest that the stirrer motor could successfully maintain throughout the entire run. It was, nevertheless, high enough to rapidly mix the sucrose/alkali mixture that was fed into the vessel. All the fermentations were carried out using the normal 6 litres of initial broth volume. Two aeration rates were used and the results compared with those of Section 6.3.2 above which involved highly aerated/agitated conditions. The aeration rates used for the 6 litre media were

12 litres/min (2 VVM) and 1 litre/min (0.17 VVM).

In the first set of experiments carried out at 2 VVM, no antifoam was added to the media. During the fermentation, a lot of foam was produced, filling the space above the medium. The pH control was affected as the sucrose/alkali mixture was trapped in the foam. An increase in the fermenter back pressure did not lead to a reduction in the foam level. The foaming resulted in the production of only 250 DSU/$cm^3$ of enzyme activity, even though the $OD_{590}$ exceeded 1.0. A repeat run gave even lower values (Table 6.5).

The second set of fermentations were carried out with antifoam in the medium. The antifoam used was Silicone concentrate A, supplied by Sigma Chemicals Co. Dorset, England. Fermentations were then carried out using the 2 VVM and 0.17 VVM aeration rates. The results are also shown in Table 6.5. At an aeration rate of 1 litre/min, the air just bubbled through the medium and caused no foaming. As a result, antifoam was not added to the other experiments carried out at this aeration rate, e.g. experiment 79-05-88 of Table 6.5.

With the exception of the enzyme yields of the experiments carried out at 2 VVM which produced a lot of foam, the enzyme activities of all the other experiments were comparable with those shown in Table 6.4, i.e. those of the 4 VVM aerated and highly agitated fermentations. The sucrose consumed, duration of the fermentation and the cell growths are also comparable, suggesting that the degree of aeration does not affect the yield of enzyme.

Table 6.5: Effects different aeration rates at 100 rpm agitation rate

| Experiment number | aeration rate l/min | antifoam added? | $OD_{590}$ | enzyme activity (DSU/cm$^3$) | sucrose added (g) | duration (h) | growth rate, $\mu$ ($h^{-1}$) |
|---|---|---|---|---|---|---|---|
| 72-02-88 | 12 | no | 1.04 | 250 | 564 | 17 | 0.389 |
| 73-02-88 | 12 | no | 0.92 | 199 | 492 | 16.5 | ** |
| 74-03-88 | 12 | yes | 0.94 | 323 | 504 | 15 | ** |
| 75-03-88 | 12 | yes | 1.01 | 306 | 600 | 16 | ** |
| 76-03-88 | 1 | yes | 1.03 | 362 | 615 | 16 | 0.314 |
| 77-03-88 | 1 | yes | 1.00 | 392 | 540 | 16 | ** |
| 79-05-88 | 1 | no | 1.05 | 413 | 660 | 16 | 0.320 |

** Growth rates not determined.

## 6.4 CONCLUSIONS

The results in this chapter showed that the type of yeast extract used affects the results of the fermentation. This was in agreement with the observations of Brown and McAvoy[67]. The Gistex Standard yeast extract supplied by Gist-Brocades, Holland, was found to be the best of all the yeast extracts tested. The next best in terms of enzyme activity was the Orhly type. The results obtained by using the DCL yeast extract which is routinely used at Fisons Pharmaceuticals Plc. gave the lowest yields of enzyme.

Using inoculum that was prepared in two stages, the lag phases of 4 - 7 hours were observed. Immediately after the lag phase, it took less than 7 - 10 hours for the cells to go through the exponential growth phase. The enzyme activities peaked just at the end of the exponential phase and then slowly decreased. This suggested some form of enzyme denaturation.

Aeration rate does not appear to affect the maximum enzyme activity. Aeration rates of 1 litre per minute and 12 litres per minute gave enzyme activities of 323 - 413 DSU/$cm^3$ under 100 rpm (Table 6.5). Similar results were obtained using 24 l/min aeration and 400 - 650 rpm agitation rates. The cell growths and sucrose consumption were not also affected. These results indicate that the yields of the fermentation are not affected by the aeration rate. Day[88] recently confirmed this proposition saying that LM fermentations are insensitive to oxygen.

Under high aeration/agitation conditions used, antifoam was required. The absence of antifoam led to excessive foam formation which caused a reduction in the enzyme yield, without affecting the cell growth to the same extent.

Finally, the inoculum incubation period did not appear to affect the final cell concentration or enzyme yield. When the inoculum was prepared in two stages as described in Chapter 5, complete cell growth took place in 10 - 15 hours. At the end of this period, the pH was 4.3 - 4.8. The $OD_{590}$ of the inoculum was normally between 0.11 and 0.18. When the incubation time was extended to between 24 - 28 hours, there was no change in these values. The effect that the length of the incubation period had on the process was on the lag phase. When the inoculum was transferred too early or too late, it just led to a longer lag phase. Nevertheless, there were a few runs that failed to

produce good cell growth. The $OD_{590}$ remained at around 0.1 even after 24 hours of fermentation. The cause of this occurrence was unknown. None of the successful runs produced $OD_{590}$ values greater than 1.30. Extension of the stationary phase only led to the consumption of more sucrose, but no increase in cell growth. It instead led to a further reduction in the enzyme activity.

# CHAPTER SEVEN

# CHAPTER SEVEN

# ANAEROBIC PRODUCTION OF DEXTRANSUCRASE

## 7.1 INTRODUCTION

*Leuconostoc mesenteroides* (LM) are facultative anaerobes. Conventional whole cell productions of dextran are performed under anaerobic fermentation conditions. Inocula used for cell-free fermentations are normally prepared anaerobically. However, not much interest has been shown in the anaerobic fermentation of LM. The highest enzyme activities reported in the literature by Schneider and co-workers[53] were obtained from highly aerated fermentations. The second best set of results in terms of enzyme yield as reported by Lopez and Monsan[65] were also carried out under aerobic conditions.

At the beginning of this research work it was initially planned to optimise aerobic fermentations. Anaerobic fermentations were then to be conducted so as to compare their results with the optimised aerobic runs. The program did not follow this initial plan. During studies on the effects of different aeration rates, problems developed. Firstly, the air flow meter available on the fermenter could not deliver enough air to the fermenter. Up to 4 VVM was required as described in Chapter 6, but only 2 VVM could be obtained. Secondly, the DO probe had exceeded its useful life and was giving inaccurate outputs. Hence new pieces of equipment were purchased. Whilst awaiting for these materials to arrive, anaerobic fermentations were carried out.

The results of these experiments turned out to be better than the best of the aerobic processes. This was contrary to the results that have generally been reported in the literature. It was therefore decided to explore anaerobic fermentations more closely. This section describes those anaerobic fermentations that were carried out. Effects of different yeast extract types and agitation rates were studied, and attempts were also made to carry out the fermentations using reduced phosphate and yeast extract concentrations in the fermentation media.

The operating parameters used in all the fermentations were a temperature of 23 ± 0.5 °C, a pH of 6.7 ± 0.05, and a fixed agitation rate of 100 rpm. No antifoam was used, except in those experiments where its effect was being investigated. The inoculum was prepared in the normal two stages, with 300 - 350 $cm^3$ of inoculum used to inoculate the fermenter. The initial volume of broth medium in the fermenter was kept constant at 6 litres for all the experiments.

Immediately after inoculation, the DO level started dropping. In under two hours, this value had dropped to zero. The pH also dropped to 6.7 in 4 - 7 hours and was automatically controlled at this value by the addition of the sucrose/alkali mixture. Both the DO and pH readings were continuously recorded using a chart recorder.

### 7.2 ANAEROBIC FED-BATCH FERMENTATIONS

Anaerobic fermentations were planned as part of the set of experiments concerned with the study of the effects of different aeration rates on the fermentation results. When these fermentations were carried out under the above conditions using the Gistex Standard extract, yields of enzyme containing in excess of 450 DSU/$cm^3$ of enzyme activity were obtained.

## 7.2.1 Results and discussions

Table 7.1 is a summary of some of the anaerobic fed-batch fermentations that were carried out. The lag phase for the anaerobic fermentations was 6 - 9 hours. As soon as the cells started growing and the sucrose/alkali addition commenced, enzyme synthesis also started. By the time the $OD_{590}$ had reached 0.3 - 0.4, the enzyme activity in the broth was about 50 DSU/$cm^3$. This increased rapidly to 400 - 500 DSU/$cm^3$ in 6 - 8 hours. Figure 7.1 is a graphical representation of a typical fed-batch experiment. It shows the cell growth, DO, enzyme activity and the total sucrose consumption values against time during the course of the fermentation. The sucrose addition rate was constant during the exponential phase as indicated by the linear relationship of the sucrose consumption curve. The total sucrose consumed was also found to be greater than for the aerobic experiments. Cell growth tended to level off at an $OD_{590}$ value of 1.2

as was the case with the aerobic runs. The growth rates calculated from these experiments were found to be slightly lower than those of the aerobic fermentations (Tables 6.4 and 6.5). These lower growth rates led to slightly longer process times.

A chart recorder was used to record the DO level in the fermenter during the fermentations. Immediately after inoculation, the DO level usually dropped to zero in under 2 hours, and remained at this value for most of the fermentation period. It was, however, observed that as the cells were approaching the end of the exponential growth phase, the DO increased slightly. This increase occurred for 2 - 4 hours and then the DO dropped back to zero where it remained until the end of the fermentation. The DO electrode was initially blamed for this but after changing it, this occurrence was found to manifest itself in almost all the experiments that were monitored. Samples of air withdrawn from the space above the medium and analysed showed a continuous reduction in the oxygen level in the air (Figure 7.2). In some cases, the oxygen content of the air above the medium was analysed to be zero, but the increase in the DO of the medium was still noticeable. Since a positive pressure was maintained in the fermenter throughout the fermentation, the above observation could not be attributed to air being sucked into the fermenter. It has not, therefore, been possible yet to put forward an explanation for this increased DO concentration.

Table 7.1: Summary of anaerobic fermentations

| Experiment number | $OD_{590}$ | enzyme activity ($DSU/cm^3$) | sucrose added (g) | growth rate, $\mu$ ($h^{-1}$) | duration of fermentation (h) |
|---|---|---|---|---|---|
| 26-02-87 | 1.12 | 451 | 858 | 0.34 | 19 |
| 27-03-87 | 1.08 | 487 | 684 | 0.31 | 18 |
| 28-03-87 | 1.08 | 475 | 744 | 0.29 | 18.5 |
| 29-03-87 | 1.10 | 488 | 726 | 0.28 | 18 |
| 30-03-87 | 1.11 | 459 | 758 | 0.31 | 19 |
| 31-03-87 | 1.06 | 475 | 672 | 0.30 | 17 |
| 47-08-87 | 1.13 | 490 | 681 | 0.32 | 17 |

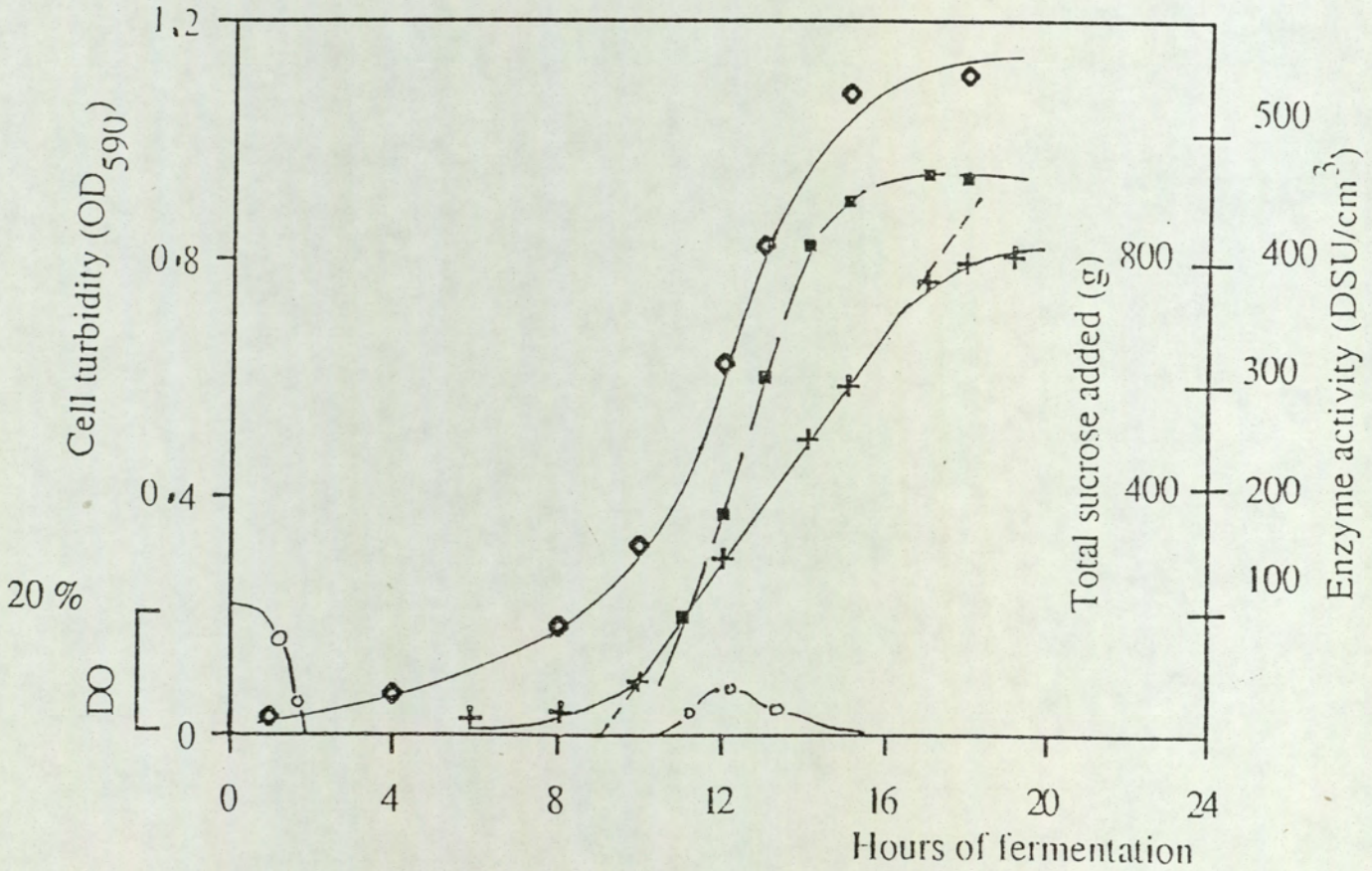



**Figure 7.1: Cell growth ( ◇ ), dissolved oxygen ( o ), total sucrose consumption ( + ) and ( ■ ) enzyme production during anaerobic fermentation - experiment 30-03-87**

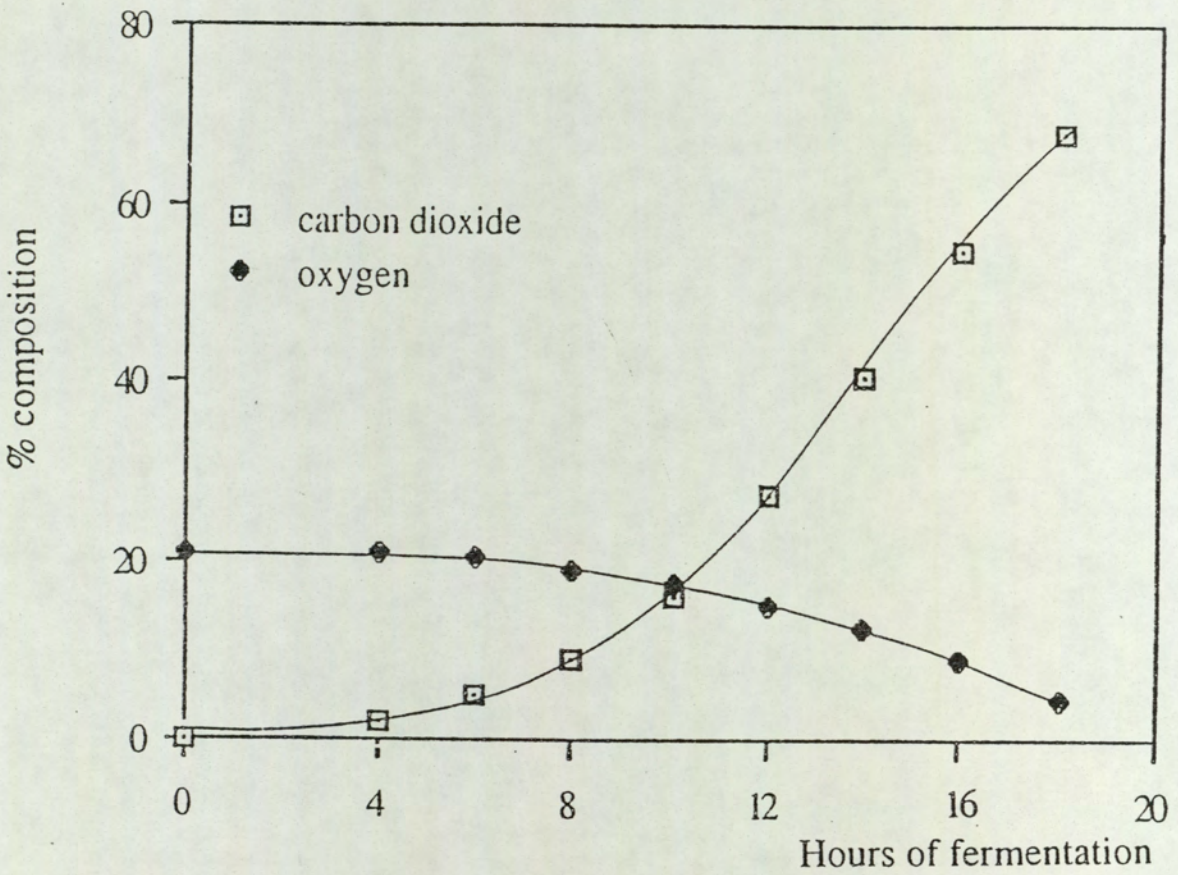



**Figure 7.2: Carbon dioxide and oxygen concentrations in fermenter exit gases during anaerobic fermentations - experiment 29-03-87**

### 7.2.2 Conclusions

At least 18 repeats of the anaerobic experiments were carried out with the Gistex Standard yeast extract as described in this section. In all cases, yields of enzyme having 450 - 500 DSU/$cm^3$ of dextransucrase activity were produced. The reproducibility of these experiments have led to these conditions being adopted as standard conditions against which to judge other experiments. Whenever a new batch of yeast extract was purchased, a standard fermentaion was carried out to establish its comparability with the "standard results".

The effects of the inoculum incubation time on the final $OD_{590}$ and enzyme activity were also investigated. The only parameter that was affected was the lag phase. As with the aerobic fermentations, there was no observable effect on the final cell growth or the enzyme yield.

## 7.3 EFFECTS OF YEAST EXTRACT TYPE

After establishing that the enzyme elaboration was higher in anaerobic than in aerobic fermentations, it was decided to repeat the experiments using the different yeast extract types, so as to find out if the results would be different under these anaerobic conditions. The Gistex, Orhly and DCL yeast extract types were used. All experiments were carried out at the 6 litre scale, with the agitation rate, temperature and pH being the same as for the anaerobic runs described in Section 7.2.

### 7.3.1 Results and discussion

The results of these runs are shown in Table 7.2. Those of a "standard" fermentation with Gistex Standard (experiment 47-08-87) have been included for comparison. Figure 7.3 also shows how the cell growth and enzyme activity varied during the course of the fermentations. The two yeast extracts compared in the graph are the Gistex Standard and the Orhly yeast extract. A number of observations are apparent from these results:

Firstly, the cell growth rate was identical for both yeast extracts, but the final cell concentrations were different for the different yeast extract types.

Table 7.2: Anaerobic fed-batch fermentations using different yeast extract types.

| Experiment number | yeast extract type | $OD_{590}$ | enzyme activity (DSU/$cm^3$) | sucrose added (g) | growth rate, $\mu$ ($h^{-1}$) | duration of fermentation (h) |
|---|---|---|---|---|---|---|
| 47-08-87 | Gistex | 1.13 | 490 | 681 | 0.32 | 17 |
| 39-06-87 | Orhly | 0.93 | 213 | 600 | 0.34 | 16 |
| 40-06-87 | Orhly | 0.81 | 284 | 528 | 0.34 | 20.5 |
| 56-11-87 | DCL | 1.06 | 226 | 933 | 0.28 | 16 |
| 57-11-87 | Orhly | 0.80 | 290 | 600 | 0.30 | 17 |
| 58-11-87 | DCL | 0.96 | 225 | 846 | 0.34 | 17 |

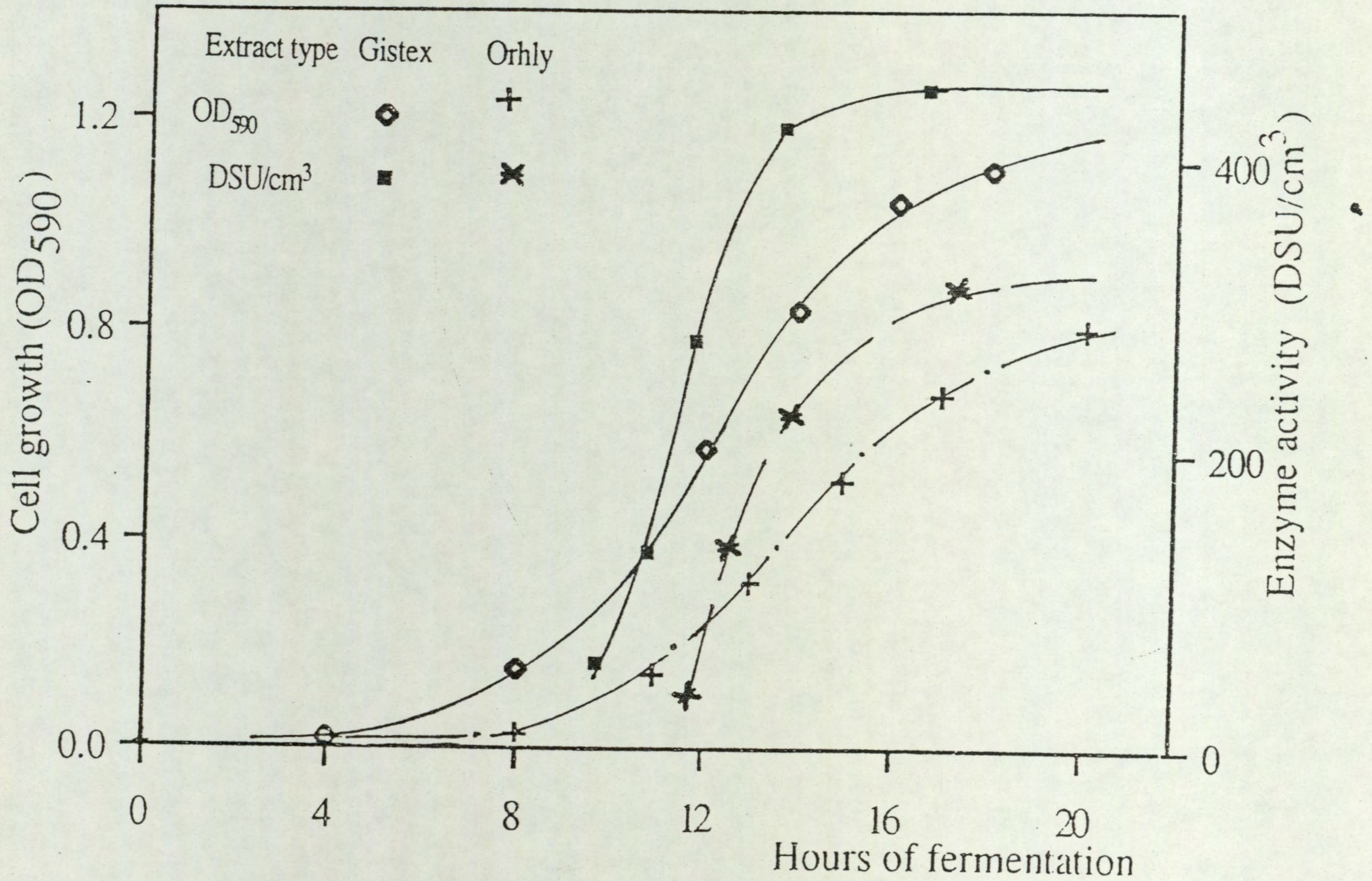



**Figure 7.3: Comparison of two yeast extract types under anaerobic fermentation conditions**

With the exception of one of the Orhly yeast experiments, the total process times were about the same. The long time for experiment 40-06-88 was due to the long lag phase that was experienced. Although the fermentation times were identical, the fermentations with the DCL yeast extract used up more sucrose than was the case for all the other types of yeast extracts. The greatest difference in the results was the final enzyme activities. They were substantially low for the Orhly and DCL yeast extracts. These results were similar to those from the shake flask and the aerobic fermentations.

No further work was carried out on the Orhly and DCL extracts. The Gistex Standard yeast extract was adopted as the extract for use in all subsequent experiments. With the exception of one batch, all other batches of the Gistex Standard produced high yields of enzyme containing more than 450 DSU/$cm^3$ of activity under the anaerobic conditions.

The batch that failed to produce high yields of enzyme was used for scale-up experiments (see Chapter 8). This yeast extract was used for 6 litre laboratory scale experiments and also found to produce the same low activities of less than 300 DSU/$cm^3$. 0.0206 g/l of folic acid were added to each of a series of fermentations in an attempt to improve the results of this low quality extract. This was the same as the amount used by Brown and McAvoy(67) in their work on dextransucrase fermentation. The activities of enzyme obtained were the same as without the added folic acid. No further work was done on the use of folic acid to enhance enzyme activity because the analytical equipment necessary for analysing the folic acid content of the yeast extract was not available. It was, therefore, not possible at this stage to identify what exactly caused the failure of this batch of yeast extract.

### 7.3.2 Conclusion

After studying the different batches of the various yeast extracts as described above, the Gistex Standard yeast extract was selected as the best type for the fermentation of LM to produce enzyme containing activities of more than 400 DSU/$cm^3$. However, since it was observed that one of the batches of the Gistex did not produce high enough enzyme yields, it was decided that all subsequent new batches be tested under the standard anaerobic conditions before being used for further investigations.

The other types of yeast extracts, notably, the Orhly and DCL types, that are routinely used for enzyme production at Fisons Pharmaceuticals Plc. did not produce as much enzyme as the good batches of the Gistex Standard. Independent tests using the Gistex Standard have successfully been carried out at Fisons Pharmaceuticals Plc. using the standard anaerobic conditions. Enzyme yields containing activities in excess of 400 DSU/$cm^3$ were obtained(90).

## 7.4 EFFECTS OF AGITATION RATE

Experiments were conducted to investigate the effects of agitation rate on the enzyme production. All the experiments were undertaken using anaerobic fermentation conditions. Other parameters like the pH, temperature and initial volume of broth used were identical to those used in all other experiments. Agitation rates of 100 to 600 rpm were used. In one set of experiments, 2 $cm^3$ of antifoam were added to the medium prior to sterilization, while on another, no antifoam was used. The aim of these experiments was to determine suitable agitation conditions that would be required during the scale-up processes.

### 7.4.1 Results and discussions

Tables 7.3 and 7.4 show the respective effects of agitation rate with and without antifoam on the fermentation results.

It was observed that as the agitation rate was increased, the final enzyme activity reduced. The effect was more drastic at agitation rates of greater than 300 rpm, when there was antifoam in the medium. At 600 rpm, the maximum enzyme activity obtained was about 133 DSU/$cm^3$ in the presence of antifoam.

At low agitation rates, the antifoam did not disperse uniformly in the medium. Instead, it formed small globules which either remained on the surface or stuck on the vessel walls. As the agitation rate was increased, these globules were broken up and dispersed in the broth medium.

Since no air was sparged into the medium, it was also decided to find out if the agitation rate could be increased without causing a lot of foaming. The reasons for these trials were partly due to the fact that the vessel to be

used for scale-up could have a higher agitation power input. The other reason was that Fisons Pharmaceuticals Plc. were interested in using any enzyme produced on a large scale for dextran production, and it was desired to remove the effects of the greasy antifoam from the downstream processing equipment. As a result, a set of experiments were carried out in which no antifoam was used. As Table 7.4 shows, the activities were higher than when antifoam was added to the medium (Table 7.3). Figure 7.4 is a graphical comparison of the activities obtained from these two sets of experiments.

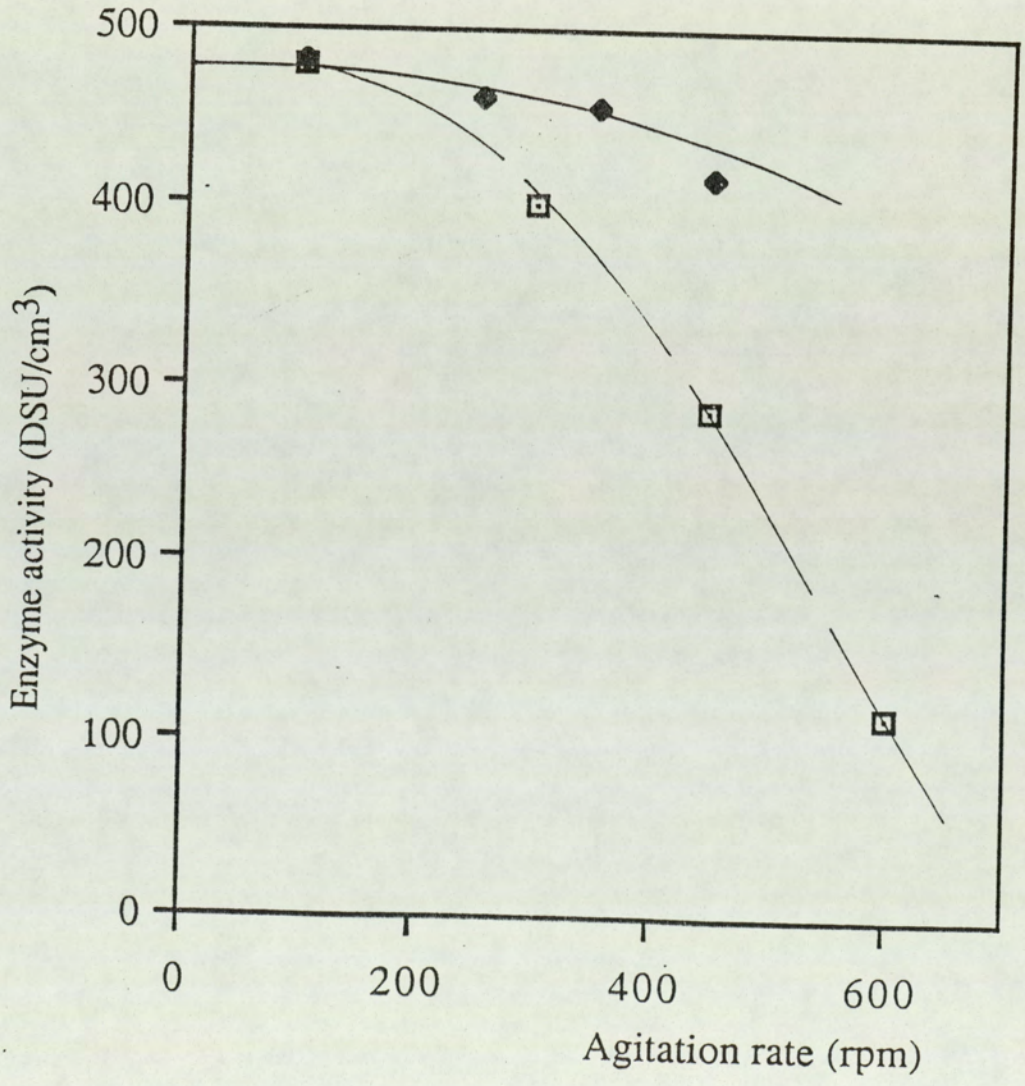



**Figure 7.4: Effect of agitation rate on enzyme activity with ( ⊡ ) and without ( ◆ ) antifoam in the fermentation medium**

Table 7.3: Effects of agitation rate on LM fermentations when antifoam was present in the medium.

| Experiment number | agitation rate (rpm) | $OD_{590}$ | enzyme activity ($DSU/cm^3$) | sucrose added (g) | growth rate, $\mu$ ($h^{-1}$) | fermentation duration (h) |
|---|---|---|---|---|---|---|
| 71-02-88 | 100 | 1.12 | 479 | 792 | 0.27 | 16.5 |
| 70-02-88 | 300 | 1.04 | 418 | 774 | 0.36 | 16 |
| 53-02-88 | 300 | 1.20 | 385 | 840 | 0.34 | 19 |
| 69-02-88 | 450 | 1.01 | 286 | 633 | 0.28 | 16.5 |
| 52-10-87 | 600 | 0.91 | 133 | 690 | 0.34 | 20 |
| 51-10-87 | 600 | 0.95 | 97 | 630 | 0.29 | 18 |

Table 7.4: Effects of agitation rate on LM fermentations when no antifoam was present in the medium.

| Experiment number | agitation rate (rpm) | $OD_{590}$ | enzyme activity (DSU/$cm^3$) | sucrose added (g) | growth rate, $\mu$ ($h^{-1}$) | fermentation duration (h) |
|---|---|---|---|---|---|---|
| 59-11-87 | 100 | 1.03 | 483 | 872 | 0.34 | 17 |
| 63-01-88 | 250 | 1.12 | 460 | 864 | * | 16.5 |
| 64-01-88 | 350 | 1.15 | 456 | 792 | 0.27 | 16 |
| 68-02-88 | 450 | 1.04 | 417 | 980 ** | 0.30 | 15 |

* Growth rate not determined.

** High sucrose consumption caused by a drift in the pH set point.

### 7.4.2 Conclusions

Two conclusions can be drawn from these sets of experiments. Firstly, the range of agitation rates used did not have any significant effect on the final enzyme yield. Secondly, the presence of antifoam reduced the enzyme elaboration without a corresponding effect on the final cell growth. At 450 rpm, for example, the enzyme activity was 417 DSU/cm$^3$ in the absence of antifoam and 286 DSU/cm$^3$ when antifoam was added to the medium (Tables 7.3 and 7.4). An examination of the sucrose consumption also showed a reduction in the amount used when high agitation rates were used with antifoam in the medium.

Surface active substances like antifoams are known to affect fermentation results. This interference can occur in a number of ways, including the affecting of the mass transfer coefficient of the system and the homogeneity of the broth. Viesturs and co-workers[94] have described in detail how these effects could alter the results of the fermentations. One possible way in which the antifoam could have affected the results is by altering the metabolism of the cells. This is indicated by the difference in the sucrose consumption. It would, therefore seem that the silicone antifoam is not the best type for the LM fermentation.

In the absence of the antifoam, the enzyme yield was not greatly affected by shear caused by agitation rates of up to 600 rpm. Activities in excess of 400 DSU/cm$^3$ were obtained under anaerobic conditions when the fermentations were agitated at 100 - 600 rpm. This represents an increase in the agitation power input per unit volume of a factor of up to 216 times that at 100 rpm (see Chapter 9). It can, therefore, be concluded that the enzyme production was insensitive to shear caused by the agitation rates used.

## 7.5 STUDIES ON THE MEDIUM COMPOSITION

Lawford *et al.*[37] reported that their enzyme production was affected by a phosphate concentration greater than 1% w/v. Other researchers have used varying amount of media components in their processes. Schneider and co-workers[53] used larger quantities of these materials (Table 5.1) to obtain high yields of enzyme activity. Experiments were conducted as preliminary tests to find out if reducing any of these components would affect the final cell growth and enzyme activity. The amount of sucrose was not altered because the initial amount used (60 g for 6 litres of medium) is very small compared to the additional sucrose used up (750 ± 50 g) in many of the experiments carried out.

For these studies, the composition of each of the parameters under investigation was halved. All others like the temperature, pH, and rate of agitation (100 rpm), and the initial broth volume of 6 litres were kept constant.

### 7.5.1 Results and discussions

Table 7.5 shows the results obtained when the amounts of the Gistex standard and potassium phosphate were reduced from 40 g/l and 20 g/l to concentrations of 20 g/l and 10 g/l respectively. The results show a reduction in both the final $OD_{590}$ and enzyme activity when the amount of Gistex was reduced. The additional sucrose used was only about half the amount normally used in the anaerobic fermentations. This was not the case with the phosphate. The cell growth always exceeded 1.0, and, as can be seen from experiment 35-40-87, the sucrose uptake was of the same order as that normally recorded. The enzyme activity was also in excess of 400 DSU/$cm^3$.

### 7.5.2 Conclusions

The results obtained by reducing the amount of Gistex compared more with those obtained with other yeast extract types than with the standard anaerobic experiments (Table 7.2). This would tend to support the hypothesis that some component in the yeast extract is responsible for these reduced yields. If it were an inhibitor, its effects would probably have been reduced when the amount of yeast extract was halved. It would appear that it is the

Table 7.5: Effects of reducing the amount of Gistex and potassium phosphate composition in the medium.

| Experiment number | component which was halved in amount | $OD_{590}$ | enzyme activity (DSU/$cm^3$) | sucrose added (g) | growth rate, $\mu$ ($h^{-1}$) | fermentation duration (h) |
|---|---|---|---|---|---|---|
| 32-04-87 * | phosphate | 1.01 | 360 | 606 | - | 12 |
| 35-04-87 | phosphate | 1.13 | 413 | 930 | - | 18 |
| 38-05-87 | Gistex | 0.78 | 316 | 408 | 0.33 | 18 |
| 41-06-87 | Gistex | 0.75 | 346 | 402 | 0.32 | 15 |

* Fermentation terminated because of temperature controller failure.

absence or scarcity of some component(s) that caused the reduction in the enzyme yield. Brown and McAvoy[67] identified folic acid as such a component.

The results also indicated that at least half of the potassium phosphate could be done away with without a corresponding reduction in the enzyme activity. A completion of this preliminary study could lead to the use of less phosphate than has been used for this project.

Stability of the enzyme in the fermentation media was not found to be affected by the phosphate concentration as was the case with aeration/agitation (see Chapter 6). The enzyme solutions obtained by using both the 10 g/l and 20 g/l potassium phosphate concentrations were stored in a frozen state for several months without loss of activity. It was not necessary to adjust the pH of the broth to 5.2 at which the enzyme is thought to be more stable.

Peptones have been used in fermentation media by several workers. Recently, these peptones were thought to improve the enzyme yield[49]. A number of peptones were obtained for trials. These included Peptone P, Special Peptone and Bacteriological Peptone, together with Caseine hydrolysate, supplied by Oxoid Ltd, Basingstoke, England. The experiments were carried out in shake flasks. In the first set of experiments, five flasks of media were prepared. In four of the flasks, the yeast extract was replaced by each of the peptones. The fifth contained the Gistex Standard yeast extract.

The pH values of the media were adjusted to 7.0 and autoclaved at 115 $^{o}$C for 20 minutes. Each flask containing 150 $cm^3$ of medium was inoculated with 15 $cm^3$ of inoculum and placed in a water bath maintained at 25 $^{o}$C. The flasks were assayed hourly. The results obtained at the end of the incubation period of 24 hours are shown in Table 7.6.

For some inexplicable reasons the Gistex flask failed to grow. An examination of the other results showed that the Bacteriological Peptone gave the highest enzyme activity. This was then selected for use in a second set of experiments.

In this second set of experiments, four flasks were used. One contained Gistex, while the rest had 25%, 50% and 75% of the Gistex replaced by the peptone. The results at the end of the incubation time of

**Table 7.6: Results of shake flask experiments using peptones.**

| Parameter investigated | cell turbidity ($OD_{590}$) | final enzyme activity ($DSU/cm^3$) | pH at the end of the process |
|---|---|---|---|
| Peptone P | 0.011 | - | 7.0 |
| Special Peptone | 0.208 | 35 | 6.2 |
| Bacteriological Peptone | 0.187 | 43 | 6.3 |
| Caseine hydrolysate | 0.06 | 5 | 6.7 |
| Gistex Standard | 0.016 | - | 7.0 |

22 hours are shown in Table 7.7. During this set of experiments, the flask containing only the Gistex produced good cell growth, and as the results show, the media in which up to 50% of the Gistex was replaced by the peptone gave better enzyme yields. The results are not conclusive because some of the flasks did not show good cell growth as have been expected. A further investigation into the use of peptones would therefore be necessary. The advantage of such a study is that, since the peptones can be more easily analysed than yeast extracts, this could give a better idea of those components in the medium that affect the results.

**Table 7.7 Shake flask experiments containing Gistex supplemented with Bacteriological peptone**

| % peptone in medium | $OD_{590}$ | enzyme activity ($DSU/cm^3$) | pH at the end of fermentation |
|---|---|---|---|
| 0 | 0.135 | 21 | 6.4 |
| 25 | 0.138 | 46 | 6.3 |
| 50 | 0.125 | 40 | 6.3 |
| 75 | 0.109 | - | 6.4 |

## 7.6 CONCLUSIONS

The following conclusions have been drawn from the experiments reported in this chapter:

1) Anaerobic fermentation have been established as the preferred method of producing high yields (450 - 500 DSU/$cm^3$) of dextransucrase. Although the growth rates are slightly lower than in aerobic processes, the enzyme elaboration was higher under non-aerated conditions.

2) The type of yeast extract used in the fermentation medium has been found to affect both the cell growth and enzyme production. The Gistex Standard yeast extract was found to be the best candidate of those studied for achieving the yields reported above.

3) Effects of agitation rates of up to 600 rpm on the 6 litre scale have been studied. The shear caused by these agitation conditions was not found to affect the final enzyme activity. However, there appeared to be a gradual decrease in the maximum activities obtained with increased agitation when antifoam was present in the medium.

4) When antifoam was present in the medium, the enzyme production was greatly reduced at agitation rates above 300 rpm which produced uniform distribution of antifoam and thus reduced the enzyme production. Cell growth was not affected suggesting that the antifoam interfered with the enzyme elaboration.

5) Preliminary experiments were carried out using half the amounts of potassium phosphate and Gistex. There was no significant difference in the results obtained with the reduced phosphate. However, both cell growth and, to a greater extent, enzyme production were affected when the amount of Gistex was reduced. This suggested that scarcity of some nutrient component as a result of the reduction in the amount of Gistex could have been the cause of the reduced enzyme activity.

6) The standard anaerobic fed-batch fermentation conditions for producing high yields of dextransucrase used in this chapter have independently been applied at Fisons Pharmaceuticals Plc. Using these conditions, enzyme preparations containing more than 400 DSU/$cm^3$ have been successfully produced[90].

# CHAPTER EIGHT

# CHAPTER EIGHT

# SCALE-UP AND MODELLING OF FED-BATCH FERMENTATIONS, AND CONTINUOUS FERMENTATIONS

## 8.1 INTRODUCTION

As indicated earlier in Chapter 4, dextransucrase fermentations have generally been carried out aerobically. The largest scale of operation reported in the literature was by Schneider and co-workers who produced enzyme on a 300 litre scale with activities of up to 300 DSU/cm3. The highly aerated conditions required, together with the inherent problems of foam control and the supply of large quantities of sterile air have made further scale-up unattractive industrially.

In Chapters 6 and 7, the importance of the type of yeast extract was established. Effects of agitation and aeration rates on the enzyme yield were also studied. Armed with these findings, it was possible to scale the process up to 1000 litres. This was achieved by using, with minor adjustments, an existing seed fermenter at Fisons Pharmaceuticals Plc. The first part of this chapter describes how the scaling-up was carried out, producing enzyme of up to 450 DSU/$cm^3$ of enzyme activity.

The subsequent sections in this chapter deal with attempts at modelling the batch processes using the ISIM continuous simulation package supplied by Simulation Sciences, Manchester, England(95).

Preliminary experiments on continuous fermentaion have also been carried out by operating the system as a chemostat. The experiments conducted so far have involved the determination of the critical dilution rate and the maintenance of a steady state at any given dilution rate. Unlike all previously cited chemostat operations for dextransucrase production, the processes reported in this section were anaerobic.

## 8.2 SCALE-UP OF THE ANAEROBIC FED-BATCH FERMENTATIONS

The scale-up was undertaken in two stages, with an 80 litre and a 1000 litre fermenter being used. The initial working volumes used were 60 and 600 litres respectively. Both fermenters had fixed speed agitators of 100 and 120 rpm respectively. Due to the scale of operation involved and the need to have an inoculum-to-medium ratio identical to that used in the laboratory fermentations, the inoculum was prepared as shown in Figures 8.1 and 8.2. MRS broth was used for the inoculum preparation. The same type of yeast extract (the Gistex Standard) was also used for all the experiments. No antifoam was added to any of the media.

The final $OD_{590}$ values of the inoculum from the third stages of preparation were 0.09 - 0.16. The temperature used during the processes was $23 \pm 2$ °C, while pH was controlled in the normal way by the addition of the sucrose/alkali mixture.

Sterilization of the 80 litre fermenter was achieved by directly injecting steam into the medium, leading to an increase in the volume of the medium. During the first run that was carried out at this scale, the increase was thought to be 20% of the material in the vessel before sterilization. The components of the medium were dissolved in 50 litres, with the hope that the increase in volume would make the total volume 60 litres. At the end of the fermentation, it was discovered that the increase in volume due to the condensation of the steam was more than 10 litres. As a result, a more accurate increase in the condensate volume was determined by carrying out a complete sterilization cycle with only water in the fermenter, and the increase was found to be 18-19 litres. In later trials, the media components were dissolved in about 40 litres before sterilization. The 1000 litre vessel had a jacket and live steam sterilization was not required. Sterilization of both fermenter systems was carried out at 118 - 121 °C for 30 minutes.

The sucrose/alkali mixtures were sterilized for 80 litres, but not for the 1000 litre fermenter experiments. The mixture for this latter scale was prepared by dissolving the sucrose in pyrogen free water. The sucrose solution was stirred continuously, and as the temperature dropped to below 25 °C, sodium hydroxide solution was slowly added.

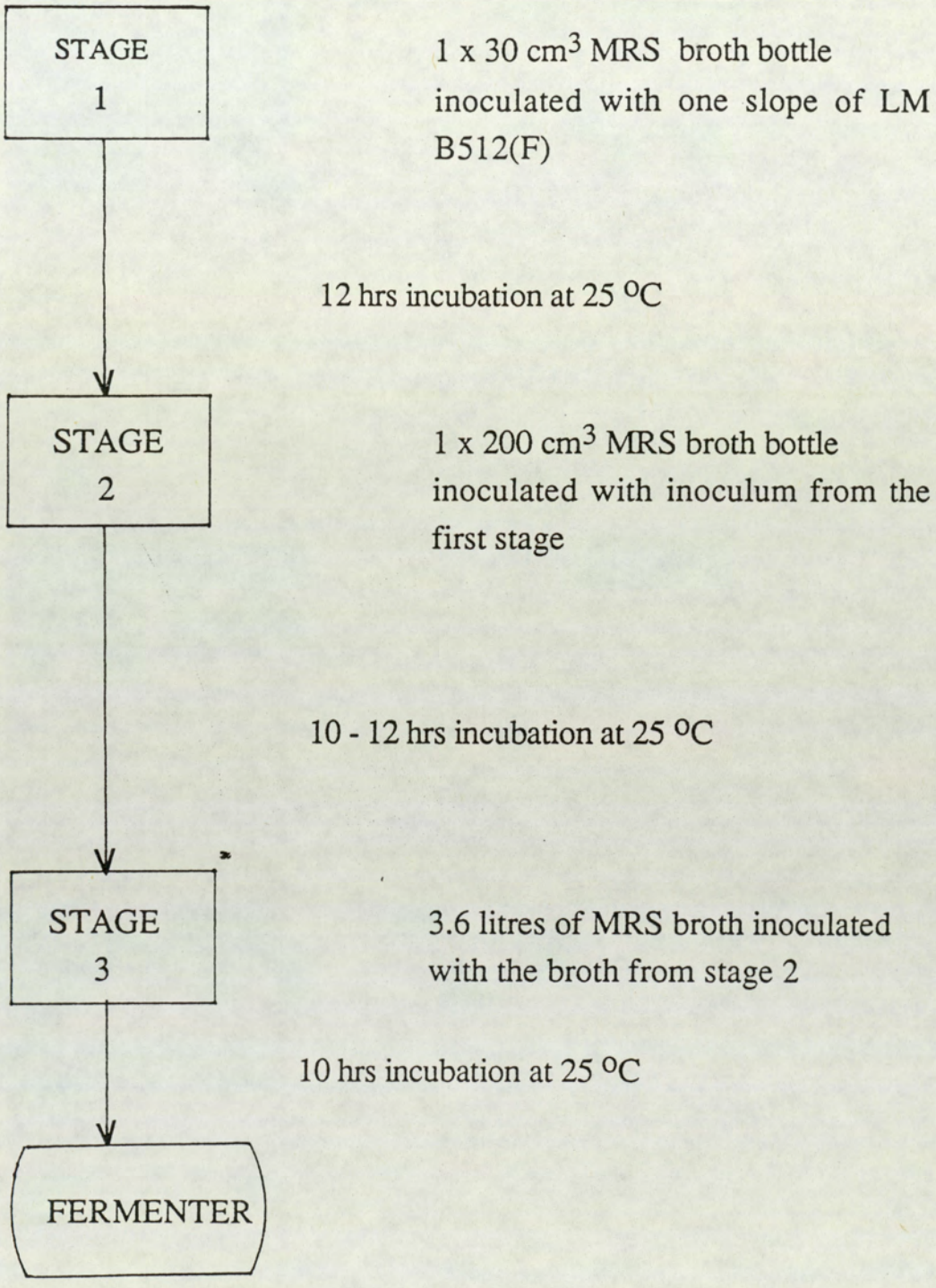



**Figure 8.1: Inoculum preparation for the 80 litre fermenter**

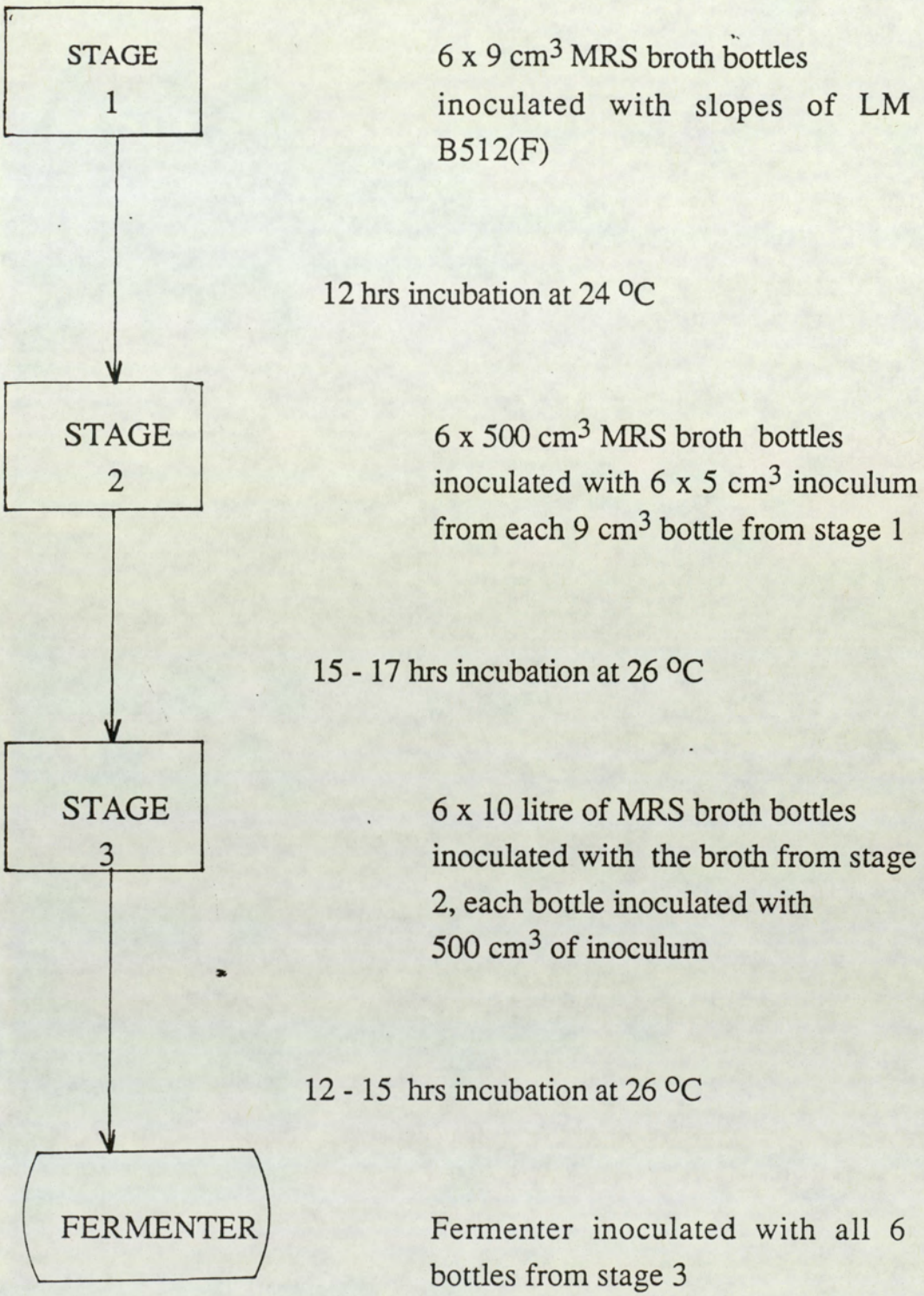



**Figure 8.2: Inoculum preparation for the 1000 litre fermenter**

When the caustic was added, the temperature rose, and it was necessary to allow the mixture to cool down before more sucrose was added. For a typical batch, 180 kg of sucrose were dissolved to make 243 litres of solution. 57 litres of 17 M NaOH were then added to give 300 litre of the sucrose/alkali mixture.

### 8.2.1 Results and discussion

Two runs were carried out at the 80 litre scale, while two sets of experiments were carried using the 1000 litre seed fermenter. The results of the 80 litre scale fermentations, together with those of the first set of experiments at the 1000 litre scale are shown in Table 8.1. Those of the second set at 1000 litres are shown in Table 8.2.

**Table 8.1: Results of scale-up of the anaerobic fed-batch fermentations**

| Experiment number | fermenter size (litres) | $OD_{590}$ | enzyme activity DSU/cm$^3$ | growth rate, $\mu$ ($h^{-1}$) | duration (h) | Temperature ($^oC$) |
|---|---|---|---|---|---|---|
| 42-06-87 | 80 | 1.08 | 310 | 0.32 | 26 | 23 ± 2 |
| 46-08-87 | 80 | 0.85 | 265 | 0.31 | 26 | 23 ± 2 |
| HZ 38T | 1000 | 0.67 | 262 | not recorded | 25 | 18 |
| HZ 39T | 1000 | 0.89 | 450 | not recorded | 25.5 | 18 - 23 |

**Table 8.2: Repeat of scale-up experiments of the anaerobic fed-batch fermentations using 1000 litre fermenter**

| Experiment number | $OD_{590}$ | enzyme activity (DSU/$cm^3$) | duration of fermentation (h) | Temperature ($^oC$) |
|---|---|---|---|---|
| 313/26 | 0.75 | 35 | 33 | 22-26 |
| 313/29 | 0.94 | 290 | 24 | 21-27 |
| 313/32 | 0.31 | 42 | 35 | 22-24 |
| 313/35 | 0.96 | 179 | 22 | 23-24 |

The results of the 80 litre pilot scale fermentation showed identical cell growth rates and fermentation times. The exact amounts of sucrose consumed in these experiments were not measured. However, the estimated totals were much smaller than expected. During the period when these runs were conducted, the fermenter was being interfaced to a computer. Feedback signals from the computer interferred with the pH control. At the beginning of the second run (experiment 46-08-87), the computer interfaced was disconnected and the pH was checked regularly with a lab meter. This time, it was realised that the available pump could not deliver enough sucrose/alkali mixture to the system. As a result the pH was controlled at 5.7 - 6.0 rather than at pH 6.7. It was also observed that the lag phases were of the order of 13 - 16 hours. This was almost double the time obtained with the small scale experiments.As mentioned above the pH meter indicated wrong values when the computer was connected to the fermenter. Thinking that these pH readings were correct, alkali was added to the medium until the meter showed the pH to be about 6.9 - 7.0. However, when samples were cross-checked later with a lab pH meter, the reading was about 7.8. The difference between the pH of the inoculum of about 4.5 and that of the medium must have accounted for the long lag phase. These problems with the pH controller, and the amount of steam that condensed in the medium could have been the cause of the lower yields. Access to the pilot scale 80 litre fermenter was on a temporary basis. After identifying the above problems it was decided to carry out trials at the 1000 litre scale.

In order to carry out these runs, a pH electrode was inserted in a recirculating loop that was fitted to the seed fermenter. This electrode was linked to a controller which activated a dosing pump that delivered the sucrose/alkali mixture to the vessel.

During the course of experiment HZ38T, the cell growth was found to be linear instead of exponential (Figure 8.3). The cell growth levelled off at an $OD_{590}$ value of 0.67. The enzyme activity was 262 DSU/$cm^3$ after 26 hours of fermentation. The temperature indicator on the vessel and another on an instrument panel showed the temperature to be within the range 23-24 $^{o}C$. During the second trial, (experiment HZ39T), the cell growth and enzyme activity profiles were the same as for experiment HZ38T. However, it was

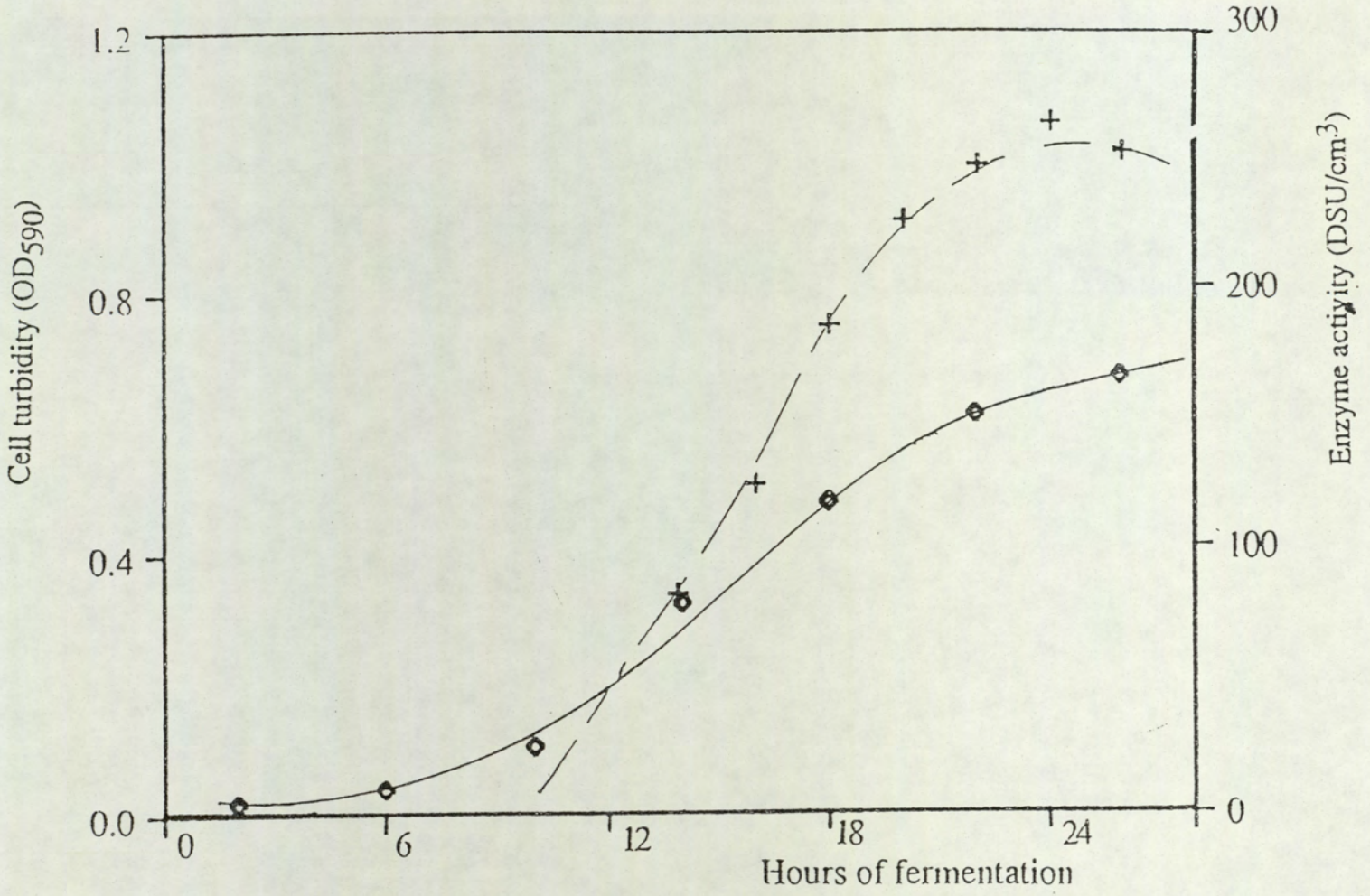



**Figure 8.3: Cell growth ( ◇ ) and enzyme production ( + ) during scale-up experiment HZ38T**

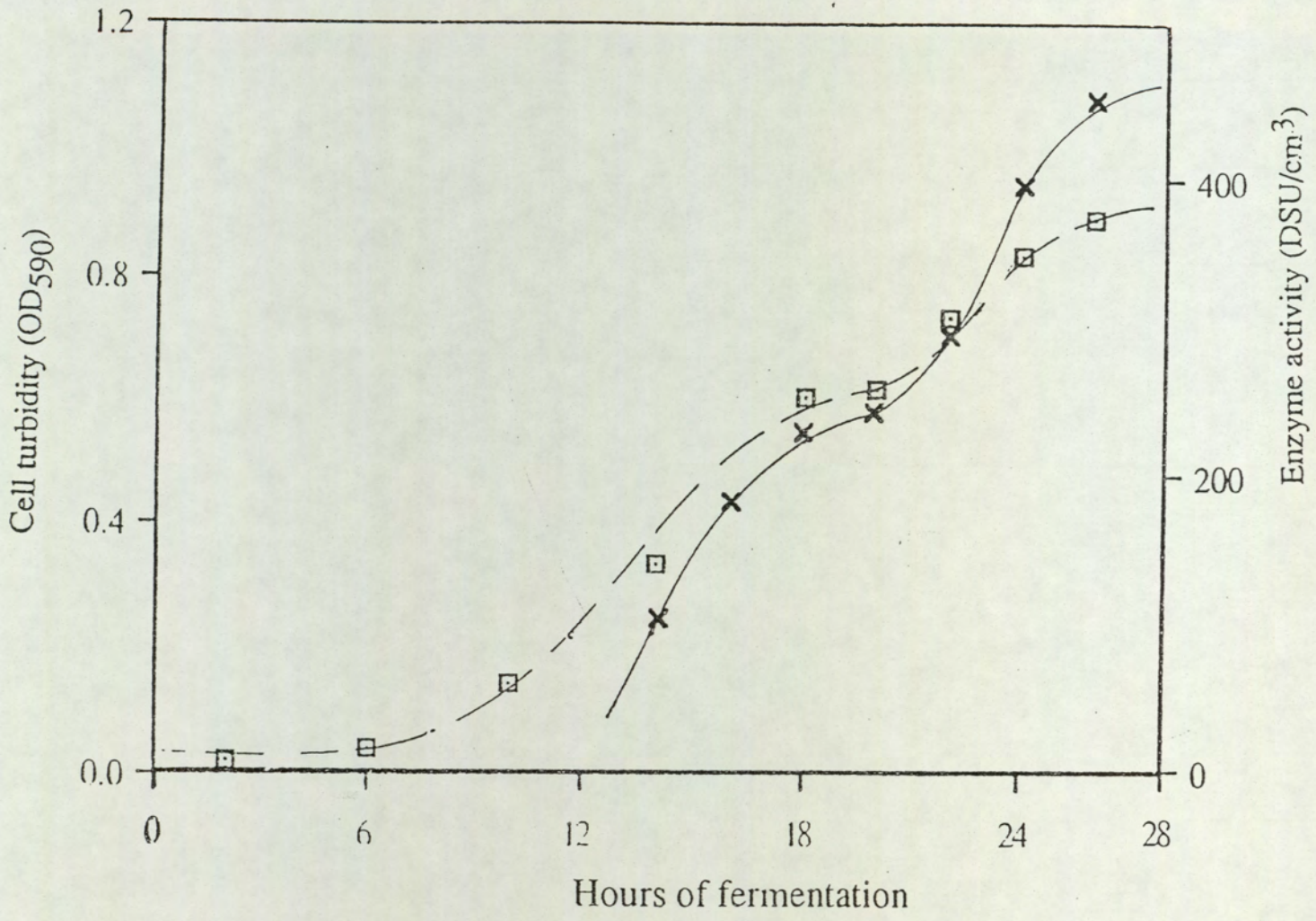



**Figure 8.4: Cell growth ( ⊡ ) and enzyme production ( ✕ ) during scale-up experiment HZ39T**

observed after 17 hours of fermentation that the temperature of the medium was actually 18 $^{o}C$ instead of the 23 - 24 $^{o}C$ shown by the instrument. Immediately, the temperature was adjusted so that the actual broth temperature became 23 $^{o}C$ while the fermenter indicators showed 29 $^{o}C$. As soon as the temperature stabilized, the enzyme activity increased rapidly to around 450 DSU/$cm^3$ (Figure 8.4). The cell growth also increased to an $OD_{590}$ value of 0.89.

The enzyme preparations from the two runs (HZ38T and HZ39T) were used for dextran synthesis at Fisons Pharmaceuticals Plc. That of experiment HZ38T was pumped into a 50,000 litre solution of 14.6% w/v sucrose. The higher activity enzyme was also pumped into a vessel containing 50,000 litres of 12.5% w/v sucrose. Dextran was synthesized from these vessels with conversions of 99% and 94% after 24 hours and 18 hours respectively. This dextran was found to be similar in structure to that produced by whole cell fermentation(95).

A second set of experiments was planned so as establish confidence in routinely producing high yields of dextransucrase. A fresh batch of Gistex yeast extract was purchased for these runs. The inoculum was prepared as described above for run HZ38T. Temperature and pH readings were cross-checked by taking samples and determining their respective values using laboratory meters. As the results in Table 8.2 show, the yields were substantially down on those of runs HZ38T and HZ39T. This was despite the fact that the inoculum grew better than in the previous runs. The lag phases were 9-10 hours, longer than the 6-7 hours encountered in the first set of experiments. In experiment 313/26, the cell growth was comparable with HZ38T (Table 8.1), however, only 33 DSU/$cm^3$ of enzyme were produced. Experiment 313/32 did not even grow to any substantial levels. The final activity after 35 hours of fermentation was 42 DSU/$cm^3$. Two of these experiments (313/26 and 313/35) gave $OD_{590}$ values of 0.94 and 0.96, and activities of 290 and 179 DSU/$cm^3$ respectively.

Since the same stock of culture was used as for other runs, the batch of Gistex was suspected as the cause of the poor yields. This fact was confirmed by carrying out small scale fed-batch processes with samples of the yeast extract. Results of two such runs , one of which was conducted at

Fisons Pharmaceuticals Plc are shown in Table 8.3. The conditions used were identical to those applied for the standard anaerobic fed-batches that yielded more than 450 DSU/$cm^3$ of enzyme activity.

**Table 8.3: Laboratory tests on Gistex used in the large scale trials**

| Experiment: | 80-05-88 | Fisons' trial |
|---|---|---|
| $OD_{590}$ | 1.09 | 0.84 |
| Activity (DSU/$cm^3$) | 270 | 205 |
| Sucrose used (g) | 567 | - |
| Growth rate ($h^{-1}$) | 0.31 | - |
| Duration (h) | 16 | 17.5 |

The results in Table 8.3 indicate that it was the particular batch of Gistex used that was responsible for the low yields of second set of scale-up experiments.

### 8.2.2 Practical aspects of the scale-up

The following are the observations that were made during the course of scaling-up the anaerobic processes:

1) It was not necessary to sterilize the sucrose/alkali added during the process. Sterility checks carried out on broths produced during the fermentations at Fisons Pharmaceuticals Plc. with non-sterile sucrose/alkali mixtures did not show any contamination.

2) Increased foam production during growth, especially when it could not be reduced by increasing the pressure in the air space above the medium, was a sign of unfavourable conditions existing in the fermenter. This was observed during the course of experiment HZ38T when the actual temperature was 18 $^{o}$C and also, during the second set of experiments at 1000 litres when poor yields were obtained. Once excessive foaming occurred, the cell growth and enzyme production soon levelled off.

3) Slow and linear growth occurred at 18 $^{o}$C. Foam formation also increased, leading to reduced cell growth and enzyme yield (see Table 8.1)

4) A reduction in the amount of inoculum used in the main fermenter from 60 to 50 litres did not affect the results of the fermentation.

### 8.2.3 Conclusions and recommendations

1 The anaerobic fed-batch fermentation of dextransucrase has been scaled-up to 1000 litres without a reduction in either cell growth or enzyme yield. Since agitation in the absence of antifoam was found to have no effects on the results, it was possible to use the available equipment without a lot of modification. The only requirements for successful scale-up were proper pH and temperature control.

2 The temperature used during the scale-up fermentation processes was $23 \pm 2\ ^{o}C$. In the case where the process was unknowingly carried out at a temperature of 18 $^{o}C$, the growth was found to be linear, with lower yields being obtained. During some of the processes, the temperature was found to go up to 27 $^{o}C$ when it was controlled manually. This did not appear to affect the overall fermentation results, so it will be necessary to determine a working range for the large scale processes.

3 It has been found that not all the batches of the Gistex gave good results. It would, therefore be necessary to test the yeast extract on a small scale before attempting to use it for large scale enzyme production.

## 8.3 MODELLING OF THE BATCH PROCESSES

The method of operation of the fed-batch fermentations described in this thesis is different from that of standard fed-batch fermentations. Fed-batch fermentations are normally carried out in two ways; one involving the addition of medium or a component of the medium at a determined rate, and the other, usually referred to as an exponential fed-batch, involving the supply of nutrient at an exponentially increasing rate so as to maintain a fixed nutrient level in the fermenter[30]. Although the addition of sucrose was allowed to vary, depending on its demand (or more appropriately on the alkali demand), it was found that the sucrose consumption was not exponential. Figure 8.5 shows that the total sucrose consumed increased almost linearly with time until the end of the exponential phase. Consequently, this process could not be modelled as an exponential fed-batch. The modelling procedure attempted in this section therefore involved the use of simple growth kinetic equations to obtain the various kinetic parameters which were then fitted into equations and the theoretical yields estimated using an ISIM continuous simulation package. The fed-batch fermentation was modelled as one with a constant nutrient feed rate equal to the average feed rate over the course of the fermentation.

### 8.3.1 Determination of kinetic parameters

Plots were made using the results of pure batch and fed-batch fermentations and the various kinetic parameters determined from these plots. Most of the graphical results presented so far have been with respect to time. In this section the different yields have also been plotted against each other.

Plots of the total cell concentration and the enzyme activity in the fermenter at any particular time against the total sucrose consumed have produced linear profiles as shown in Figures 8.6 and 8.7 respectively. The total sucrose consumed was taken as the amount used since the beginning of the fermentation divided by the initial broth volume of 6 litres, and expressed as grams per litre. This is because the sucrose level in the fermenter remained approximately constant during the course of the fermentation process. It was not possible to analyse the fermenter broth continuously, however, samples that were analysed showed the sucrose concentration to be 0.3 - 0.7% w/v.

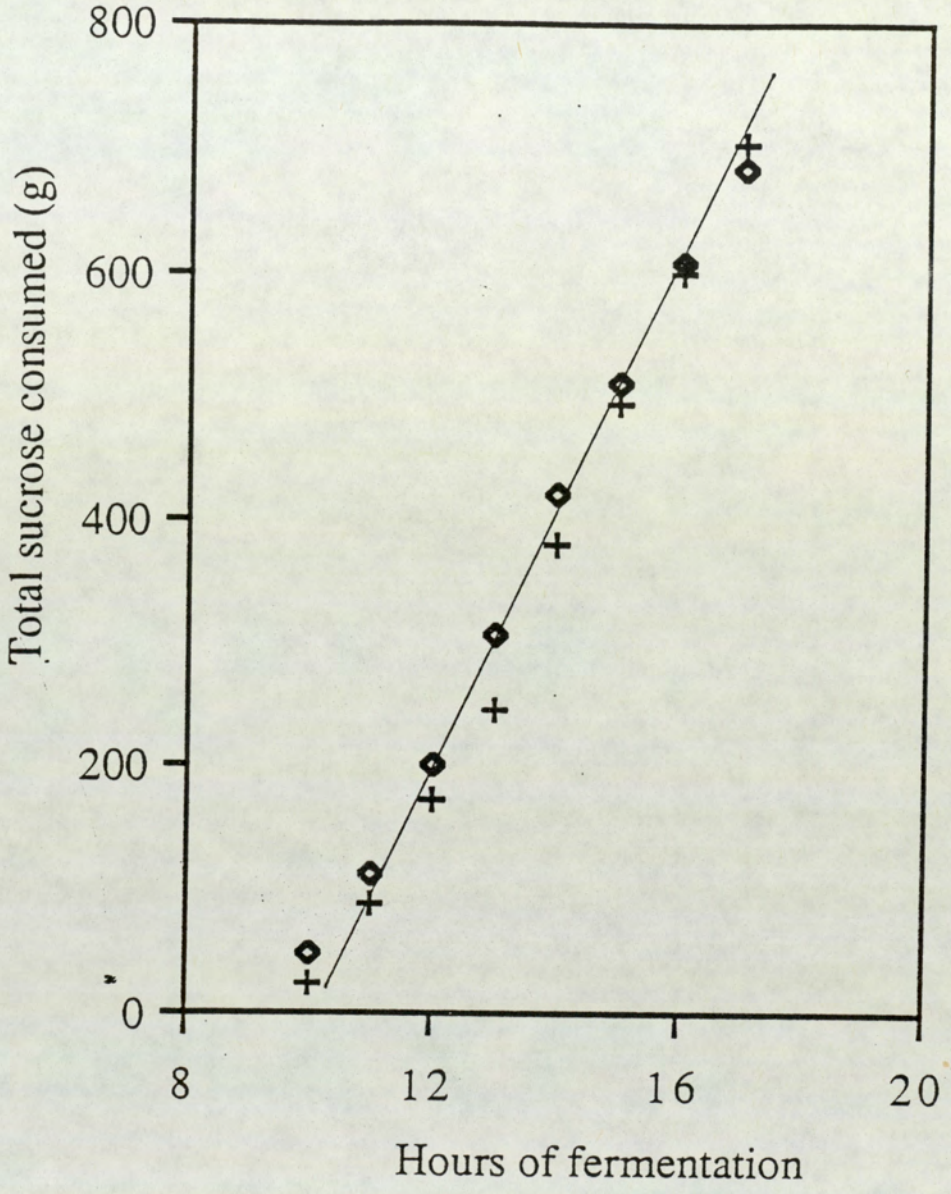



**Figure 8.5: Total sucrose consumption against time during anaerobic fed-batch fermentations - experiment 27-03-87 ( ◇ ) and 28-03-87 ( + )**

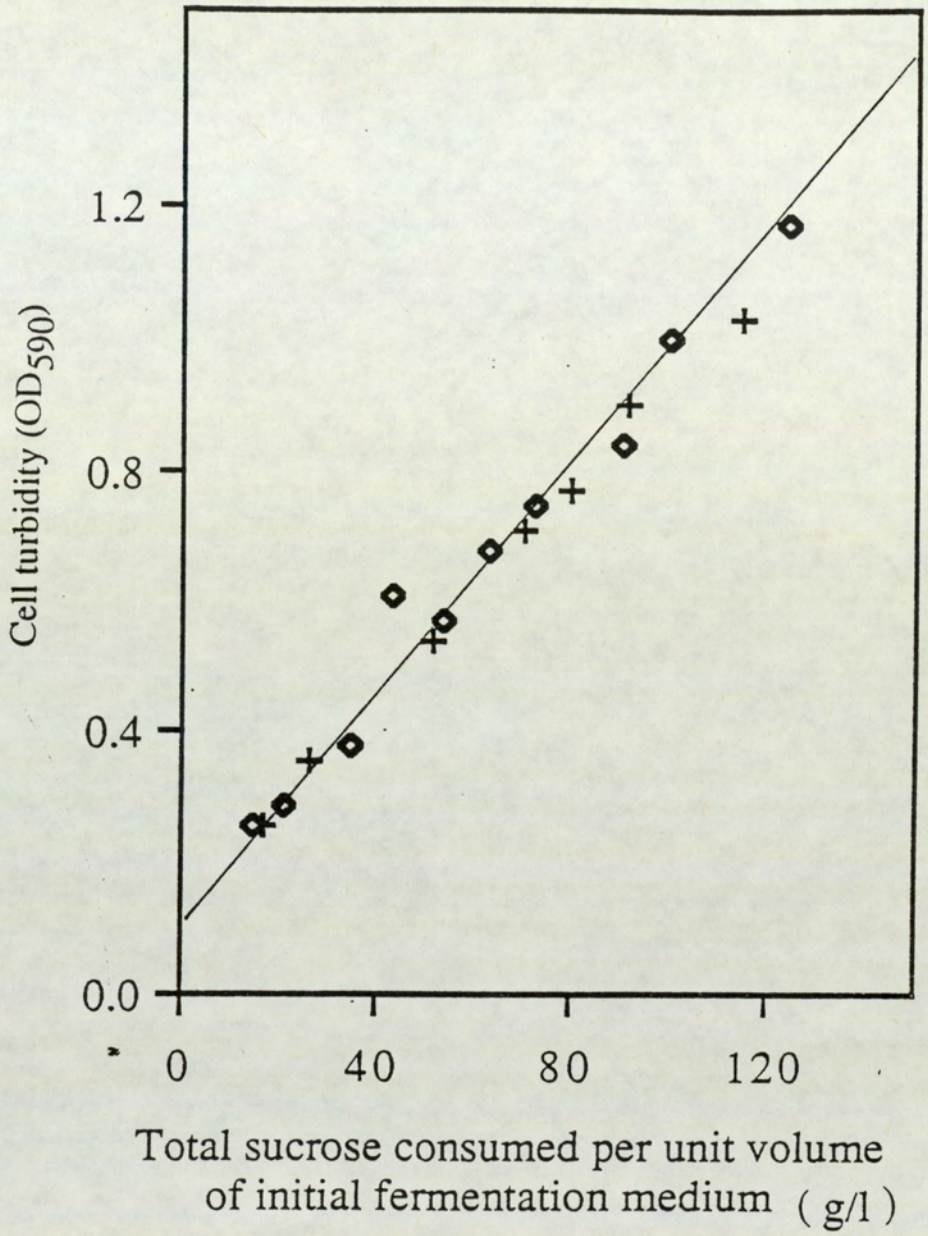



**Figure 8.6: Cell turbidity against total sucrose consumed per unit initial fermenter broth volume during anaerobic fed-batch fermentations - experiment 27-03-87 (+) and 28-03-87 (◇)**

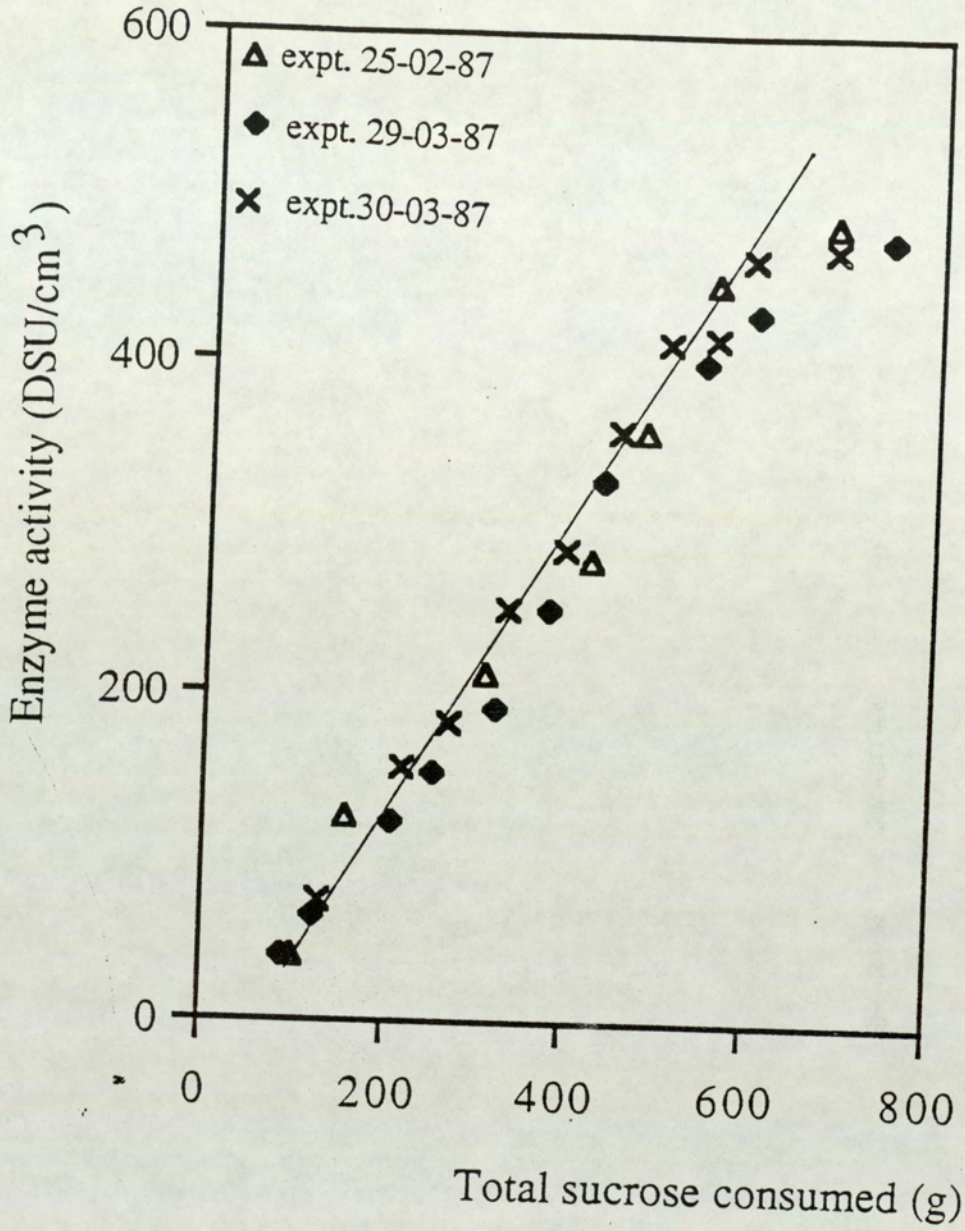



**Figure 8.7:** **Enzyme production against total sucrose consumption during anaerobic fermentations**

The slopes of these plots are the biomass yield coefficient ($Y_{x/s}$) and product (enzyme) yield coefficient ($Y_{p/s}$) with respect to sucrose respectively. Although Figure 8.6 is plotted in terms of $OD_{590}$ values, the $Y_{x/s}$ value was obtained by using the cell dry weight calibration curve in Figure 5.1 to convert the $OD_{590}$ values to grams per litre of cell dry weight. The $Y_{x/s}$ value has been determined from a number of plots to be 0.10 ± 0.03 g dry cell/g sucrose under anaerobic fed-batch fermentations. Pure batch fermentations were also carried out using 2% w/v and 5% w/v sucrose concentrations in the medium. Similar plots of the cell dry weight ($x - x_o$) against the total sucrose consumed ($s_o - s$) also produced linear profiles with identical slopes as the $Y_{x/s}$ values obtained from the fed-batch (see Figures 8.8 and 8.9).

The slopes of the graphs of total enzyme activity against sucrose consumed under standard anaerobic conditions, ($Y_{p/s}$), have been found to be 5.0 ± 0.8 DSU/cm$^3$/g/l of sucrose used, i.e. 5.8 * $10^6$ DSU/kg sucrose (see Figure 8.7). It was not possible to verify these values using batch fermentations because of the low enzyme activities that were obtained. The maximum activities for the 1%, 2% and 5% w/v sucrose containing batch fermentations were 25, 53 and 160 DSU/cm$^3$ respectively. These yields were obtained at the end of the fermentations when the sucrose levels were almost zero. Therefore, it was not possible to obtain reliable enzyme versus sucrose profiles in the batch runs.

The results of fed-batch fermentations were also used to determine the enzyme productivity of the cells, ($Y_{p/x}$). This was done by plotting the enzyme activity in the medium at any time against the total cell concentration at that same time. An example of such a plot is shown in Figure 8.10, which shows the relationship between enzyme activity and $OD_{590}$ for a number of fed-batch experiments. $Y_{p/x}$ was calculated by expressing the $OD_{590}$ values in terms of cell dry weight using Figure 5.1. The slopes of the enzyme activity against cell dry weight, ($Y_{p/x}$ values), have been found to be about 67 ± 6 DSU/cm$^3$ of enzyme activity/g/l of dry cells.

The determination of the $K_s$ value for the system was the main difficulty in obtaining growth parameters for the fermentation of LM. $K_s$ is the concentration of the limiting substrate, (sucrose), in the medium that will

produce a cell growth rate, $\mu$, equal to half the maximum growth rate, $\mu_{max}$. The growth rate for LM was found to be $0.34 \pm 0.08\ h^{-1}$. These same values were obtained under fed-batch and pure batch conditions using the different sucrose levels above. From these results, it was concluded that the growth rate obtained was the maximum possible under the experimental conditions. To find out a value for $K_S$ the initial sucrose level was reduced. Two problems were encountered when this was attempted. Firstly, the sucrose was totally used up before any appreciable cell growth could be obtained and the enzyme production was virtually zero. Secondly, the HPLC equipment used for the analysis of the sucrose was not sensitive enough to accurately detect the small sucrose concentrations. Sucrose concentrations of less than 0.2% w/v, (2 g/l), were not detected by the HPLC system. A value of 1.1 g/l has been reported as a $K_S$ value for dextransucrase, using sucrose as the carbon source[49]. This value of 1.1 g/l has been used in the modelling of the process.

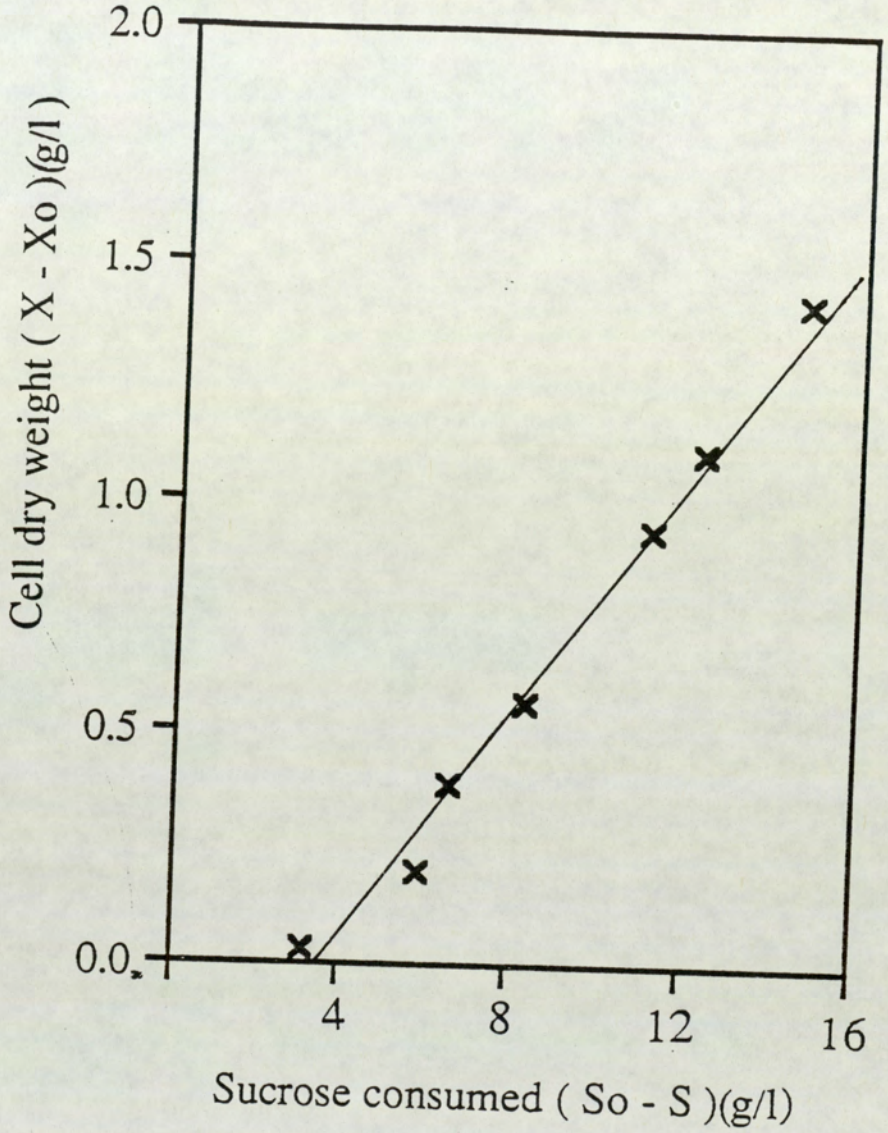



**Figure 8.8: Cell dry weight against total sucrose consumed during a 2 % w/v batch fermentation (experiment 43-07-87)**

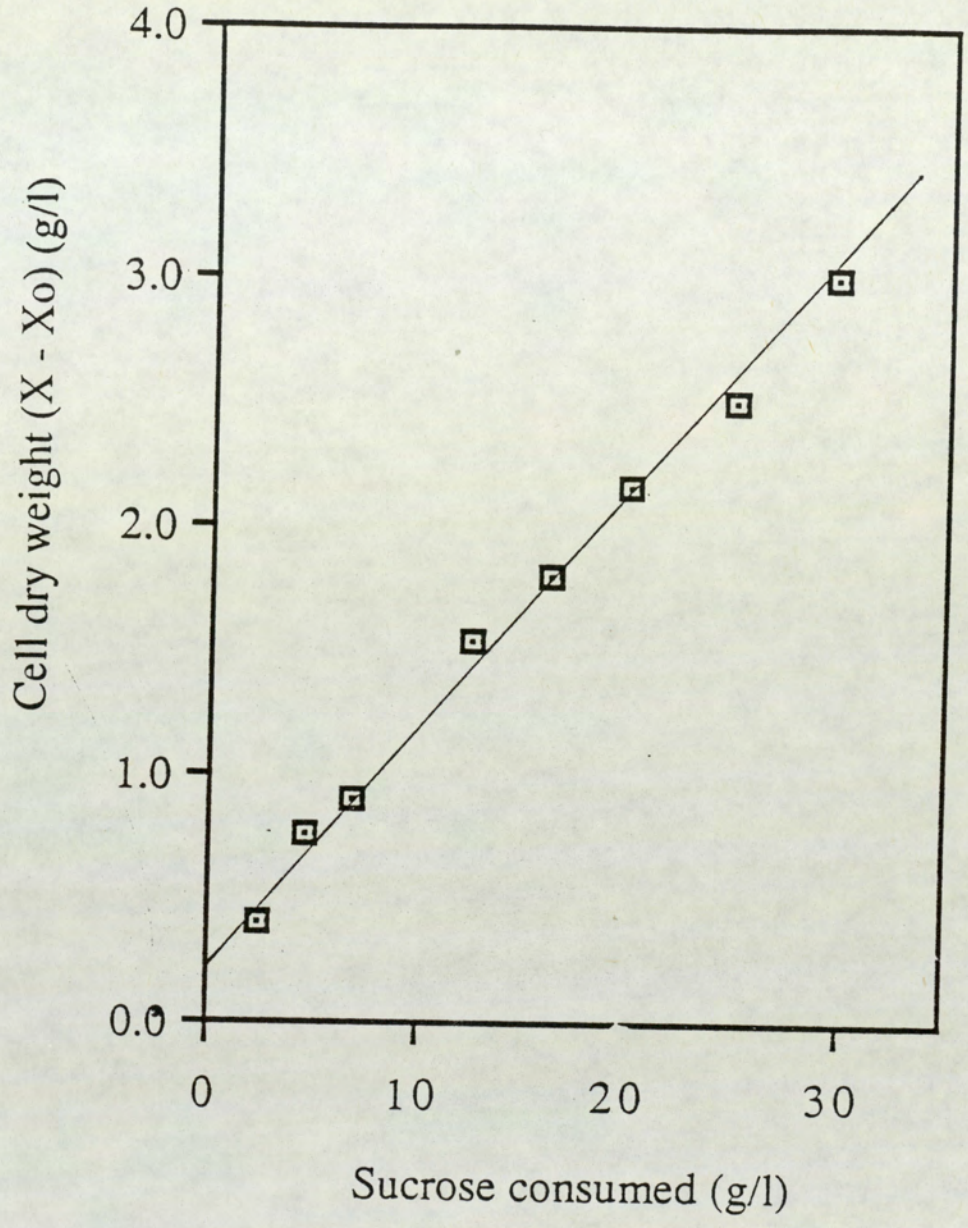



**Figure 8.9:** Cell dry weight against total sucrose consumed during a 5 % w/v batch fermentation (experiment 44-07-88)

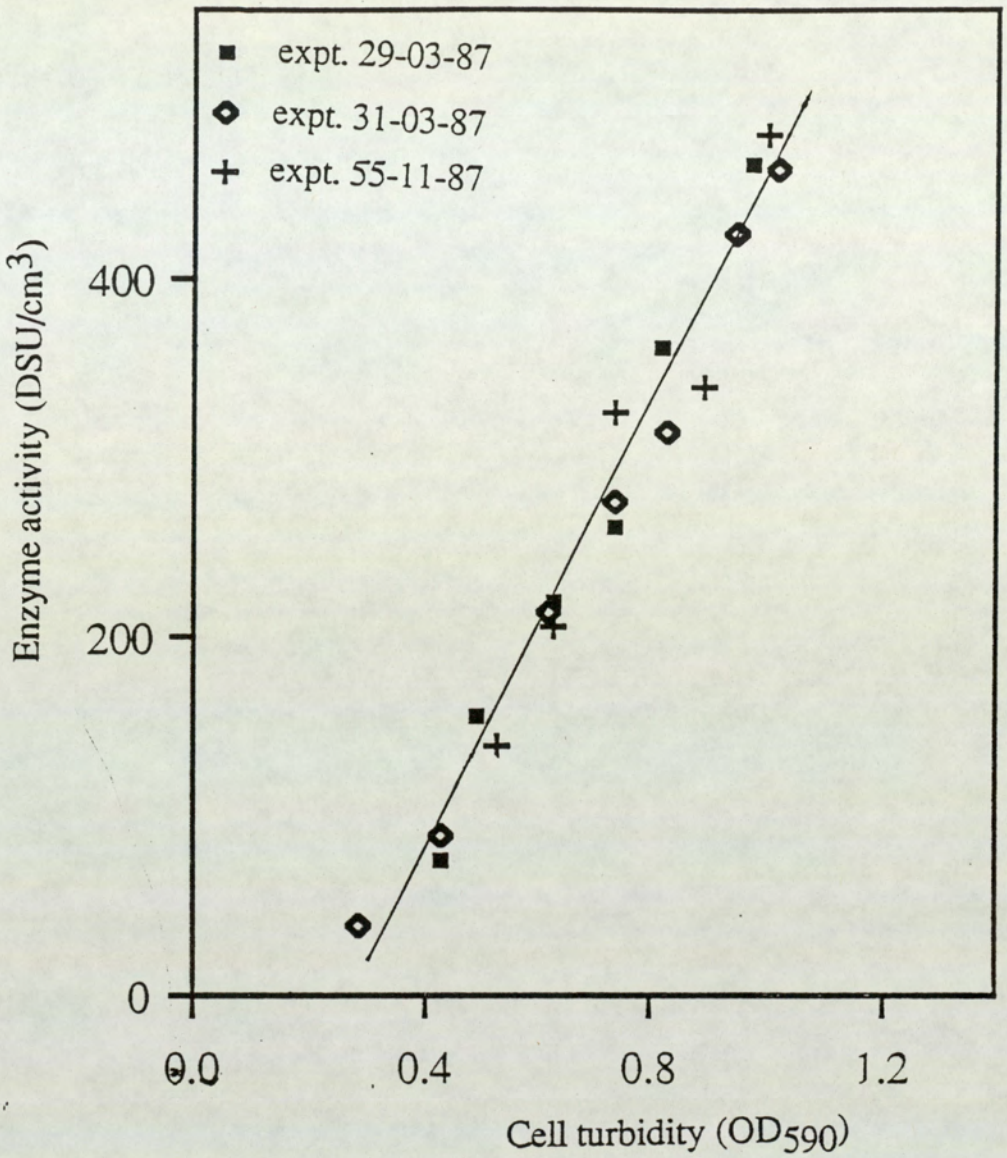



**Figure 8.10: Enzyme production against cell turbidity (cell productivity) during anaerobic fed-batch fermentations**

### 8.3.2 Model equations and results

A number of simplified assumptions were made in order to attempt a simulation of the fermentation process:

(1) The cell growth was assumed to follow simple Monod kinetics in which there is no inhibition. Sucrose was considered to be the only limiting substrate component. Therefore the cell growth equation used was:

$$\frac{dx}{dt} = \mu x \qquad 8.1$$

where

$$\mu = \frac{\mu_{max} S}{K_s + S} \qquad 8.2$$

(2) Addition of sucrose leads to an increase in the fermenter volume. Ignoring the amount of sample taken out during analysis, the rate of increase of fermenter volume is:

$$\frac{dV}{dt} = F \qquad 8.3$$

where F is the average sucrose/alkali input rate.

(3) Sucrose was considered to be the only growth limiting nutrient component. It is used only for cell growth and enzyme production. Maintainence energy has not been taken into consideration in the model. The model equation used for sucrose consumption was thus:

$$\frac{ds}{dt} = FC_s - \left(\mu x/Yx/s + \frac{1}{Yp/s}\ \frac{dp}{dt}\right) \qquad 8.4$$

(4) The enzyme production is growth related. Effects of yeast extract type on the final yield of enzyme were ignored, because the availability of the right type of yeast extract was assumed. Therefore, the enzyme production rate was expressed as follows:

$$\frac{dp}{dt} = \mu x/Y_{p/x} \qquad 8.5$$

These equations have been used in a model program and applied to the ISIM package for the simulation of the fermentation processes. A sample program, together with the results of the simulations are shown in Appendices 1A and 1B. Actual experimental results have been included for comparison.

## 8.4 CONTINUOUS FERMENTATION

This was carried out by operating the fermenter as a chemostat. The work done so far has mainly involved the determination of suitable dilution rates and attempts at maintaining steady state conditions at any dilution rate. The processes were carried out anaerobically using a 350 $cm^3$ vessel. The actual working volume was 335 $cm^3$. The medium composition was identical to that used in the batch processes (see Table 5.1), with the exception that the sucrose concentration was 2% w/v instead of 1% w/v. The additional feed also contained 2% w/v sucrose. The higher sucrose level was the same as that used by Paul *et al.*(69) who reported 70 DSU/$cm^3$/h. By using this sucrose concentration, the results could easily be compared with theirs.

The medium was prepared, its pH value adjusted and then put into the

fermenter. After inserting the pH probe and connecting all the additional tubing, the whole assembly was autoclaved at 115 - 119 $^{o}$C for 25 minutes. After autoclaving and cooling, the vessel was connected to the fermenter control module and the temperature controlled at 23 $^{o}$C. Agitation was by means of a magnetic stirrer at 200 rpm. 15 $cm^3$ of inoculum were used to inoculate the fermenter. After 7 - 9 hours, the cells started growing. At this point, the feed addition pump was switched on and the feed rate maintained at a fixed value until steady state conditions were achieved. At steady state, the cell concentration ($OD_{590}$) remained relatively steady. This condition was maintained for several hours before altering the feed rate so as to obtain a different dilution rate. The initial feed rate used was such that the dilution rate was less than the growth rate of 0.34 $\pm$ 0.08 $h^{-1}$ obtained for the batch processes. The dilution rates were then slowly increased until cell wash out occurred. It was after this point that the fermentation was terminated.

### 8.4.1 Results and discussion

To date, three continuous fermentations have been carried out. The first one was aborted because of blockage of the feed tubes. The results of the next two runs are shown in Table 8.4. The results of experiment 61-01-88 show that the optimum dilution rate is between 0.29 and 0.36 $h^{-1}$. This point is not quite clear from the results of experiment 66-02-88 where the highest activities were obtained at dilution rates of 0.27 - 0.28 $h^{-1}$. Fluctuations in feed flow rate due to partial blockage of the feed supply tubings made the maintenance of a fixed dilution rate very difficult. The flow rate was checked periodically using a flow tube in the feed line and the necessary pump setting adjustments made. Due to the small working volume, slight changes in the feed flow rate caused a displacement from the stationary phase and it took more than three hours to bring the system back to steady state. A change of the system to one with a larger fermenter capacity is currently planned to help minimise this problem.

One interesting fact that appears to have emerged from the above results is that dilution rates greater than 0.37 $h^{-1}$ caused cell washout. This dilution rate is of the same order as the growth rates obtained under the fed-

batch conditions. They are, however, lower than the 0.53 $h^{-1}$ and 0.6 $h^{-1}$ dilution rates used by Lawford *et al.*[32] and Paul *et al.*[69] respectively. Their experiments were aerobic while those described here were anaerobic. It is hoped that with the purchase of a better fermenter system, it will be possible to produce yields at least as high as those of Paul *et al.*

**Table 8.4: Steady state results of continuous fermentation**

| Experiment number | Dilution rate ($h^{-1}$) | $OD_{590}$ | Enzyme activity ($DSU/cm^3$) | Duration (h) |
|---|---|---|---|---|
| 62-01-88 | 0.25 | 0.139 | 8 | 25.5 |
| | 0.295 | 0.141 | 13 | 18 |
| | 0.33 | 0.185 | 13.5 | 11.4 |
| | 0.36 | 0.208 | 15 | 18 |
| | 0.38 | 0.071 | - | 8 |
| 66-02-88 | 0.20 | 0.124 | 11 | 10 |
| | 0.23 | 0.222 | 24 | 20 |
| | 0.28 | 0.200 | 17 | 8 |
| | 0.37 | 0.028 | - | 10 |

## 8.5 CONCLUSIONS

Scale-up of the anaerobic fermentation of LM has been successfully carried out. Operating at a 1000 litre scale, enzyme of up to 450 DSU/$cm^3$ has been produced under the anaerobic conditions. In the course of the scale-up experiments, the quality of the yeast extract used was once more found to have significant effects on the fermentation results, confirming the results reported in Chapters 6 and 7 on the effect of the type of yeast extract. The yeast extract that caused the failure of some of the large scale experiments was a different batch of Gistex standard, showing the need to test each batch of yeast extract on a small scale before using it for large scale fermentations. In the long run, it might be necessary to carry out a complete study of the actual requirements of LM so that a more defined medium could be formulated.

Other points that emerged from the scale-up trials included the possibility of using non-sterile sucrose/alkali mixtures and the need to define the limits of the fermentation operating parameters. It was realised that there was no contamination when the sucrose/alkali mixture was not sterilized. At a temperature of 18 $^oC$, the cell growth was virtually linear and the enzyme production was only about 250 DSU/$cm^3$. Trials at 28 $^oC$ produced less than 400 DSU/$cm^3$ of enzyme in the laboratory. It would therefore be necessary to carry out more experiments so as to more accurately define the operating temperature. For the time being, a temperature of 22 - 25 $^oC$ would seem good enough for the process. The same exercise could be used to define an optimum pH range. During scale-up, the pH of the medium was between pH 6.5 and 7.0 throughout the process. It went up to 7.0 immediately after the addition of the sucrose/alkali mixture, otherwise the pH was between 6.5 and 6.8 during a large part of the fermentation process.

An attempt has been made to model the batch processes. The simulations have produced results comparable with the experimental results.

Preliminary continuous fermentation experiments have shown that under anaerobic conditions, cell washout occurred at dilution rates greater than 0.37 $h^{-1}$. Steady state conditions have been achieved for each dilution rate. However, due to the fact that the feed pump available could not maintain a constant feed rate, the steady state conditions could not be

maintained for as long as were desired. Having selected a suitable yeast extract type, and studied the effects of aeration, agitation and antifoam, it is anticipated that successful continuous fermentations can be achieved with a better controlled fermenter system. An optimisation of the sucrose concentration in the feed medium as well as the dilution rate could lead to continuous high enzyme activity production.

# CHAPTER NINE

# CHAPTER NINE

# DISCUSSIONS, CONCLUSIONS AND RECOMMENDATIONS FOR FUTURE WORK

## 9.1 INTRODUCTION

The aim of this research work was to investigate the best conditions that could be used to produce high yields of dextransucrase by batch and continuous fermentation techniques. Chapter 6 described some preliminary experiments that were carried out, based mainly on the information available in the literature. These included the use of fructose, glucose and sucrose as the carbon sources, and aerobic fed-batch fermentations. Chapter 7 described the experiments that were carried out under anaerobic fed-batch conditions. These produced broths containing enzyme activities of 400 - 500 DSU/cm$^3$. Effects of agitation rate with and without antifoam, and the type of yeast extract used in the medium on the yield of biomass and enzyme were also studied. In Chapter 8, the anaerobic fermentation techniques were scaled-up. Enzyme was successfully produced at 80 litre and 1000 litre scales with yields of up to 450 DSU/cm$^3$ obtained.

The first sections of this chapter discuss the results of the aerobic and anaerobic fermentations. Effects of yeast extract type, agitation rate and antifoam on the results of the anaerobic fed-batch fermentations are also described. The results of the scale-up processes, together with the problems encountered are also outlined. The conclusions drawn from the various experiments are then presented in the subsequent section.

Different workers have used different media compositions for the production of dextransucrase. The medium composition used for this research work was the same as that used by Schneider *et al.*(53). It was not possible to carry out detailed work on the media due to the time available for this research work, however preliminary experiments were carried out with reduced yeast extract and phosphate levels in the fermentation medium (see Chapter 7). A number of other observations like the linear growth at reduced temperature during the scale-up processes were also noted. The last section of

this chapter describes those aspects of the fed-batch fermentations that require additional attention. Completion of the project by investigating the continuous fermentation conditions is also included as future work.

## 9.2 EFFECTS OF AERATION ON THE YIELD OF DEXTRANSUCRASE

Fermentations of LM were carried out under different aeration conditions. In one set the DO level in the fermenter was maintained at 40 - 70% of the initial saturation value as recommended by Schneider and co-workers[53]. These DO levels were achieved by aerating the media at 4 VVM and agitating it at 400 - 650 rpm (see Section 6.3). Under these conditions, the enzyme activities obtained were typically around 350 DSU/cm$^3$.

Another set of aerated fermentations was carried out in which the agitation rate was maintained at 100 rpm, while the aeration rate was altered from one experiment to the other. As the results in Table 6.4 showed, the activities were of the same order as those obtained when higher conditions of aeration and agitation were used.

When no aeration was applied to the fermenter, the enzyme activity produced was much higher than in the aerated experiment. Table 7.1 showed typical results obtained from anaerobic experiments using Gistex Standard yeast extract. These experiments were carried out under 100 rpm agitation conditions. The enzyme activities were 450 - 500 DSU/cm$^3$ as compared to about 350 DSU/cm$^3$ under aerobic conditions.

A possible explanation for the difference in the level of enzyme produced under aerated and non-aerated conditions could be a change in the metabolism of the *Leuconostoc mesenteroides*. This was indicated by the amounts of sucrose consumed under the different conditions. The total consumption under the anaerobic fermentation conditions was between 680 and 860 g for the 6 litre scale fermentations (see Table 7.1). Under aerobic conditions, the consumption was typically 500 - 600 g for the same working volume. Energy required by growing cells is provided in the form of ATP (Adenosine triphosphate) molecules[28,96,97]. The number of ATP molecules produced in the presence of oxygen greatly exceed those produced

under anaerobic conditions. Since more ATP molecules are available when the fermenter is aerated than when it is not aerated, the organism obtains the required metabolic energy by breaking down the sugars, fatty acids and amino acids with the aid of enzymes[97]. It would, therefore, appear that under anaerobic conditions, fewer ATP molecules are available, and as a result larger amounts of enzyme (possibly including dextransucrase) are secreted into the medium to break down the sucrose to glucose which is then oxidised to provide the required energy. The above phenomenon could explain why there is a higher sucrose consumption and more enzyme production in the anaerobic than in the aerobic processes.

Another reason that has been suggested for the greater production of dextransucrase under anaerobic conditions is the transport mechanism involved in the enzyme elaboration. The transport of dextransucrase is thought to be more efficient in the presence of carbon dioxide[88]. Therefore more enzyme would be produced under anaerobic conditions where more carbon dioxide is present than under aerobic conditions. The presence of oxygen in aerated systems may also present a disadvantage in that it could oxidise the S-H (sulphur-hydrogen) bonds in the enzyme structure[22].

The transport mechanism theory does not, however, explain the difference in the sucrose consumption observed. It would therefore appear that it is a difference in the metabolism of LM that leads to the production of different levels of enzyme under aerobic and non-aerobic fermentation. The validity of this supposition could be tested with detailed studies of the metabolism and energetic of the culture.

## 9.3 EFFECTS OF ANTIFOAM

The silicone concentrate antifoam was used in all experiments where excessive foam formation was expected. These included the aerobic fermentations and some of the anaerobic runs where high agitation was employed. Antifoam was used in both sets of aerobic fermentations described in Section 9.2 above. When some of the experiments at a fixed 100 rpm agitation were aerated at 12 l/min without antifoam in the medium, excessive foaming occurred. The average yield of enzyme under these conditions was 224 DSU/cm$^3$ as compared to 315 DSU/cm$^3$ when antifoam was present

(Table 6.4). It was therefore apparent that excessive foaming affected the enzyme yield. Excessive foaming refers to the conditions when the surface of the broth medium was totally covered by several centimeters of foam. An increase in the fermenter back pressure, that is, the pressure of the air space above the medium, did not lead to a reduction in the foam level. Whenever excessive foaming occurred, samples taken from the fermenter showed the presence of a high concentration of air bubbles. Air bubbles are known to provide high surface areas which intensify the dissolution of oxygen present in the fermenter air space and thus make the processes more aerobic[94]. This was usually followed by a levelling off of the enzyme production.The silicone antifoam kept foaming to a minimum when it was added to the medium before sterilization. 2 $cm^3$ of the antifoam were sufficient for this purpose.

Anaerobic fermentations in which mild agitation conditions were applied did not require antifoam. However, when tests were conducted using different agitation rates, the antifoam was found to affect the results (Figure 7.4). Since air was not sparged into the medium, the increased agitation enabled a more accurate study of the effects of antifoam to be made. At low agitation, the antifoam formed globules which remained on the surface of the medium or stuck to the fermenter walls. As the agitation rate was increased, the silicone concentrate was seen to be dispersed in the medium. When this happened, the enzyme production reduced drastically without a corresponding decrease in the other results of the LM fermentation. Viesturs and co-workers[94] have reported that surface active substances like antifoams can influence the physiological function of cells by causing morphological changes in the cell structures. This could be the way in which the silicone concentrate, which is a synthetic antifoam, affects the enzyme elaboration. Under highly aerated and agitated conditions it could, therefore, be that a combination of both antifoam and oxygen are responsible for the lower activities of enzyme obtained than for slowly agitated anaerobic fermentations.

## 9.4 EFFECTS OF STIRRER SPEED ON DEXTRANSUCRASE PRODUCTION

The New Brunswick fermenter that was used for the 6 litre laboratory scale experiments had an agitator which was capable of operating at 100 - 1200 rpm. However, it was discovered that the motor did not maintain agitation rates greater than 800 rpm for considerable lengths of time. When the fermenter was aerated at 4 VVM, agitation rates of up to 650 rpm were sufficient to maintain the DO level at 40-70% of the initial saturation value. This DO range was that recommended by Schneider *et al.*(53). When it was realised that milder aeration/agitation conditions favoured higher enzyme production, no more attempts were made to work at higher agitation rates. Effects of agitation on the cell growth and enzyme yield were carried out under anaerobic conditions. Agitation power input per unit volume of the fermenter under non-aerated conditions is as follows(98)

$$\frac{P}{V} = N_p \rho N^3 Di^2 \qquad \text{..... 9.1}$$

where $N_p$ is the power number of the impeller, and is constant for any given agitation conditions.

N the impeller speed

Di the impeller diameter, and

$\rho$ the density of the medium.

From the above equation, it can be seen that the power per unit volume (P/V) is proportional to $N^3Di^2$ (assuming the other parameters remain constant over these agitation conditions).

The agitation rates that were used in the anaerobic fermentations ranged from 100 - 600 rpm. According to equation 9.1 above, the P/V value at 600 rpm will be 216 times that at 100 rpm.

$$\frac{(P/V)_2}{(P/V)_1} = \left(\frac{N_2}{N_1}\right)^3 = \left(\frac{600}{100}\right)^3 = 216 \qquad \text{---- 9.2}$$

where subscript (1) represents the conditions at 100 rpm and (2) those at 600 rpm respectively. As the results in Table 7.4 showed, there was no significant decrease in the fermentation yields over this agitation range. The final enzyme activity obtained in all the experiments conducted over these sets of conditions were above 400 DSU/cm$^3$. The exception was when antifoam was present in the medium as described in Section 9.3 above. The larger scale fermenters had fixed speed agitators (120 rpm). Information on the dimensions of their impellers was not available, so it was not possible to compare the power inputs with that of the small scale fermenter. No alterations were made on the stirrer speeds. The results obtained from these experiments indicated that these agitation rates did not affect the enzyme yield or cell growth.

It can, therefore, be concluded that the stirrer speeds used did not affect the enzyme yield.

## 9.5 COMPOSITION OF FERMENTATION MEDIUM

It can be argued that the main investigations carried out during this research program have been on the medium for producing the high yields of enzyme dextransucrase. Anaerobic fermentation conditions have been shown to be beneficial both in producing higher yields of enzyme than have previously been obtained, and in its ease of scale-up. The effects of the silicone antifoam that was used in the medium have also been investigated. Other components of the medium that have been studied included the type of carbon source, the composition of the phosphate, and the type and composition of the yeast extract. As mentioned earlier, the medium composition was identical to that used by Schneider and co-workers[53] (see Table 5.1).

Shake flask experiments were conducted so as to confirm that surcrose is needed for dextransucrase production. Glucose, fructose and sucrose were used as the carbon sources. Only those media that contained sucrose produced enzyme. When some of the fructose and glucose based media were dosed with sucrose, some dextransucrase was produced, confirming that the sucrose induced the elaboration of the enzyme.

Schneider *et al.*(53) used a 2% w/v di-potassium hydrogen ortho-phosphate concentration in their media. Preliminary tests were carried out in which the amount of phosphate was halved. The yield of enzyme was not affected (Table 7.6). These results indicated that the phosphate concentration in the medium could be reduced without affecting the fermentation results.

In their work, Schneider and co-workers used a 4% w/v yeast extract concentration (Table 5.1). Other workers have used varying amounts of yeast extracts, some supplemented with peptones. Brown and McAvoy(67) identified that different yeast extract types affected the enzyme yield, and attributed this difference in the performance of yeast extract to be due to the different levels of folic acid in them. Different types of yeast extracts were tested. These included the Gistex standard, Orhly, Oxoid and the Distillers Co., Ltd (DCL) types of yeast extracts. The Gistex standard produced the highest enzyme activity (450 - 500 DSU/$cm^3$ of dextransucrase activity). This was followed by the Orhly, Oxoid and DCL types respectively. The DCL yeast extract gave the lowest enzyme activities (200 - 250 DSU/$cm^3$). These activities were obtained from anaerobic fermentations which were agitated at 100 rpm with no antifoam added to the medium. All other conditions such as temperature and pH were identical to those described for the standard fermentations in Chapter 7. Similar trends were obtained by using some of these yeast extracts under aerobic conditions.

Although the Gistex was found to be the best type, one batch failed to produce any good cell growth or enzyme activity. Attempts were made to improve the results from this batch of extract by adding folic acid, but were unsuccessful.

Yeast extracts are non-homogeneous and are very difficult to analyse. Recently, trials were started in which specific peptones that are more easily analysed were used as nitrogen sources. The object was to select the best

peptone to be used, and then analyse the media before and after the fermentation so as to determine the components of the peptone that had been used up. A number of peptones were purchased from Oxoid and tested. One of them, Bacteriological Peptone was found to produce the best results of those tested (Table 7.7). Various amounts of this peptone and Gistex were added to the fermentation media. The results (see Table 7.8), showed that media in which 25% of the Gistex was replaced by the peptone gave the best results in terms of cell growth and enzyme activity. The pattern of this results shows that a combination in which less than 25% of the Gistex is replaced by the peptone would be the best combination which could then be analysed.

## 9.6 CONTINUOUS FERMENTATIONS

Studies have been started on continuous fermentation using a 350 $cm^3$ chemostat. Steady state conditions were achieved at different dilution rates, but these could not be maintained for more than 15 hours because of the unstable nature of the particular fermenter system. A newer chemostat is being purchased and this problem should easily be overcome.

Cell washout was found to occur at dilution rates greater than 0.4 $h^{-1}$. The dilution rates needed for continuous anaerobic fermentation should, therefore, be similar to the growth rates of 0.3 - 0.35 $h^{-1}$ determined from the batch experiments. These are much lower than the 0.53 $h^{-1}$ and 0.6 $h^{-1}$ used by Lawford *et al.*(37) and Paul *et al.*(69) respectively. The maximum steady state enzyme activity obtained from the preliminary continuous fermentations was 25 DSU/$cm^3$/h at 0.23 $h^{-1}$ dilution rate. This was maintained for up to 20 hours. Higher dilution rates were more difficult to maintain for such periods of time because the available pump could not maintain a constant feed rate.

## 9.7 TEMPERATURE AND pH OPERATING RANGES

A pH of 6.7 has been widely reported as the optimum value for the production of high yields of dextransucrase (see Chapter 4). Higher pH values caused the enzyme to denature. At lower pH values, dextran

production increases, making the medium to become very viscous.

During the course of this research work, it was observed that enzyme preparations containing more than 400 DSU/$cm^3$ were kept in the freezer for up to three months at pH values of 7.0 - 7.3 with a loss of only about 50 DSU/$cm^3$ activity. When the pH was reduced to 4.8 - 5.5, the same stability was obtained. Samples that were centrifuged lost more than 50% of the activity when stored under similar conditions. The enzyme is therefore more stable in the presence of the cells.

It was possible to maintain the pH at 6.7 ± 0.05 in the laboratory. However, during scale-up, the pH fluctuated between 6.5 and 7.3. This did not appear to affect the results (see Chapter 8), thus indicating that it is possible to define a pH range over which the process can be controlled without affecting the results. Unfortunately it has not been possible to investigate this further within the time period of this work.

Alsop[40] carried out tests at different temperatures and concluded that 23 °C was the optimum for the production of dextransucrase. This temperature was used throughout this project. However, during the scale-up processes, it was very difficult to control the temperature at this set point using the control system that was available on the vessel. Also, during some of the scale-up experiments the cell growth was found to be linear and enzyme activity low when the temperature was 18 °C (see Chapter 8).

An anaerobic fed-batch experiment that was carried out at 28 °C produced 430 DSU/$cm^3$ of enzyme activity. The cell growth was faster but the total sucrose consumed was the same as for the other anaerobic fermentations. The inoculum preparation, broth volume used, agitation and pH were the same as for the standard fed-batches described in Chapter 7. The indication from this experiment is that temperatures higher than 23 °C could be applied. 25 °C has been used by several workers, and some have even worked at 30 °C (see Chapter 4). A completion of this study by my colleague, Ms R Pennell, who is continuing this work, will help identify the practical working temperatures that can safely be used for the enzyme production.

## 9.8 CONCLUSIONS

The following conclusions can be drawn from the results of this research work:

1 Anaerobic fermentation of *Leuconostoc mesenteroides* B512(F) has for the first time been shown to produce higher yields of dextransucrase than the yields obtained under aerobic fermentation conditions. Yields of enzyme containing 450 - 500 DSU/$cm^3$ and 300 - 390 DSU/$cm^3$ have been obtained using fed-batch anaerobic and aerobic fermentation conditions respectively. These were obtained by working on a 6 litre scale.

2 Scale-up of the anaerobic process has also been successfully carried out at 80 and 1000 litre scales with enzyme preparations containing activities of up to 450 DSU/$cm^3$ being obtained. Some of the enzyme that was produced at the 1000 litre scale has also for the first time been used for the industrial production of dextran by the enzymic route at Fisons Pharamceuticals Plc., Holmes Chapel. Up to 99% conversion of sucrose to dextran was achieved. The dextran was analysed and found to be identical to that produced by the traditional whole cell fermentation process.

3 Studies have been carried out on different types of yeast extracts used in the fermentation medium and shown to significantly affect the fermentation results. Of those studied, the Gistex standard type of yeast extract was found to be the best for the production of dextransucrase. Variability of batches of the Gistex has also been found to affect the cell growth and, to a greater extent, the enzyme production.

4 Effects of stirrer speed were studied using the 6 litre scale working volume. Increased agitation from 100 rpm to 600 rpm, i.e. an increase in the agitator power input per unit volume of the medium by a factor of up to 216, did not reduce the yield of enzyme obtained under anaerobic conditions to below 400 DSU/$cm^3$. It can therefore be concluded that under these conditions, the stirrer speeds used did not affect the enzyme production.

5 The silicone concentrate antifoam used in the fermentation medium affected the amount of enzyme produced. Studies using anaerobic conditions showed that when the antifoam was present, increased agitation led to much lower yields than the corresponding enzyme activities obtained when the antifoam was not present. Since the increased agitation led to better mixing of the antifoam in the medium, it can be concluded that the reduced enzyme activity was as a result of an interference by the antifoam. However, this antifoam was found to be quite effective in keeping the foam to a minimum. A volume of 2 $cm^3$ of the antifoam was found to be sufficient to control foaming in the 6 litre fermentations.

6 The final cell concentration and enzyme activity have been found to be independent of the length of the inoculum incubation period and the size of the inoculum used. Only the lag period has been affected. Low levels of inoculum gave longer lag phases. Incubation times of less than 8 hours did not produce a good inoculum and this also led to long lag periods. Incubation times of between 10 and 15 hours have been found to be suitable for each stage of the inoculum preparation.

7 None of the fermentations produced $OD_{590}$ values in excess of 1.3. An extension of the stationary phase only led to more sucrose consumption, but no additional cell growth or enzyme production. In fact, a lengthening of the stationary phase was found to lead to a reduced final enzyme activity, especially in the aerobic processes.

## 9.9 RECOMMENDATIONS FOR FUTURE WORK

1 Continuation of this research topic by carrying out studies on continuous fermentation techniques.

2 Optimisation of the anaerobic batch process by:

(a) Carrying out a detailed investigation of the nutrient requirements of

LM. Emphasis should be on the yeast extract, especially the components of the yeast extract that are necessary for the production of high yields of dextransucrase. Two possible methods of carrying out this study have been mentioned in this report. These are the Brown and McAvoy[67] technique concerning the use of folic acid and the use of peptones to supplement the yeast extract. Such a study should include a study on the quantities of each of the components that would be required. Also investigations into the optimum amounts of phosphate and sucrose added could lead to a better formulated fermentation medium.

(b) Studying the use of other antifoams.

(c) Completing the study on the practical ranges of pH and temperature that could be used during industrial processes.

(d) During the course of the anaerobic processes described in this report, oxygen was found to be present in the air samples collected from the fermenter. The fermentations were therefore not absolutely oxygen free. It would be useful to find out the effects of purging all the oxygen either with nitrogen or carbon dioxide before carrying out the fermentation.

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## NOMENCLATURE

| | | |
|---|---|---|
| ATP | Adenosine triphosphate | |
| $C_S$, $S_R$ | Substrate concentration in reservoir | g/l |
| D | Dilution rate | $h^{-1}$ |
| $D_i$ | Impeller diameter | m |
| DNA | Deoxyrhibonucleic acid | |
| DO | Dissolved oxygen concentration | % |
| DSU | Dextransucrase unit | |
| E | Enzyme concentration | units/$cm^3$ |
| EDTA | Ethylenediamine tetraacetic acid | |
| ES | Enzyme-substrate complex | |
| F | Feed flow rate | l/h |
| FAD | Flavin adenin dinucleotide | |
| FMN | Flavin mononucleotide | |
| Km | Michaelis-Menten constant | |
| $K_S$ | Substrate saturation constant | |
| N | Agitator speed | revs/s |
| NAD | Nicotinamide adenine dinucleotide | |
| NADP | Nicotinamide adenine dinucleotide phosphate | |
| $OD_{530}$ | Optical density at 530 nm | |
| $OD_{590}$ | Optical density at 590 nm | |
| p | Product concentration | g/l |
| Pm | Maximum productivity | |
| Po | Overall productivity | |
| qi | Metabolic quotient | |
| s | Substrate concentration | g/l |
| t | Time | h |

| | | |
|---|---|---|
| $t_d$ | Doubling time | h |
| V | Volume | l |
| V' | Enzyme reaction rate | |
| $V_{max}$ | Maximum enzyme reaction rate | |
| VVM | Volumetric air flow rate per unit volume | l/h/l |
| x | Cell concentration | g/l |
| $x_o$ | Initial cell concentration | g/l |
| $Y_{a/b}$ | Yield coefficient of component (a) with respect to (b) | |

**Greek symbols**

| | | |
|---|---|---|
| $\alpha$ | Cell death rate | $h^{-1}$ |
| ß | Product destruction rate | $h^{-1}$ |
| $\rho$ | Density | $kg/l^3$ |
| $\mu$ | Cell growth rate | $h^{-1}$ |
| $\mu_{max}$ | Maximum cell growth rate | $h^{-1}$ |

# APPENDICES

# APPENDIX 1A

## COMPUTER SIMULATION PROGRAMS

## SIMULATION PROGRAM FOR FED-BATCH FERMENTATION

```
: Program for simulating fed-batch fermentations
:
: Constants for differential equations
:
  CONSTANT UMAX=0.32, KS=0.11, YPS=0.1, YPX=75
:
: Control parameters for the simulation
  CONSTANT CINT=0.5, T=5, TFIN=20, ALGO=0
:
: Model description
: Initial conditions
  INITIAL
:
  S=10
  P=0
  X=0.16
  FS=0.2
  V=6
  RATE=0
:
: Model equations
  DYNAMIC
:
  V'= FS
  X'= RX
  P'=RP
  S'= 600x FS/V + RS
  RX= UxX
  U = UMAX xS/(KS+S)
  RS = -(RX/YXS + RP/YPS)
  RP = YPxUxX
:
: Control section to determine the end of the
: exponential phase and stop sucrose addition
:
  IF(.NOT.COMP(U-RATE)) FS=0; RATE = U
: Simulation results
:
  OUTPUT T,X,S,P
  PREPARE T,X,S,P,U
```

## SIMULATION PROGRAM FOR BATCH FERMENTATION

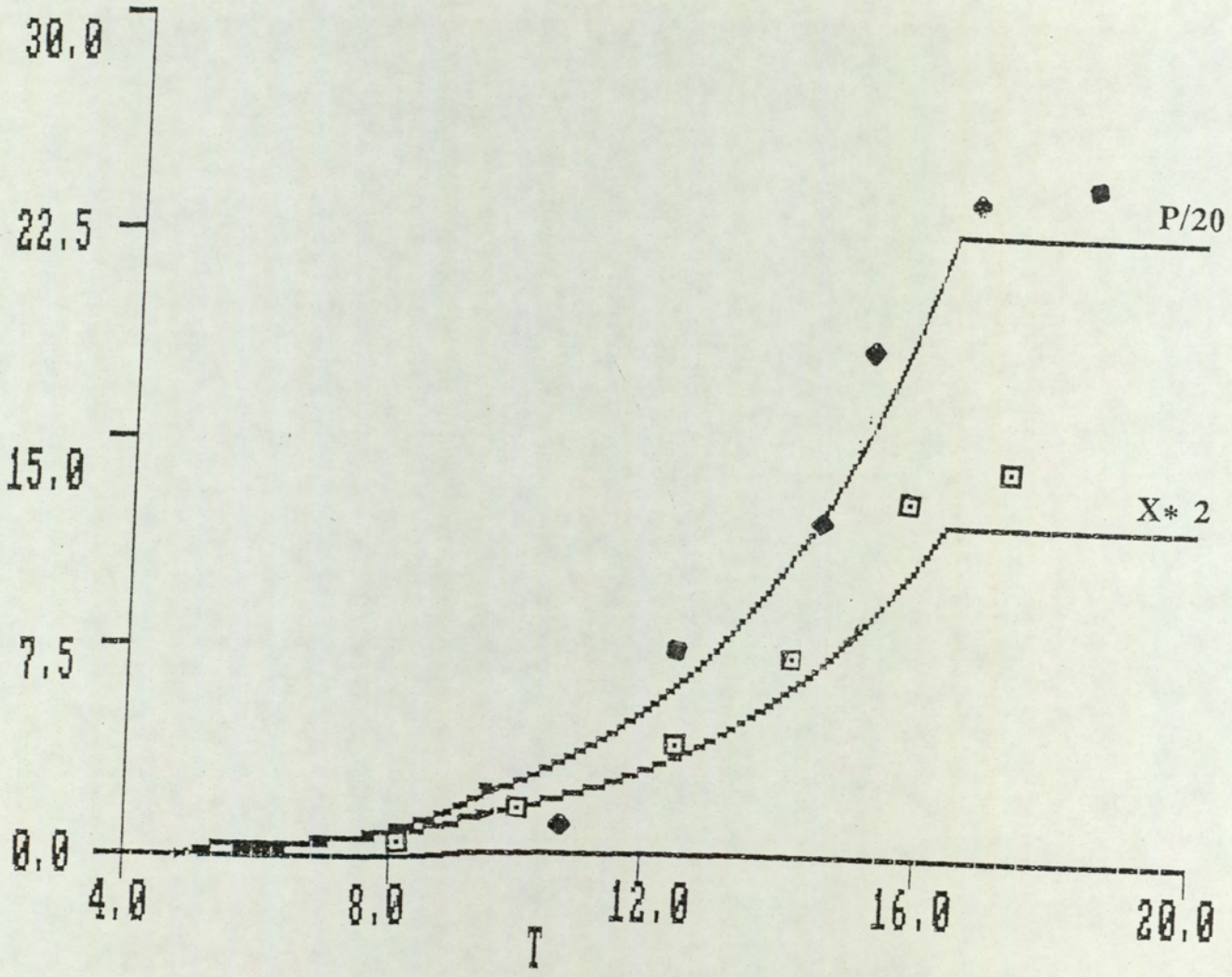
```
: Program for simulating batch fermentations
:
: Constants for differential equations
:
  CONSTANT UMAX=0.32, KS=0.11, YPS=0.1, YPX=75
:
: Control parameters for the simulation
  CONSTANT CINT=0.5, T=5, TFIN=20, ALGO=0
:
: Model description
: Initial conditions
  INITIAL
:
  S=10
  P=0
  X=0.16
:
: Model equations
  DYNAMIC
:
  X'= RX
  P'= RP
  S'= RS
  RX= UxX
:
  U = UMAX xS/(KS+S)
  RS = -(RX/YXS + RP/YPS)
  RP = YPxUxX
:
: Simulation results
:
  OUTPUT T,X,S,P
  PREPARE T,X,S,P,U
```

## LIST OF SYMBOLS USED IN THE SIMULATION

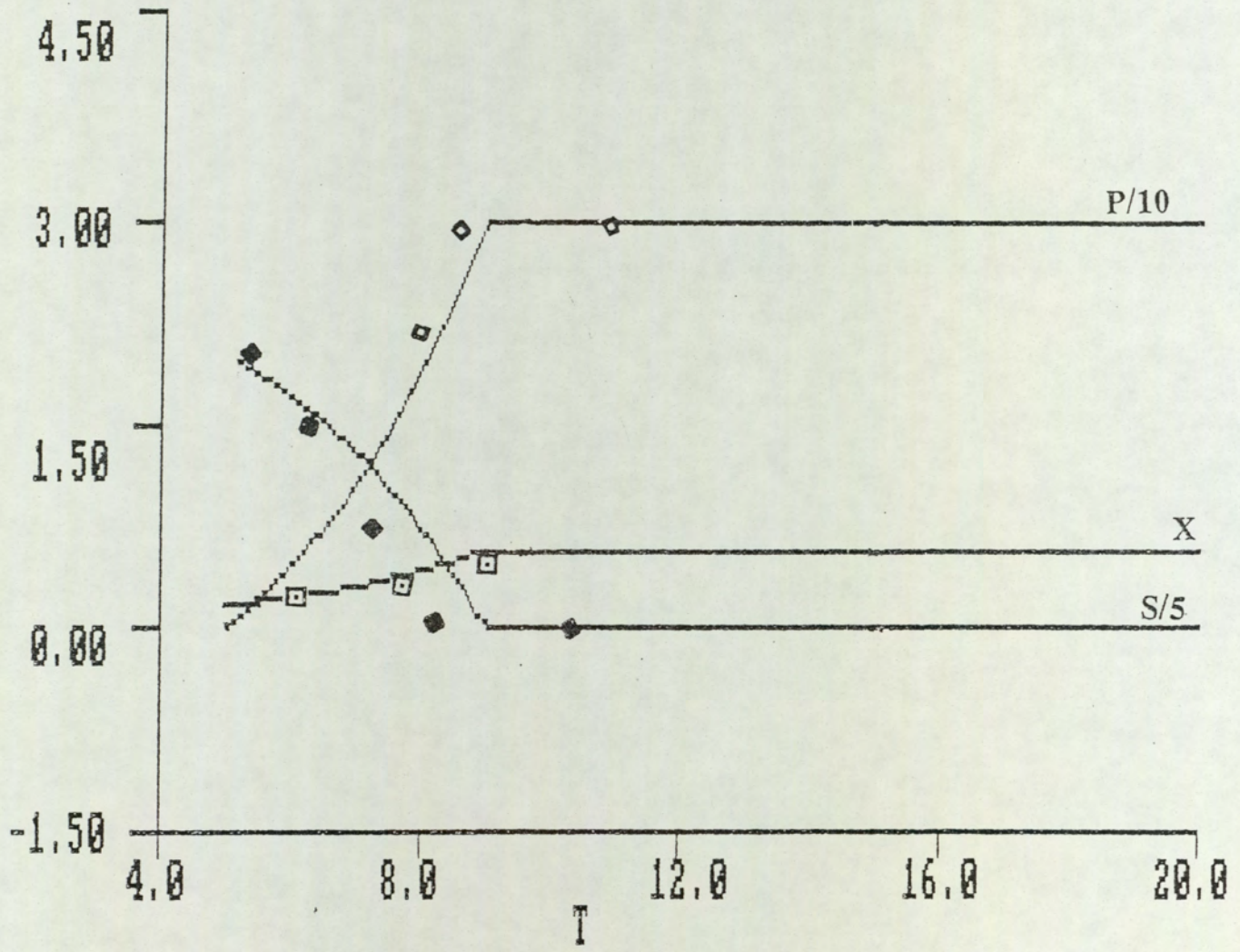
| | | |
|---|---|---|
| ALGO | Integrating routine to be used for simulation | |
| CINT | Communication interval for output of results | |
| FS | Sucrose input rate | g/l/h |
| KS | Enzyme saturation constant, i.e. Ks | |
| P | Product concentration | g/l |
| P',RP | Product formation rate | g/l/h |
| S | Substrate concentration | g/l |
| S',RS | Substrate utilization rate | g/l/h |
| T | Time | h |
| TFIN | Simulation end time | h |
| U | Growth rate ($\mu$) | $h^{-1}$ |
| UMAX | Maximum growth rate ($\mu_{max}$) | $h^{-1}$ |
| V | Volume | l |
| X | Cell concentration | g/l |
| X',RX | Rate of increase of cell concentration | g/l/h |
| YPS | Yield coefficient of enzyme with respect to substrate | |
| YPX | Yield coefficient of enzyme with respect to cells | |
| YXS | Yield coefficient of cells with respect to substrate | |

# APPENDIX 1B

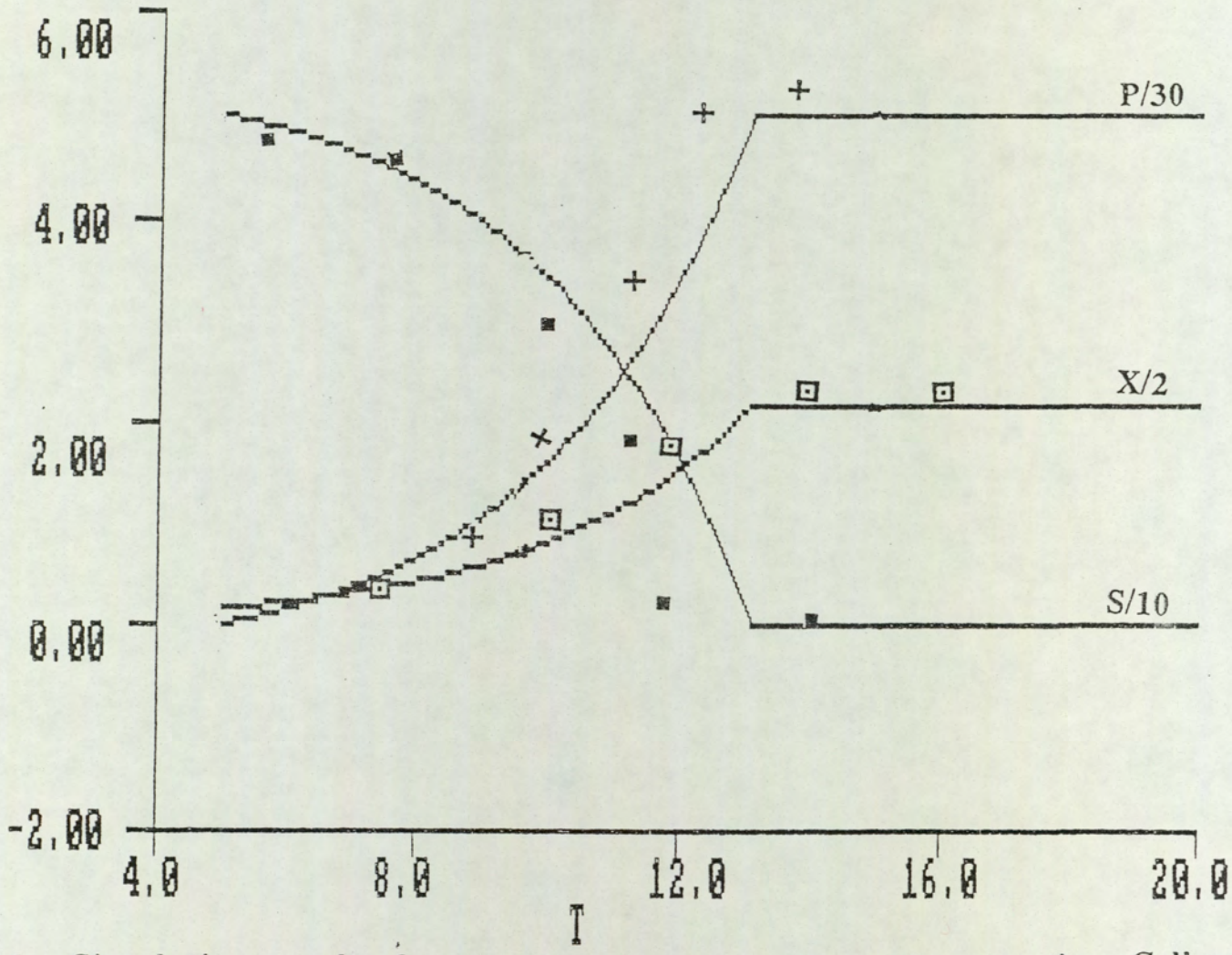
# SIMULATION RESULTS




A1: Simulation results for a fed-batch fermentation using a constant sucrose input rate of 20g/l/h. Results of experiment 26-02-87 showing cell growth (⊡), and enzyme production ( ◆ ) included for comparison




A2: Simulation results for a 1 % w/v sucrose batch fermentation. Cell growth ( ⊡ ), enzyme production ( ◇ ) and residual sucrose concentration ( ◆ ) for experiment 43-07-87 included for comparison




A3: Simulation results for a 5 % w/v sucrose batch fermentation. Cell growth ( ⊡ ), enzyme production ( + ) and residual sucrose concentration ( ■ ) for experiment 44-07-87 included for comparison