COMBINED BIOREACTION AND SEPARATION IN A SIMULATED COUNTER-CURRENT CHROMATOGRAPHIC BIOREACTOR-SEPARATOR SYSTEM

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SUMMARY

The objective of this work has been to investigate the principle of combined bioreaction and separation in a simulated counter-current chromatographic bioreactor-separator system (SCCR-S). The SCCR-S system consisted of twelve 5.4cm i.d x 75cm long columns packed with calcium charged cross-linked polystyrene resin. Three bioreactions, namely the saccharification of modified starch to maltose and dextrin using the enzyme maltogenase, the hydrolysis of lactose to galactose and glucose in the presence of the enzyme lactase and the biosynthesis of dextran from sucrose using the enzyme dextransucrase.

Combined bioreaction and separation has been successfully carried out in the SCCR-S system for the saccharification of modified starch to maltose and dextrin. The effects of the operating parameters (switch time, eluent flowrate, feed concentration and enzyme activity) on the performance of the SCCR-S system were investigated. By using an eluent of dilute enzyme solution, starch conversions of up to 60% were achieved using lower amounts of enzyme than the theoretical amount required by a conventional bioreactor to produce the same amount of maltose over the same time period. Comparing the SCCR-S system to a continuous annular chromatograph (CRAC) for the saccharification of modified starch showed that the SCCR-S system required only 34.6-47.3% of the amount of enzyme required by the CRAC.

The SCCR-S system was operated in the batch and continuous modes as a bioreactor-separator for the hydrolysis of lactose to galactose and glucose. By operating the system in the continuous mode, the operating parameters were further investigated. During these experiments the eluent was deionised water and the enzyme was introduced into the system through the same port as the feed. The galactose produced was retarded and moved with the stationary phase to be purge as the galactose rich product (GalRP) while the glucose moved with the mobile phase and was collected as the glucose rich product (GRP). By operating at up to 30%w/v lactose feed concentrations, complete conversions were achieved using only 48% of the theoretical amount of enzyme required by a conventional bioreactor to hydrolyse the same amount of glucose over the same time period. The main operating parameters affecting the performance of the SCCR-S system operating in the batch mode were investigated and the results compared to those of the continuous operation of the SCCR-S system.

During the biosynthesis of dextran in the SCCR-S system, a method of on-line regeneration of the resin was required to operate the system continuously. Complete conversion was achieved at sucrose feed concentrations of 5%w/v with fructose rich products (FRP) of up to 100% obtained. The dextran rich products were contaminated by small amounts of glucose and levan formed during the bioreaction.

Mathematical modelling and computer simulation of the SCCR-S system operating in the continuous mode for the hydrolysis of lactose has been carried out.

KEY WORDS Bioreactor-separator, Chromatographic, Saccharification, Lactose hydrolysis, Dextran biosynthesis.

DEDICATED TO MY PARENTS

Mr and Mrs Shieh

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CHAPTER 1

INTRODUCTION

Chromatography offers great potential as a separation and purification technique. This technique is already used extensively in the analytical field and has large scale applications in the pharmaceutical, cosmetic and food industries. Although chromatography has been used for hundreds of years for separation, it was not until the early 1960s that the application of chromatographic systems as chemical reactor-separators was first investigated simultaneously by Magee[1] and Dinwiddie[2] in the USA and Ronginski *et al.*[3] in the former USSR. Its potential however as a combined bioreactor separator has yet to be exploited.

The most important components of bioprocess activities are the bioreaction and downstream processing steps[4]. Both of these aspects are technically challenging and are a high cost activity due to the nature of bioprocess streams which are often complex and sensitive to operating parameters such as temperature, pH, the type of solvent used and shear force[5]. Despite the major developments in downstream processing, the downstream process still accounts for 50-80% of the total production cost[6]. The key to reducing the production cost is to limit the number of process steps in the overall process and therefore the demand to improve the performance of bioprocessing within the industry is increasing. One approach to improve performance is to intensify the process by integrating the reaction and product recovery into a single step. The main feature of this approach is for the bioreaction and product purification to take place in the same unit. This feature is of great advantage in bioreaction systems where substrate or product inhibition to the reaction is present and where the bioreaction can be reversible. In the case of product inhibition and reversible bioreaction, separating the product from the reaction zone as soon as the products are formed enhances the rate of reaction and the yield.

The objective of this research work was to investigate the principle of combined bioreaction and separation in a preparative scale simulated counter-current chromatographic bioreactor-

separator system (SCCR-S) and also to mathematically model and simulate the SCCR-S system. The SCCR-S system consisted of twelve columns each of 5.4cm i.d and 75cm long and packed with calcium charged cross-linked polystyrene resin. Three bioreactions were chosen for the study, namely, the saccharification of modified starch to maltose using the enzyme maltogenase which is a heat stable, high maltose producing, exo-acting alpha-amylase; the hydrolysis of lactose in the presence of the enzyme lactase from *Aspergillus oryzae* and the biosynthesis of dextran from sucrose using the enzyme dextransucrase. The dextransucrase enzyme required for the biosynthesis of dextran experiments was not commercially available and therefore it was necessary to carry out large scale fermentations and purification to produce the amounts of dextransucrase required.

Starch hydrolysis is one of the major enzyme catalysed processes in industrial operations today especially in the production of sugars such as maltose, glucose and fructose. The production of high purity maltose is very expensive using a conventional batch bioreactor since the maximum conversion that can be achieved is about 80% for reaction times in excess of 70 hours. This bioreaction was used to study the behaviour and effects of the operating parameters on the performance of the SCCR-S system for the continuous saccharification of modified starch and simultaneously separating the unconverted starch from the maltose to produce high purity maltose. High purity maltose may be consumed at high concentration levels without elevating the blood glucose level and is therefore suitable for diabetics.

Lactose is the principal carbohydrate in milk and whey occuring at a concentration of about 5%w/v. This diasaccharide can be hydrolysed into glucose and galactose by means of enzymes known as lactases (ß-galactosidase). These sugars exhibit better digestibility, higher solubility and greater sweetening power than lactose. This bioreaction was used to study and compare the batch and continuous modes of operation of the SCCR-S system. Galactose which is a product of the bioreaction strongly inhibits the activity of the enzyme lactase. By separating the galactose from the reaction zone, its inhibition effect on the

enzyme can be reduced thus using less amount of enzyme than required by a conventional batch process.

Dextran is mainly used in the pharmaceutical industry as a blood volume expander, blood flow improver and in the manufacture of iron dextran used in the treatment of anaemia. Dextransucrase enzyme from the bacterium *Leuconostoc mesenteroides* NRRL B512(F) is the biocatalyst of choice for the synthesis of clinical grade dextran. Robyt and co-workers[7] have shown that the fructose molecules produced during the reaction interfere with dextran chain growth by acting as an acceptor which causes premature release of the growing dextran chains from the dextransucrase enzyme molecules. This phenomena is most pronounced at high sucrose concentrations. Alsop[8] reported that at sucrose concentrations of 20%w/v, nearly half of all the dextran produced had a molecular weight below 5000 daltons. The objective of any dextran process is to maximise the yield of high molecular weight dextran (>150,000 daltons) since low molecular weight dextran has few commercial uses. Although high sucrose concentrations produce high amounts of low molecular weight dextran, the high sucrose throughputs are important to reduce equipment and operating costs.

Ideally, a production method that minimises the effect of the acceptor byproduct, fructose, at high sucrose concentrations is required. This can be achieved by simultaneously separating the fructose from the reaction medium. Barker and co-workers[9,10,11] achieved this bioreaction and separation by using batch chromatographic bioreactor-separator systems of various sizes. They found that up to 80% more high molecular weight dextran can be obtained by separating the fructose from the reaction medium. Setford[12] studied the integration of bioreaction and separation in a rate-zonal centrifuge and found that up to 100% more clinical dextran with molecular weights between 12,000 and 98,000 daltons was obtained at 20%w/v sucrose concentration than for the conventional process. This was due to the removal of acceptor fructose molecules from the sedimenting reaction zone by the action of centrifugal forces. This research attempted to apply the same principle using continuous preparative chromatography.

The research work is presented in this thesis as follows:

A survey of the literature on chromatographic reactors with emphasis on chromatographic bioreactor-separators as opposed to chemical reactor-separators is presented in Chapter 2.

In Chapter 3, a literature survey on general enzyme biotechnology together with general information relating to the carbohydrates used in this work is presented.

Chapter 4 describes the analytical equipments and techniques used in this research, the enzyme assay techniques and the kinetics of the enzymes used.

Chapter 5 concentrates on the SCCR-S system: the operating principle, the characterisation and the operating procedure of the SCCR-S system.

The results obtained from studying the effects of the operating parameters that affect the performance of the SCCR-S system under simultaneous bioreaction and separation for the saccharification of modified starch to maltose are presented in Chapter 6. A comparison of the performance of the SCCR-S and a continuous annular chromatographic reactor-separator is also presented.

Chapter 7 describes the bioreaction and separation experiments involving the hydrolysis of lactose when the SCCR-S system was operated in both the batch and continuous modes. The results obtained are used to compare the performance of the SCCR-S operating in the batch and continuous modes of operation.

Chapter 8 describes the production and purification of the enzyme dextransucrase and includes the results of preliminary work on the biosynthesis of dextran in the SCCR-S system.

The mathematical modelling and computer simulation of the SCCR-S employed as a combined bioreactor-separator for the hydrolysis of lactose is outlined in Chapter 9.

Finally Chapter 10, contains the main conclusions of the research work and lists recommendations for further investigations.

CHAPTER 2

CHROMATOGRAPHY AND CHROMATOGRAPHIC REACTION AND SEPARATION

2.1 INTRODUCTION

Chromatography is described as a separation or refining process which is effected by the differential migration velocities of components distributed between mobile and stationary phases. The mobile phase could be gaseous or liquid while the stationary phase could be solid or liquid retained on a solid support. The choice of the stationary phase depends on the different affinities of the components for it. The separation of the components from a mixture is achieved by utilising one or more of the following separation mechanisms: adsorption, size exclusion, ion exchange or partition chromatography. The classification of chromatography into the types of phase employed and mode of operation have been well documented[13,14]. The elution techniques of discharging solutes from a chromatographic column can be classified into frontal analysis, displacement and solution development. This classification is also extensively documented[15,16].

The major contribution to the development of gas chromatography was by Martin and Synge[17] whose work was recognised by the award of the Noble Prize. Gidding and other workers[18] later made major contributions to the understanding of chromatographic operation through their theoretical contributions.

A chromatographic reactor-separator can be defined as a column or system in which one or more components are converted partially or totally to different chemical (biochemical) species and the products formed separated simultaneously by the chromatographic principle. In such systems, the presence of a chemical or biochemical catalyst is required.

Most of the previous work in the field of chromatographic reaction-separation has been limited to gaseous reaction applications. Most of the studies have been mainly concerned

with the kinetics and mechanism of chemical reactions for analytical purposes[19,20] and improving the equilibrium conversion in reversible chemical reactions[20-24].

In this Chapter, the principles, terminology, theories of chromatography and recent developments in the field of chromatographic reaction and separation are reviewed.

2.2 PRINCIPLES OF CHROMATOGRAPHY

Separation in chromatography is due to the different migration velocities of the components through chromatographic column resulting from the relative distribution of each component between the mobile and stationary phases. This can be expressed for component (i) by the distribution coefficient Kdi, defined as:

$$Kdi = \frac{\text{Concentration of component i in the stationary phase}}{\text{Concentration of component i in the mobile phase}}$$

The mechanism on which the principle of chromatography is based is that which determines the regularities of the movement and spreading of the chromatographic zone. This mechanism includes convective transportation, diffusion and sorption equilibrium. Although the chromatographic process inseparably involves the above processes, it is the distribution coefficients of the components that determine the possibility of separation in the column. The formation of a velocity profile in a column is due to the geometrical irregularities of the chromatographic bed and the hydrodynamic properties of the fluids. Therefore, the efficiency of chromatographic separations depends on the distribution coefficient differences, the uniformity of the bed and mobile phase, the speed of establishing a sorption equilibrium and on the rate of longitudinal diffusion of the solute in both phases.

2.3 CHROMATOGRAPHIC THEORY

The separation of components in a mixture injected into a chromatographic column results from the relative affinities of the components for the column packing. From the chromatogram, generally regarded as the solute concentration against time profile (Figure 2.1) measured by a detector from the introduction of the sample to its emergence from the

2.1) measured by a detector from the introduction of the sample to its emergence from the outlet of the column, important parameters such as retention time (T_{Ri}), retention volume (V_{Ri}), distribution coefficients (Kd_i) and the resolution can be calculated.

The retention time is defined as the average time a molecule takes to travel the length of the column while the retention volume is defined as the mobile phase volume required to completely elute a given component. The latter is the product of the retention time and the mobile phase flowrate. The resolution measures the degree of separation and can be calculated from the equation.

$$Rs = \left(\frac{2(T_{R2} - T_{R1})}{W1 + W2} \right) \qquad 2.1$$

Where R_S = resolution

W1, W2 = peak width at the base in units of time (Figure 2.1)

Equation 2.1 was transformed by Purnell[25] to incorporate the fundamental parameters as shown by Equation 2.2.

$$Rs = \left(\frac{1}{4}\right)\left(\frac{\alpha - 1}{\alpha}\right)\left(\frac{K_2}{1 + K_2}\right)(N^{1/2}) \qquad 2.2$$

Where α = Separation factor

N = Number of theoretical plates in the column

K_2= Capacity factor of the most retarded component = $Kd_2\left(\frac{Vs}{Vo}\right)$

V_o = Void volume and V_S = stationary phase volume.

The fundamental retention Equation which relates the retention volume (V_{Ri}) and the distribution coefficient Kd_i is given by:

$$V_{Ri} = V_o + Kd_i V_S \qquad 2.3$$

2.3.1 Theory of Band Broadening

A narrow band feed injected into a chromatographic column is subjected to non-uniformities of flow, diffusion spreading and variations in the rate of solute redistribution between the two phases resulting in broadening. This should be kept to a minimum since it has direct effect on the separation.

Giddings[18] reviewed various concepts and developed mathematical models to describe band broadening. The most popular of these are the "theoretical plate concept" and the "rate theory".

The concept of the theoretical plate was originally introduced into distillation theory in an attempt to find a quantitative method of expressing the efficiency of a distillation column. Understandably, Martin and Synge[17] introduced a similar concept to chromatographic columns. They considered a chromatographic column to consist of a number of layers of packing each equivalent to a theoretical plate (HETP). The mathematical model was simplified by making assumptions such as, complete equilibrium between the phases, constant distribution coefficient (Kd) through the column, negligible diffusion and mobile phase regarded as discontinuous.

Gluekauf[26] converted the discontinuous model by Martin and Synge[17] into a continuous one by reducing the plate volume to an infinitesimaly small value to develop the most commonly used Equation relating the retention time(T_{Ri}) and variance to an "apparent number" of theoretical plates (N*) as shown by Equation 2.4.

$$N^* = 8\left(\frac{T_{Ri}}{W^{h/e}}\right)^2 \qquad 2.4$$

Where $W^{h/e}$ is the band width measured at height equal to the peak height divided by the base of the natural logarithm, e, Figure 2.2.

The most significant error with the plate model arises from the assumption of plate-wide equilibrium and it also fails to take account of the contributions made by molecular

structure, temperature, molecular distribution, sorption phenomenon and flow patterns towards zone spreading. Nevertheless, this model is useful for characterisation of zone spreading and column efficiency.

Unlike the plate model, the rate theory model includes mass transfer and longitudinal terms. This model was first proposed by Lapidus and Amundson[28] and later modified by Van Deemter *et al.*[27] to include axial diffusion and finite rates of mass transfer contribution to take the form of Equation 2.5.

$$H = A + \frac{B}{V} + Cs'V + Cm'V \qquad 2.5$$

Where, H = plate height

A = eddy diffusion term

B = longitudinal diffusion term

Cm', Cs' = resistance to mass transfer in the mobile and stationary phases respectively.

V = mobile phase velocity

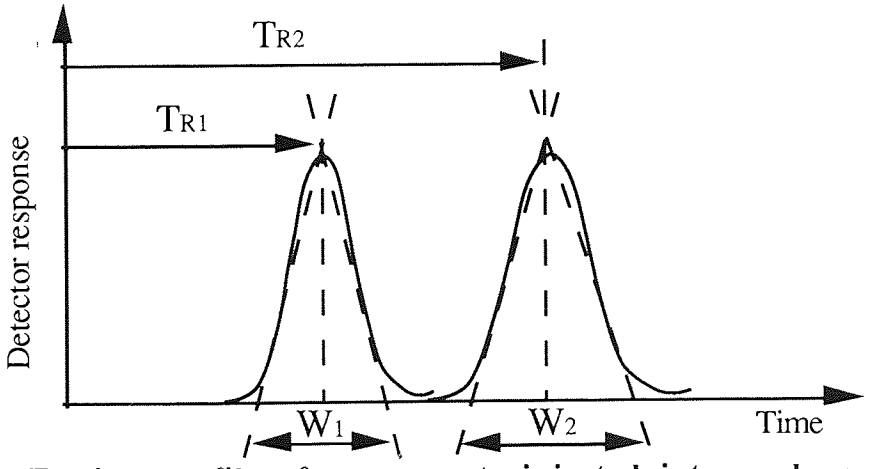


Figure 2.1: Elution profile of components injected into a chromatographic column

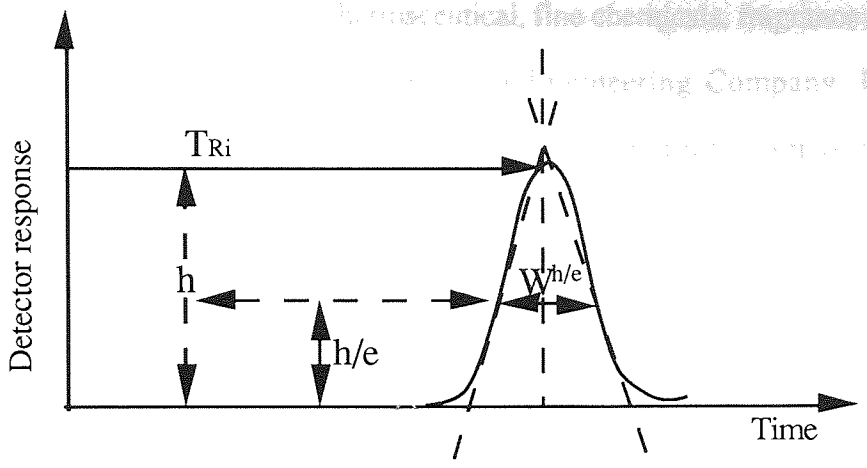


Figure 2.2: Elution profile of a sample

2.4 CONTINUOUS CHROMATOGRAPHY

The large-scale development of chromatographic processes was biased towards the batch mode during the 1960s and 70s. Recently a number of alternative continuous systems have been developed. The term "continuous" refers to any system which employs continuous introduction of the feed stream. The continuous chromatographic systems fully utilise the available mass transfer area, offer constant product quality and usually do not require product recycling. They fall into two broad categories, the cross-current processes where the mobile phase moves almost perpendicularly to the direction of the stationary phase[29] and counter-current processes where the mobile and stationary phases move in opposite directions. The counter-current processes are classified according to the principle they employ to obtain the counter-current movement and the classes are, moving bed systems[30,31], moving column systems[32], moving feed point systems[33] and simulated moving bed systems[34,35].

A comparison of the solute concentration profiles in a co-current and counter-current system for the separation of a binary mixture are shown by Figures 2.3 and 2.4.

2.4.1 Some Industrial Chromatographic Separation Processes

Elf-Aquainte, France[36] markets six preparative gas chromatographs using 10mm to 500mm diameter batch columns. It also markets liquid batch chromatographs of up to

30cm diameter which are used in the pharmaceutical, fine chemicals, fragrance and flavours and biotechnology industries. The Finish Sugar Engineering Company, Finland, has achieved much in terms of large-scale batch chromatographic separations in the carbohydrate field. Seven columns of of 3.6m diameter by 12m high have been used for the desugarization of molasses at a throughput of 60,000 tons/year[37].

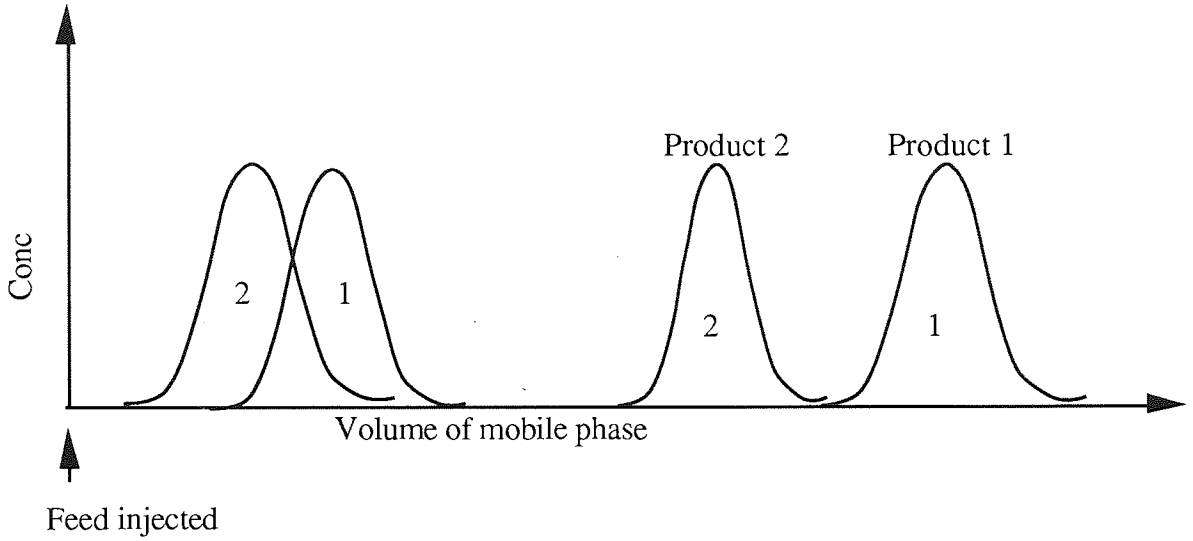


Figure 2.3: Repeated batch co-current operation

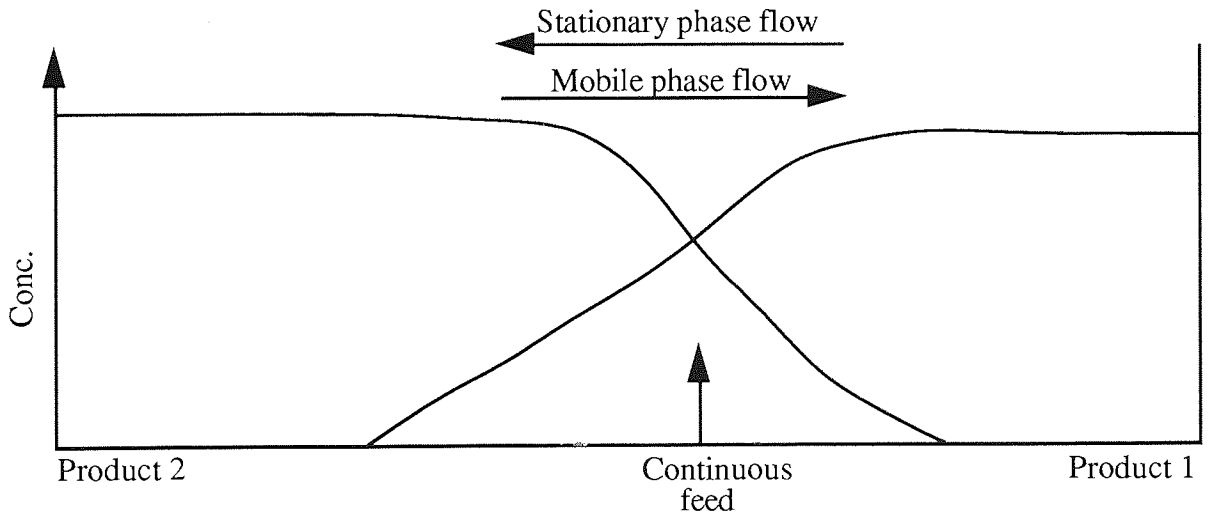


Figure 2.4: Continuous counter-current

Figures 2.3 and 2.4 show the concentration profiles for the separation of a binary mixture in a co-current and counter-current systems.

Universal Oil Products (UOP) has developed simulated moving bed processes and over 40 plants are believed to be operating world-wide using the Sorbex principle[34,35] with a total capacity of over 3.5 million tons/year.

Advance Separation Technologies Inc., Lakeland, USA, recently developed a simulated moving bed system consisting of 30 columns for use in the purification of fermentation products[38]. It is understood that there are plants using this process world wide.

Ishida *et al.*[39] have developed a simulated moving bed which has been used for multicomponent separations.

The Oak Ridge National Chemical division, Tennessee, USA[40] has had the most notable success in applying the cross-current chromatographic principle to the separation of metal ions in solution.

2.5 CHROMATOGRAPHIC REACTION AND SEPARATION

During the past three decades several publications dealing with preparative application of chromatographic reactors have been published. These have been limited to the chemical field mainly for gases[1,2,24]. Due to the advantages of simultaneous reaction and separation in a chromatographic reactor-separator, most of the studies carried out have been targetted on the kinetics and mechanism of chemical reactions for analytical purposes[19,20] and also on improving the equilibrium conversions for some industrial chemical reactions[1,2,23,24]. Some of the industrial chemical reactions studied were the dehydrogenation of cyclohexane into benzene, butylenes into butadien and the Haber process. These were carried out on small batch chromatographic columns and showed that conversions greater than those predicted by thermodynamics could be achieved.

It was not until the early 1970s that researchers started investigating the application of continuous chromatographic systems as chromatographic reactors in the chemical field. Most of the work has been on counter-current moving bed systems[41-45]. Cho *et al.*[46]

studied the acid catalysed hydrolysis of aqueous methyl formate and simultaneously separating the products formic acid and methanol in a rotating annular chromatographic reactor.

Most of the work on continuous chromatographic reactors has also been concerned with the development of mathematical models to predict the reactor behaviour and performance[41-43,45]. The models developed have generally assumed plugflow of both the solids and the counter propagating carrier in an isothermal reactor, with various assumptions about adsorption rates and isotherms, reaction kinetics and dispersion.

Despite all the work done on chromatographic reactor-separators in the chemical field, the potential of these systems in the biochemical field has still to be exploited. Based on the literature, the only applications of chromatographic reaction and separation in the biochemical field are by Hashimoto *et al.*[47,48] who devised a continuous process involving moving bed system for the production of high purity fructose and Barker *et al.*[11,49-50] who studied the applications of batch and continuous chromatographic systems as chromatographic bioreactor-separators. Details of these systems are reviewed in the subsequent Sections.

2.6 CHROMATOGRAPHIC CHEMICAL REACTION AND SEPARATION

2.6.1 Principles of Chromatographic Chemical Reaction and Separation

The principles of chromatographic chemical reaction and separation can best be illustrated by considering two types of reactions. The reaction types are:

(a) $A \rightleftharpoons B + C$

(b) $A + B \rightleftharpoons C + D$

To illustrate these principles it is assumed that the adsorption isotherms are linear, that the adsorption equilibrium is instantaneously established and the affinity of any component, i, for the packing may be expressed by its distribution coefficient, Kdi.

For reaction type (a),

$$A \rightleftharpoons B + C$$

When a pulse of A is injected and $Kd_A<Kd_B<Kd_C$, then as illustrated in Figure 2.5, the products B and C are formed continuously and appear as tails at the rear of the unreacted moving band of component A. The overlapping between the products B and C regenerates some of A thus restricting the extent of conversion and purities. Similarly, when $Kd_C<Kd_B<Kd_A$ (Figure 2.6), the inadequate separation between B and C presents similar problems and indicate that high conversions require very long columns. The ideal situation however is when $Kd_B<Kd_A<Kd_C$ or $Kd_C<Kd_A<Kd_B$ (Figure 2.7). The products will move in opposite directions hence enabling higher conversions to be achieved with high product purities[51].

For reaction type (b) where,

$$A + B \rightleftharpoons C + D,$$

it is only possible when $Kd_C \neq Kd_A = Kd_B \neq Kd_D$. Any other combination of the distribution coefficients will result in poor conversions and product purities. An example of this type of reaction is the esterification of acetic acid to ethyl acetate using ethanol[52,53]. A conversion of 90% was obtained in the chromatographic reactor compared to 67% equilibrium conversion using conventional reactor.

Matsen *et al.*[24] stated that chromatographic reaction and separation can only be advantageous under certain conditions. These conditions are:

(1) The equilibrium constant for the reaction must be small;

(2) Reaction rates should be high enough so that separation of products rather than the rate of reaction limits the extent of reaction;

(3) At least two products must be formed which are chromatographically separated;

(4) Reactants must not be separated in the system. For all the practical purposes this limits one to a single reactant or two where one also serves as the eluent or carrier.

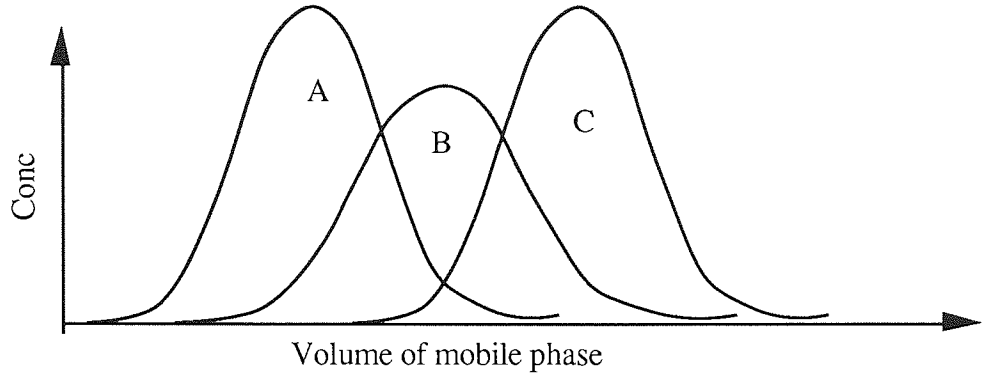


Figure 2.5: When $Kd_A < Kd_B < Kd_C$

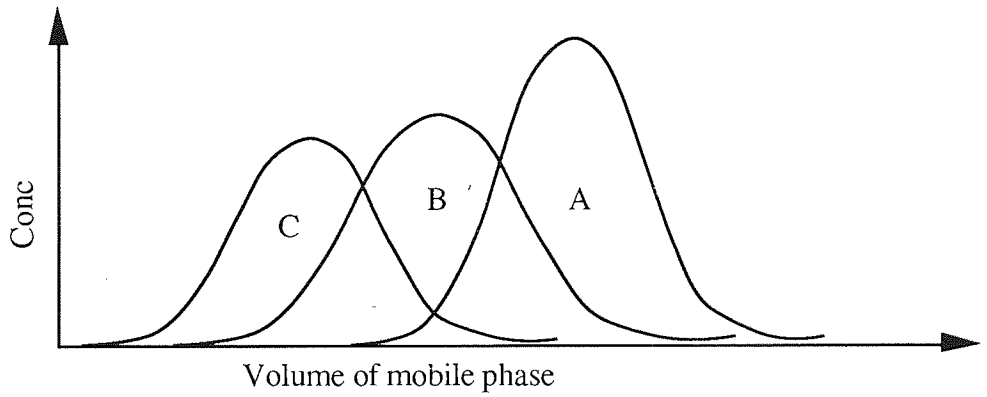


Figure 2.6: When $Kd_C < Kd_B < Kd_A$

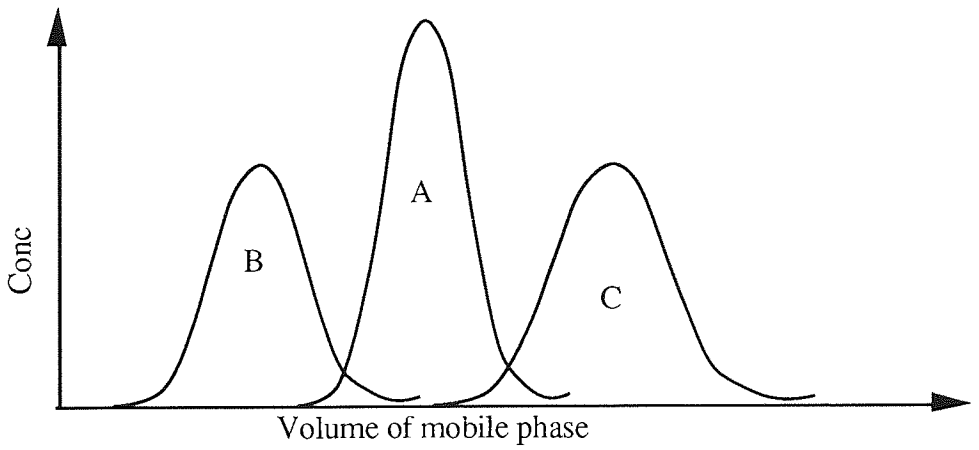


Figure 2.7: When $Kd_B < Kd_A < Kd_C$

Figures 2.5, 2.6, and 2.7 show the concentration profiles when partial conversion is under pulse chromatographic conditions

2.6.2 Factors Affecting Chromatographic Chemical Reactor-Separator Performance

Previous workers[10,24] in the field of chromatographic chemical and separation identified the following factors to affect the performance of chromatographic chemical reactor-separators:

(1) Flow Rate

Increasing the flowrate reduces the residence time in the system and therefore results in decreased conversions and low purity products. Matsen *et al.*[24] found that at low flow rates the conversions were higher and independent of flow rates indicating that adsorption, reaction and desorption were fast under the conditions studied and that the extent of reaction was equilibrium limited.

Mile *et al.*[54] studied the dehydrogenation of cyclohexane to benzene and observed an enhancement of product yield above the equilibrium value at an optimum flowrate. This feature is indicative of the displacement of equilibrium by the chromatographic process.

(2) Pulse Size

Matsen *et al.*[24] studying the dehydrogenation of cyclohexane to benzene found that increasing the pulse size with all other operating conditions constant results in a decrease in the degree of conversion. Roginski *et al.*[55] stated that an absolute yield of product should reach a maximum after which it becomes independent of sample size.

(3) Repetitive Pulses

When repetitive pulse injections are used, the additional variable of pulse frequency needs to be considered. Gore[56] found that the maximum average conversion in a chromatographic reactor was sensitive to the frequency of injection. Matsen *et al.*[24] in their work on the dehydrogenation of cyclohexane found that at very low frequencies, conversions were the same as for single pulses and that conversion dropped off steadily when optimum frequency was passed.

(4) Reactor Length

Increasing the reactor-separator length will increase the degree of conversion and improve the product purities. This is due to the increase of residence time, surface area and in the case of equilibrium reactions or product inhibited reactions, peak overlap is greatly reduced.

2.7 CHROMATOGRAPHIC BIOREACTION AND SEPARATION

Although a lot of research work has been carried out on the application of chromatographic systems as chromatographic reactor-separators in the chemical field, there has been very few publications in the field of bioreaction and separation involving batch or continuous systems. The application of liquid chromatographic systems as chromatographic bioreactor-separators did not occur until in the 1980s. Zafar[9] reported that there are three possible methods of contacting enzymes with substrate in a bioreactor-separator;

(1) The enzyme is mixed with the eluent and the reaction takes place in the mobile phase while the stationary phase acts only as an adsorbent.

(2) The enzyme is mixed with the substrate just before being introduced into the chromatograph. The reaction occurs in the mobile phase as in the first one.

(3) The enzyme is immobilised directly onto the stationary phase or on another solid support. When the enzyme is immobilised on another solid support, this is either mixed with the adsorbent or the immobilised enzyme and the adsorbent are packed in alternate columns.

Method (1) is appropriate when the enzyme and the substrate are separable in the system. Mixing the enzyme in the eluent ensures the presence of the enzyme throughout the column. This ensures intimate contact between the enzyme and the substrate.

Method (2) is suitable when the enzyme and the substrate are not separable in the system. This method has the advantage that it requires less enzyme than method (1).

The different types of chromatographic bioreactor-separator systems reported in the literature will be reviewed in detail in this section.

The principles of chromatographic chemical reaction and separation and the factors affecting this type of reactor-separator have been shown to also apply to bioreactor-separators by Zafar[9] studying biosynthesis of dextran from sucrose using the enzyme dextransucrase in batch chromatographic columns.

2.7.1 Batch Chromatographic Bioreactor-Separator System

Barker *et al.*[9-11] studied the biosynthesis of the macromolecule dextran and the variable by-product, fructose from sucrose in the presence of the enzyme dextransucrase. They used batch chromatographic columns varying in size from 0.97 to 5.4 cm diameter and 30 to 200 cm in length. The arrangement of their equipment was as shown by Figure 2.8.

During the biosynthesis, the fructose was retarded immediately as it was produced by complexing with the calcium ions on the resin while the dextran formed was size excluded and migrated with the mobile phase. By employing the combined bioreaction and separation approach, they found that at 20% w/v sucrose concentrations over 77% of the dextran produced had molecular weights in excess of 157,000 daltons while only 43.7% of the dextran produced using conventional fermentation techniques at similar conditions was over 157,000 daltons. The improvements in the yield was due to the instantaneous separation of the acceptor by-product, fructose, which terminates the dextran chain length growth from the reaction medium. Modelling and simulation of this system was successfully carried out.

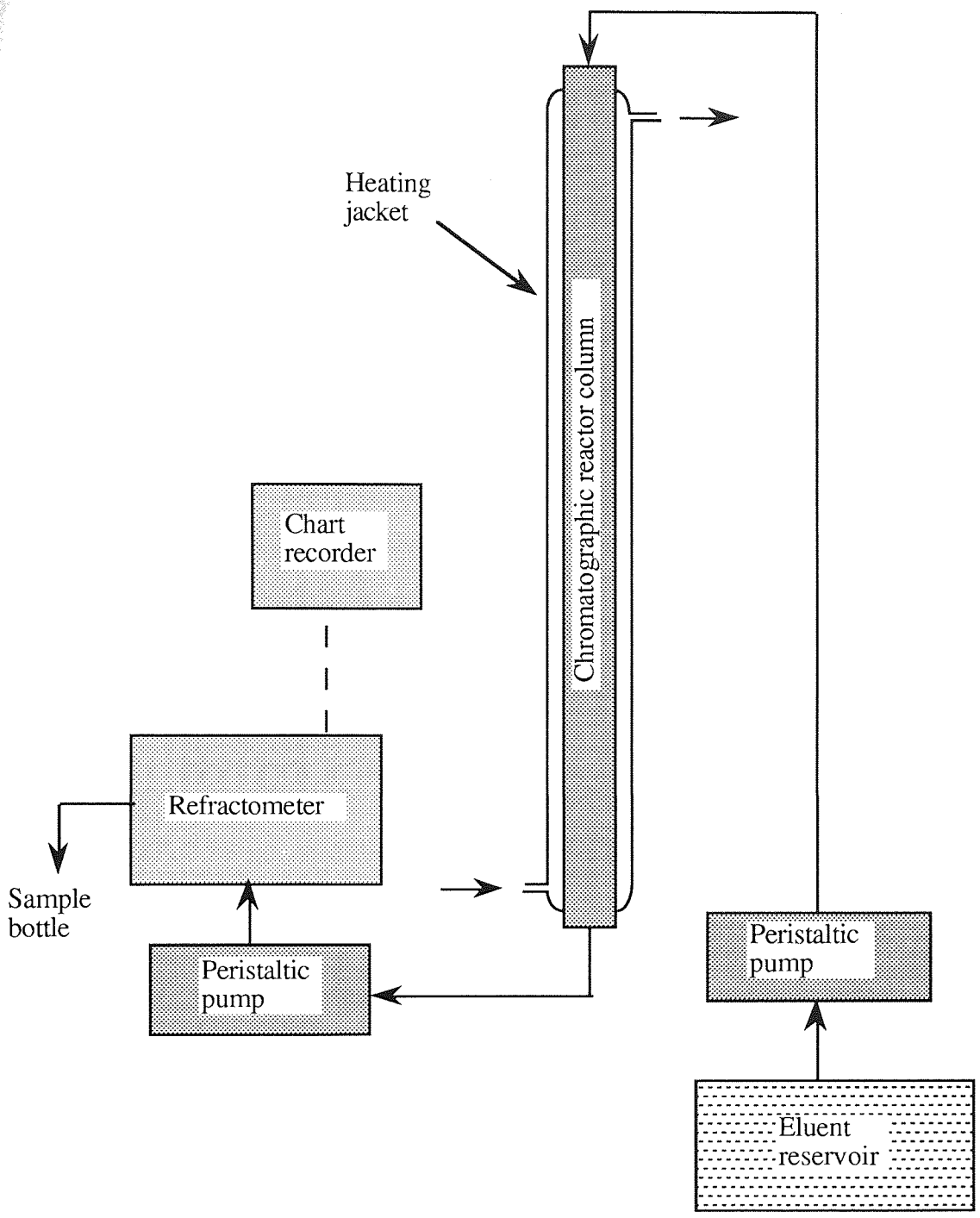


Figure 2.8: Arrangement of the equipment used by Zafar et al.[9-10]

The non-linear differential equations obtained from the mass balance for sucrose, fructose and dextran were solved using finite difference analysis. The mass balance equation for sucrose was as shown in Equation 2.6.

$$(1 + (\frac{1 - e}{e})Kds)\frac{\delta S}{\delta t} + U\frac{\delta S}{\delta Z} + \frac{SVmax}{S + Km} = 0 \qquad 2.6$$

Where,

Kds = Distribution coefficient of sucrose
S = Sucrose concentration
Z = Distance
t = Time
Km = Michealis-Menten Constant
Vmax = Maximum reaction Velocity
U = Mobile phase Velocity
e = Voidage

Barker *et al.*[57] are currently studying the optimization of batch chromatographic bioreactor-separators for various carbohydrate systems.

2.7.2 Continuous Chromatographic Bioreactor-Separator Systems

The known applications of these systems in the field of biotechnology are those of Hashimoto *et al.*[47-48] and Barker *et al.*[49-50].

2.7.2.1 The Hashimoto *et al.* Systems

Hashimoto *et al.* investigated three types of bioreaction in different modes of continuous chromatographic bioreactor-separator operation.

(1) The isomerisation of fructose using an immobilised glucose isomerase in a simulated moving bed counter-current system (Figure 2.9).

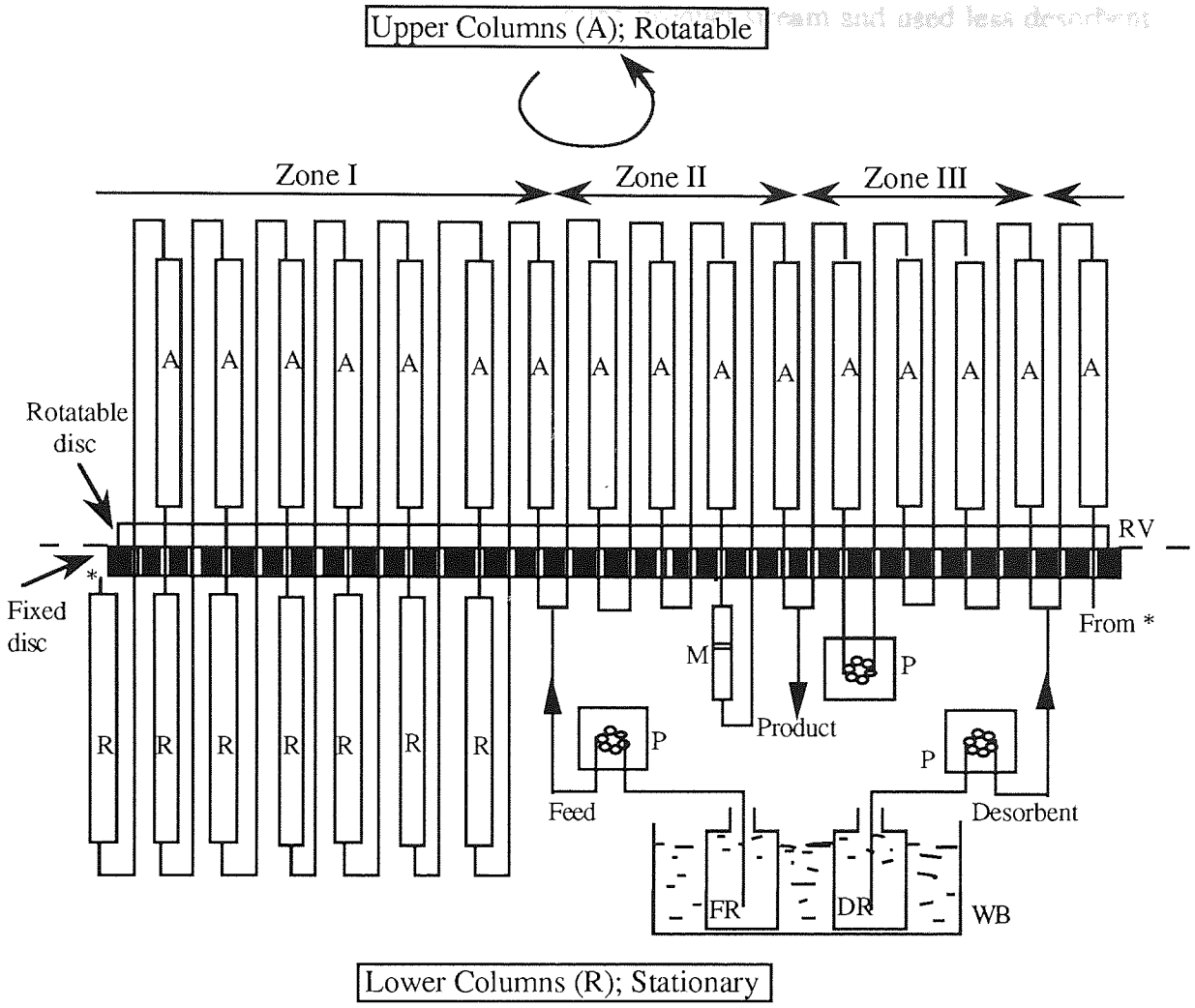
(2) The repeated use of free coenzyme adenosine 5-triphosphate (ATP) by immobilised hexokinase (HK) and immobilised pyruvate kinase (PK) in a simulated moving bed continuous chromatographic reactor (Figure 2.10)

(3) The continuous conversion of maltose to glucose by repeated used of the enzyme glucoamylase in a gel chromatographic column (Figure 2.11).

Hashimoto *et al.*[47] used a continuous counter-current moving bed system for the production of high fructose syrup (45-65% fructose), using alternate columns packed with a Zeolite packing in the Ca^{2+} form and columns containing immobilised glucose isomerase.

Figure 2.9 illustrates a schematic diagram of the apparatus used by Hashimoto *et al.* The system consisted of a total of 16 adsorption columns packed with Y zeolite (Ca^{2+} form) divided into three zones, I, II and III with 8, 4 and 4 columns respectively in each zone. The adsorption columns were 1.38 cm diameter and 10.2 cm long. There were 7 reactors packed with the immobilised glucose isomerase located in zone I and each reactor was 1.38 cm in diameter and 18.0 or 10.2 cm long.

The adsorption columns and the reactor columns were alternately connected to form a closed loop as shown by Figure 2.9. The continuous counter-current contact between the liquid and the adsorbent was simulated using a multiport rotary valve. The rotary valve consisted of two stainless steel discs, each bearing 32 holes. The adsorption columns were connected to the upper disc and both rotated counterclockwise at regular intervals (2 or 3 mins) and the reactor columns were kept stationary connected to the stationary lower disc (Figure 2.9)



KEY

RV - Rotary valve
DR - Desorbent reservoir
FR - Feed reservoir
WB - Water bath

A - Adsorption columns
R - Reactors
P - Constant feed pump
M - Flowmeter

Figure 2.9: Schematic illustration of the Hashimoto *et al.*[47] continuous moving bed system

An equimolar reaction mixture of glucose and fructose was fed into the system and passed through zone I. Fructose in the mixture was adsorbed with small quantity of glucose. The glucose content of the solution leaving the adsorption columns becomes higher than the equilibrium level and was introduced into a reactor.

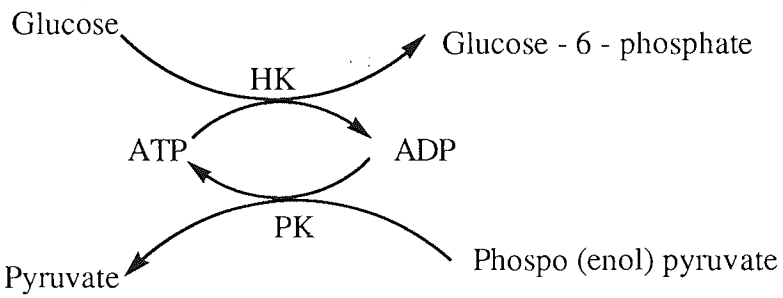
By introducing the mixture through the adsorption columns and reactors alternately in zone I, most of the glucose in the mixture was converted to fructose. The fructose adsorbed in zone I was transferred to zones II and III by movement of the adsorption columns. The fructose adsorbed was desorbed in zone III as the product. This process was capable of

producing up to 45-65% fructose purities in the product stream and used less desorbent than the equivalent fixed bed batch process.

Although this system as a whole functioned as a chromatographic bioreactor-separator, the actual reaction and separation steps were decoupled due to the use of separate reactor and adsorption columns.

Hashimoto *et al.*[47] developed two mathematical models, intermittent moving bed and continuous moving bed models for calculating concentration profiles of glucose and fructose in these type of systems. The validity of these models was experimentally confirmed.

The second biochemical reaction investigated by Hashimoto *et al.*[48] is shown in the following reaction scheme;



Where HK = Hexokinase , PK = Pyruvate kinase

The enzymes HK and PK were immobilised onto Amberlite XAD-2 through physical adsorption. The resin adsorbed ATP more strongly than the substrates and the products. The column configuration and the principle of operation of the simulated moving bed system used for this work is shown in Figure 2.10. The system consisted of six columns connected to form a loop with one feedstream and two product withdrawal points. Each column was 5cm long and 1.5cm in diameter. The operation of the system was as shown in steps 1, 2 and 3 of Figure 2.10.

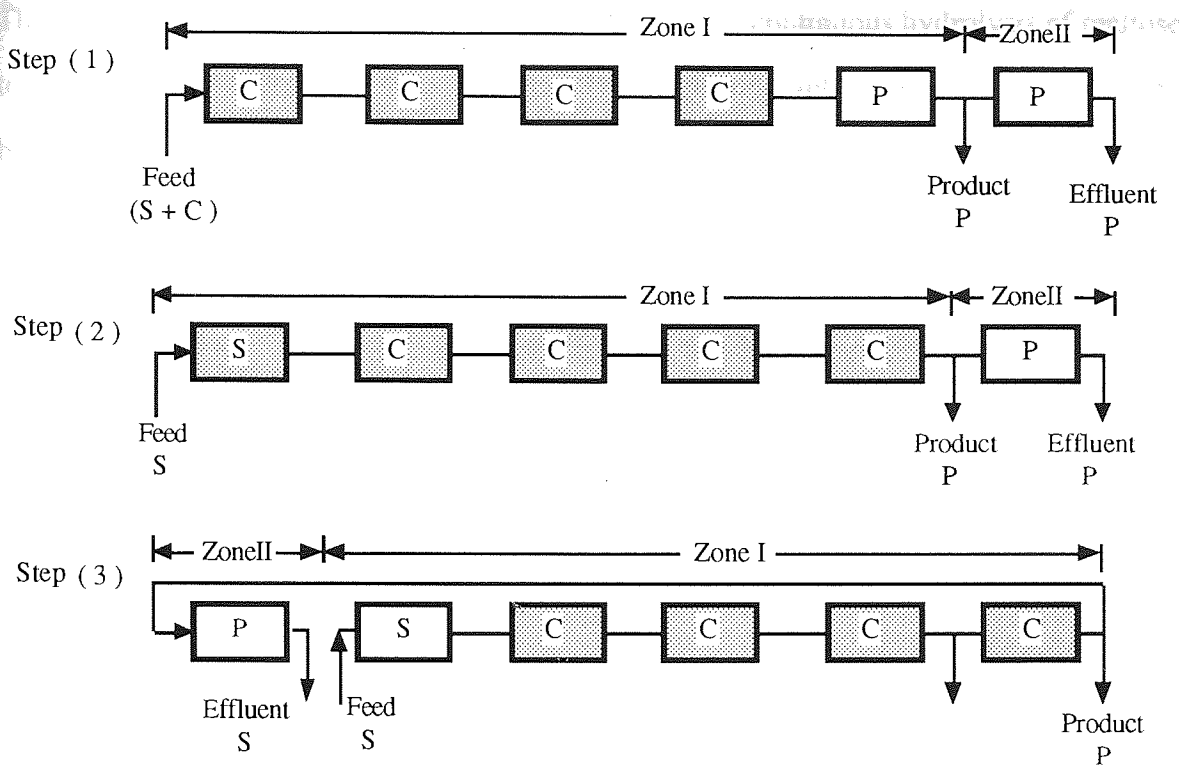


Figure 2.10: Schematic representation of the operation of the simulated moving bed for the repeated use of coenzyme[48] P = product, S = substrate and C = coenzyme

In step 1, the mixture of coenzyme and substrate solutions were fed to make the adsorption zones of the coenzyme in four columns.

In step 2, the feed was changed to the solution with only the substrate.

In step 3, the feed point and the product withdrawal points were moved to the next column in the direction of the liquid flow. The substrates and the products moved faster than the coenzyme. The products were recovered into the product stream in zone II. This step was repeated after a preset time determined by the time taken by the coenzyme to migrate a distance of one column.

The substrates were converted continuously to the products without further addition of the coenzyme if there was no leakage of the coenzyme.

The system was demonstrated to be capable of repeated use of the coenzyme. However, more experimental data was needed to evaluate the viability of the system.

The third reaction studied by Hashimoto *et al.*[48] was the continuous hydrolysis of maltose to glucose using the enzyme glucoamylase. The chromatographic system was constructed by connecting two columns packed with Bio-Gel P-10. The columns were of the same dimension, 1.5cm diameter and 10cm in length. The principle of operation of the reactor is shown in Figure 2.11. The operation of the reactor was based on the fact that enzyme migrates faster than substrate and products in a gel chromatographic column because the molecular weight of the enzyme is generally much higher than those of its substrate and product. By changing the position of the feed inlet point and the direction of the flow of the mobile phase (steps 1-6) repeated use of the enzyme was achieved.

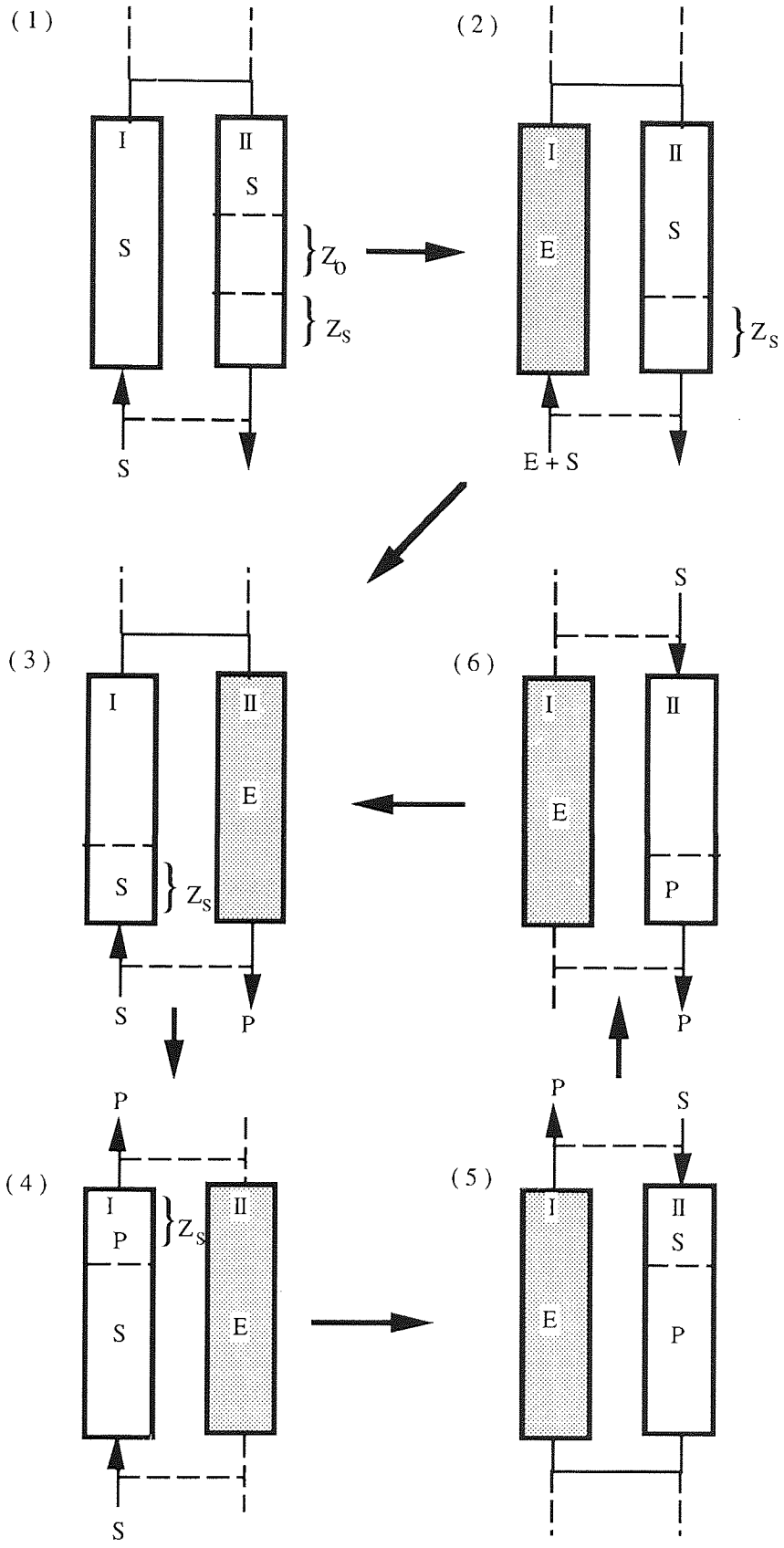


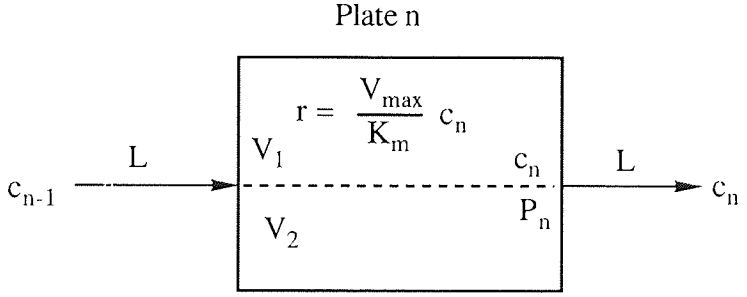
Figure 2.11: Schematic representation of the principle of repeated use of free enzyme for simultaneous bioreaction and separation of maltose into glucose[48]

2.7.2.2 The Barker *et al.* Systems

Semi-continuous moving port counter-current separators were developed by Barker and Co-workers[58-59]. These systems were used initially for the physical separation of industrial carbohydrate feedstocks and products with purities of over 90% were obtained.

One of these systems was modified[16,49] and used to study the inversion of sucrose into glucose and fructose and the simultaneous separation of the products. Complete conversions were obtained at feed concentrations up to 55%w/v. From this study, they found that only 34% of the theoretical amount of enzyme required by a conventional batch process was used. The columns in this system were packed with cross-linked polystyrene resin in the Ca^{2+} form (KORELA VO7C).

Akintoye[16] modelled and simulated this system using the theoretical plate concept. He obtained three first order differential equations which were solved analytically using Laplace transforms. The equation obtained from the sucrose mass balance across a theoretical plate was as shown in equation 2.7.



$$V_1\frac{dC_n}{dt} + V_2\frac{dP_n}{dt} = LC_{n-1} - LC_n - r\,V_1 \qquad 2.7$$

Where, V_1 = plate volume of the mobile phase
V_2 = plate volume of the stationary phase
L = mobile phase flow rate
V_{max} = maximum initial reaction velocity
Km = Michaelis-Menten constant
C_n = concentration of solute in the mobile phase
P_n = concentration of solute on the mobile phase

This equipment was used for this research and therefore will be discussed in detail in a later section.

Barker and Co-workers[50,60] also studied the simultaneous bioreaction and separation for the first time in a continuous rotating annular chromatograph (CRAC) by inverting sucrose to glucose and fructose using the enzyme invertase. They also studied the saccharification of modified starch to maltose in the CRAC.

The CRAC consisted of two concentric cylinders which formed an annulus 140cm long, 29.7cm external diameter and 1.2cm wide giving an annular volume of 14.5dm^3. The chromatographic resin used was DOWEX 50W-X4 in the Ca^{2+} form with a mean particle size of 150μm. The principle of operation of the CRAC is illustrated in Figure 2.12.

The CRAC was modelled and simulated[50] for the inversion of sucrose to glucose and fructose. Equation 2.8 shown below was derived from the mass balance for sucrose and solved by the finite difference method. Similar equations were obtained for glucose and fructose.

$$\omega\frac{\partial c_S}{\partial \theta} + \omega\left(\frac{1-\varepsilon}{\varepsilon}\right)\frac{\partial q_S}{\partial \theta} + v\frac{\partial c_S}{\partial z} + \frac{c_S V_{max}}{c_S + K_m + \frac{c_S^2}{K_I}} = D_z\frac{\partial^2 c_S}{\partial z^2} \quad . \qquad 2.8$$

Where, C_S = concentration of sucrose
Z = axial distance
D_Z = axial diffusivity
L = bed length
θ = angular coordinate
V= intestitial velocity
e = voidage
ω = rotation rate
K_I = inhibition constant

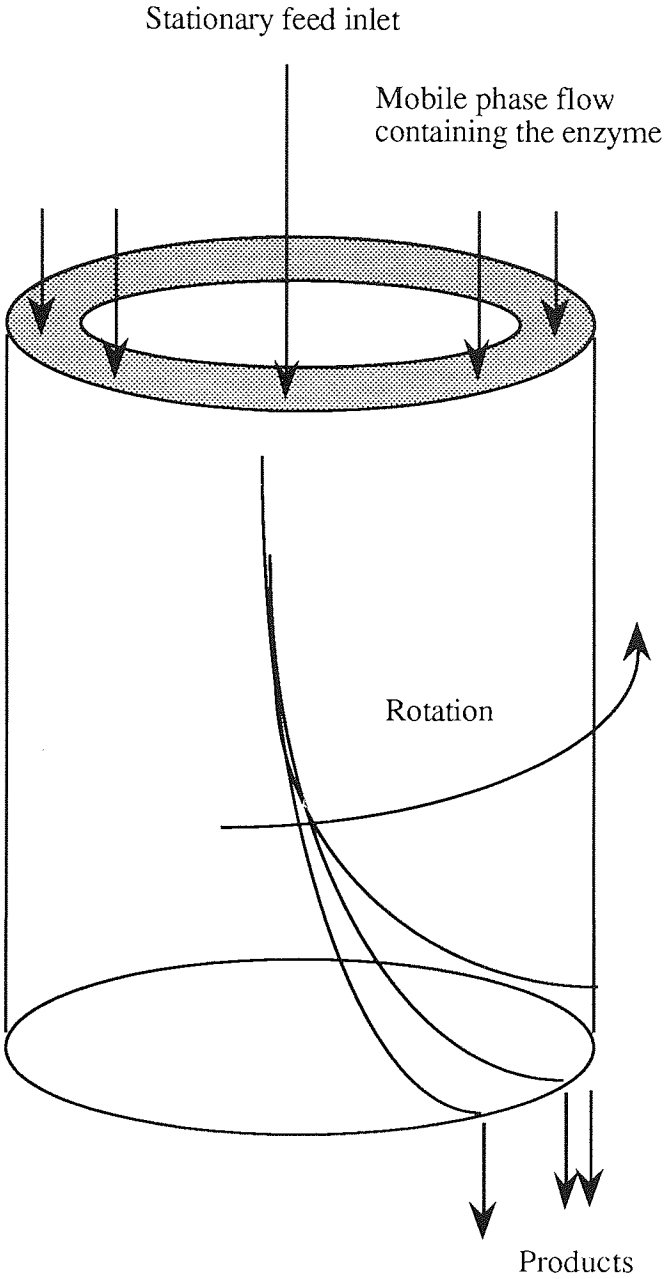


Figure 2.12: Operating principle of the continuous annular chromatographic bioreactor-separator[50]

CHAPTER 3

ENZYME BIOTECHNOLOGY AND CARBOHYDRATES

3.1 ENZYME BIOTECHNOLOGY

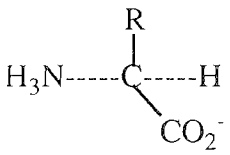
3.1.1 Introduction

Enzymes are known as biological catalysts. They accelerate specific chemical reactions and they function in dilute aqueous solutions under mild conditions of temperature and pH. They are protein in nature and produced by living cells[61]. Enzymes are highly specific in the type of reaction they catalyse. Some enzymes require additional chemical components called cofactors for their activity (e.g Fe^{2+}, Mn^{2+} or Zn^{2+}), or a complex organic molecule called a coenzyme.

Enzymes are classified into six different groups depending upon the reaction they catalyse. These are oxidoreductases, transferases, hydrolases, lyases, isomerases and ligases. Enzymes are sold on an activity basis which gives an indication of how much enzyme is required to achieve a desired product yield.

3.1.2 Structure and Properties of Enzymes

All enzymes consist primarily of a protein molecule which in essence is a 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. These are linked in specific sequence

as defined by the genetic information of the cell. This forms what is known as the primary structure and contains the active sites of the enzyme [61-62]. 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) consisting of a number of polypeptide chains are associated by means of secondary forces to form multi-subunit enzymes.

Most enzymes have particular and specific conformations in their native and active states. Disruption of the specific conformation leads to a reduction or loss of biological activity. This loss in activity can be caused by exposing the enzyme molecule to heat, extreme pH changes, air-liquid interfaces or by treatment with other denaturing agents.

Enzymes have molecular weights ranging from 12,000 to over a million [63]. They are therefore very large compared with the substrate or functional groups they act upon.

3.1.3 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 accurate and quick to carry out. The most widely used are spectrophotometric, fluorescence, electrode, polarimetric, and sampling methods [64].

(1) Spectrophotometric Method: This involves the absorption of light either in the visible or ultraviolet regions of the spectrum by the product or substrate. Usually, the

amount of light absorbed varies with the amount of product or substrate in the reaction medium.

(2) Electrode Method: This 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 enzyme activity.

(3) Polarimetric Method: This method is 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. 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 by HPLC or colourimetric techniques. HPLC techniques are widely used because they have the advantage of simultaneously analysing both the substrate and the product.

(5) Other Methods : Other commonly used methods include fluorescence and manometric methods. Fluorescence methods are used when one of the reaction components is a fluorescent substance. Manometric methods are useful when the reaction involves either gas uptake or gas production.

When the above methods are used, the activity of the enzyme is expressed as the initial rate of the reaction.

3.1.4 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 the enzymes are pH and temperature. The presence of certain chemicals like alcohols may also affect enzyme stability.

All enzymes have an optimum pH range over which their activity is maximum. Small changes from the optimum pH temporarily inactivates 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 results in gradual permanent inactivity. The optimum pH for some enzymes may vary with the substrate type and composition.

High temperature completely inactivates a majority of enzymes. Lower temperatures slow the rate of enzyme activity but is fully restored as the temperature is increased to its optimum [61]. Enzyme reaction rate increases with temperature according to the Arrhenius relationship. Stability of the enzyme is also affected by increased temperature.

3.1.5 Enzyme Extraction and Purification

Enzymes generally occur either within cells (intracellular) or outside cells (extracellular) and their extraction and purification depend on the type of enzyme produced. Figure 3.1 shows a generalised process for the recovery and purification of enzymes from cells.

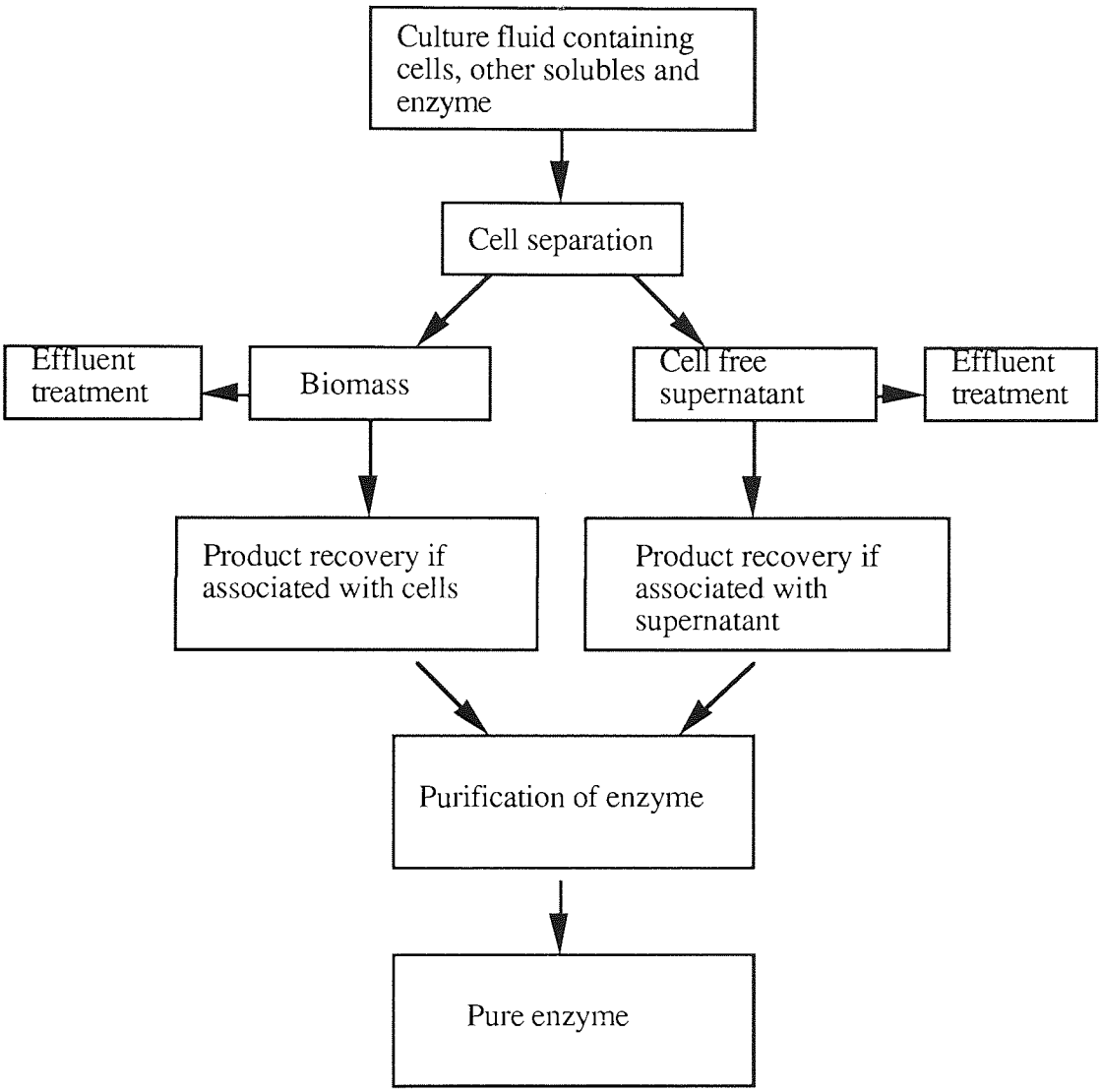


Figure 3.1: A generalised process for the recovery and purification of enzymes

3.1.5.1 Enzyme Extraction

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 extraction is necessary. For intracellular enzymes a certain degree of cell disruption is required. The methods of cell disruption and extraction widely used include:

(A) Physical Methods

(i) Grinding or agitation: with abrasives such as glass beads.

(ii) Liquid shear: a suspension of cells is passed at high pressure through a small orifice into a chamber at atmospheric pressure. The sudden drop in pressure causes disruption of the cells.

(iii) Sonication: Ultrasonic treatment is applied to cause the cells to disintegrate.

(iv) Freezing and thawing.

(v) Solid shear: The cell paste is frozen (usually to -20°C) and forced through a small hole at high pressure. The hole acts as an abrasive causing the cells to disrupt.

(B) Chemical Methods

(i) Alkalis: most cells are disrupted under conditions of high alkalinity (pH 11.5-12.5) and will be useful for extracting the enzyme if it is stable under these conditions.

(ii) 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.

(iii) 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.

(iv) Osmotic Shock: The cells are washed in buffer to free them from the growth medium and resuspended in, say, a 20% sucrose solution. The cells are centrifuged and then resuspended in water at about 4°C. This causes an increase in the cell osmotic pressure and leads to the release of certain cell constituents.

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

3.1.5.2 Enzyme Purification

Enzymes occur in complex mixtures. They are usually present in mixtures containing cells, growth medium components as well as other enzymes. Generally, the cost of extracting and purifying an enzyme is a major cost component in the overall cost of producing the enzyme. The process adopted for purifying an enzyme should be that which does not adversely affect the enzyme. A brief description of the different techniques used for the purification of enzymes are described below.

(i) Centrifugation

This method is normally used as the first step in the purification process. It is used to remove the cell matter which could be whole or disrupted. At present this is one of the elemental separation techniques that compose biotechnological downstream processing and wide varieties of these types of separators are available.

(ii) Precipitation

This technique involves the precipitation of either the enzyme or the impurities. Due to the large volume of supernatant involved, the precipitation of the enzyme is generally preferred. Salts such as ammonium sulphate which are cheap, non-toxic, highly soluble and in some cases have stabilising effects are widely used for enzyme precipitation[72]. Organic solvents, for example, polyethylene glycol (PEG) have also been used. The problems with organic solvents include their tendency to denature proteins, and are flammable.

(iii) Crossflow Membrane Filtration

Crossflow membrane filtration processes are increasingly gaining popularity and application in biotechnological downstream processing. This type of filtration prevents cake formation at the filter surface by sweeping away the particles with tangential shearing forces. This type of filtration is used for cell removal, selective separation of various soluble components in fermentation broth and concentration of the final enzyme mixture.

Membrane filtration processes are classified according to the range of size of particles the membranes reject and also by the driving force exerted on each membrane.

- Microfiltration: This is used when the particles to be removed are greater than 0.1μm with very low pressure (<10psi).

- Ultrafiltration: This exploits the differences in molecular weight (10^3-10^6MW). The operating pressure is slightly higher than that of microfiltration.

- Reverse Osmosis (RO): R.O membranes are capable of rejecting salt ions but do require very high operating pressures to function.

(iv) Chromatography

The traditional purification techniques such as precipitation are unable to achieve high levels of purity of enzymes. In recent years, purification of enzymes by column chromatography has become the most effective of all enzyme purification methods. The mechanisms of separation depend in different cases on adsorption, ion exchange, specific affinity to immobilised ligands or molecular sieving effects. The different chromatographic techniques that are used for purifying enzymes are :

-Gel Filtration : This is limited mainly to the fractionation of macromolecules by the exclusion principle. High molecular weight compounds elute first and because of diffusion into and out of the stationary phase, the low molecular weight compounds are retained longer.

-Ion-Exchange Chromatography : 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. This is a powerful purification technique due to high protein capacity which can be used and to the variation in the elution procedure that is possible. Many types of ion-exchange materials are available. The most common are the cellulose ion-exchangers, diethyl-aminoethyl (DEAE) and carboxymethyl

(CM) cellulose, anion and cations. Ion exchange resins are water soluble polymers containing anionic or cationic groups such as - SO_3-,COO- or NH_3^+.

-Affinity Chromatography : This is a highly specific technique which relies on the specific interaction of an enzyme with an immobilised ligand. The interaction tends to be reversible to enable the release of the product by change in pH or ionic strength of the eluent. This has been most commonly used in situations such as enzyme antibody, antigen nucleic acid, hormone and vitamin purification. Affinity chromatography is particularly useful in removing unwanted but low concentration contaminating enzymes. A comprehensive review on affinity separation has been carried out by Chase [65].

Other purification techniques include chromatofocussing where proteins are separated according to their iso-electric points, Hydrophobic Interaction Chromatography (HIC) which is particularly suitable for samples in the presence of high salt concentrations such as a precipitation step with ammonia sulphate and Fast Protein Liquid Chromatography (FPLC) which is used for products that are extremely potent and in small quantities e.g. hormones, peptides and monoclonal antibodies.

3.1.6 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 substrate. Kinetic studies represent just one of a range of tools available for investigating the structure and function of enzymes. A range of complex mechanistic models have been developed [66] and the most commonly used is the Michaelis-Menten model (Equation 3.1),

$$V=\frac{Vmax[S]}{Km+[S]} \qquad 3.1$$

where, V = Initial reaction velocity
Vmax = Maximum Velocity
Km = Michaelis - Menten constant
[S] = Substrate Concentration

Equation 3.1 is for systems where the substrate concentration is in excess and there is no inhibition of the enzyme. In systems where there is inhibition, Equation 3.1 is modified to give Equation 3.2

$$V=\frac{Vmax[S]}{Km+\frac{[S]^2}{Ki}+[S]} \qquad 3.2$$

where, Ki is the inhibition constant.

The direct linear plot method[67] can be used to calculate the values of Vmax and Km from experimental data. Other graphical methods such as the Lineweaver-Burk plot and the Edie-Hofstee[62] plot can also be used to calculate the values of Vmax and Km from experimental data using the Michaelis-Menten relationship expressed in different forms.

To use the Lineweaver-Burk plot, the Michaelis- Menten model is expressed as shown by Equation 3.3

$$\frac{1}{V}=\frac{Km}{VmaxS}+\frac{1}{Vmax} \qquad 3.3$$

This equation is a linear relationship and therefore by plotting 1/V against 1/S, the values of Km and Vmax can be obtained. The Edie-Hofstee plot uses the Lineweaver-Burk equation as the starting point and multiplies both sides by VmaxV to give a relationship for V as shown by Equation 3.4.

$$V = Vmax - \frac{KmV}{S} \qquad 3.4$$

From the plot of this equation the values of Km and Vmax can also be obtained.

Three main criteria are considered when considering the commercial suitability of an enzyme. The relative importance of these criteria will vary with the application envisaged. These criteria are : -

(1) The rate of reaction (Catalytic activity)

(2) The extent of reaction (Equilibrium constant)

(3) The duration of usable activity (Stability)

3.1.7 Maltogenase

Maltogenase is a thermostable maltogenic amylase produced by a strain of *Bacillus* modified by recombinant DNA techniques. This enzyme is commercially produced by Novo Nordisk of Denmark[68]. A strain of *Bacillus stearothermophilus* is used to produced a thermostable maltogenic amylase. This strain is difficult to grow and the enzyme yield is poor. Using established recombinant DNA techniques, the gene responsible for producing the amylase is combined with a plasmid carrier and then transferred to a strain of *Bacillus subtilis* host which is easier to handle and gives acceptable yields of the enzyme.

Maltogenase is an exo-acting maltogenic alpha-amylase which catalyses the hydrolysis of α-(1,4) glucosidic linkages in amylose, amylopectin and related glucose polymers to produce mainly maltose with small amounts of glucose.

3.1.7.1 Properties and Action pattern of Maltogenase

The maltogenic amylase produced by the original *B. stearothermophilus* strain and that produced by the "Modified" *B. subtilis* host were shown to be identical[68] and therefore have the same properties and action pattern. The molecular weight of the enzyme is about 70,000 daltons with an iso-electric point of 8.5.

The optimum temperature of maltogenase is 60-65°C. The effects of temperature on the activity and stability of this enzyme are shown in Figures 3.2 and 3.3. The optimum pH for the enzyme is 5.3. The effects of pH on the enzyme activity is shown in Figure 3.4.

The action pattern of the enzyme maltogenase has been studied by Outtrup *et al.*[68]. They found that unlike ß-amylase which successively removed maltose from the non-reducing chain ends in a stepwise manner until the molecule is degraded or until a branch point (α(1,6) glucosidic linkage) is approached maltogenase in the initial stages of hydrolysis

produced maltose, some maltotetraose and maltotriose. As the reaction progressed the maltotriose and maltotetraose disappeared with some glucose being formed.

They found that amylopectin was more completely degraded with maltogenase than with ß-amylase inspite of the absence of an endo-alpha-amylase activity and debranching activity. This suggests that maltogenase can hydrolyse α(1,4) glucosidic links closer to the branch points than can soybean ß-amylase.

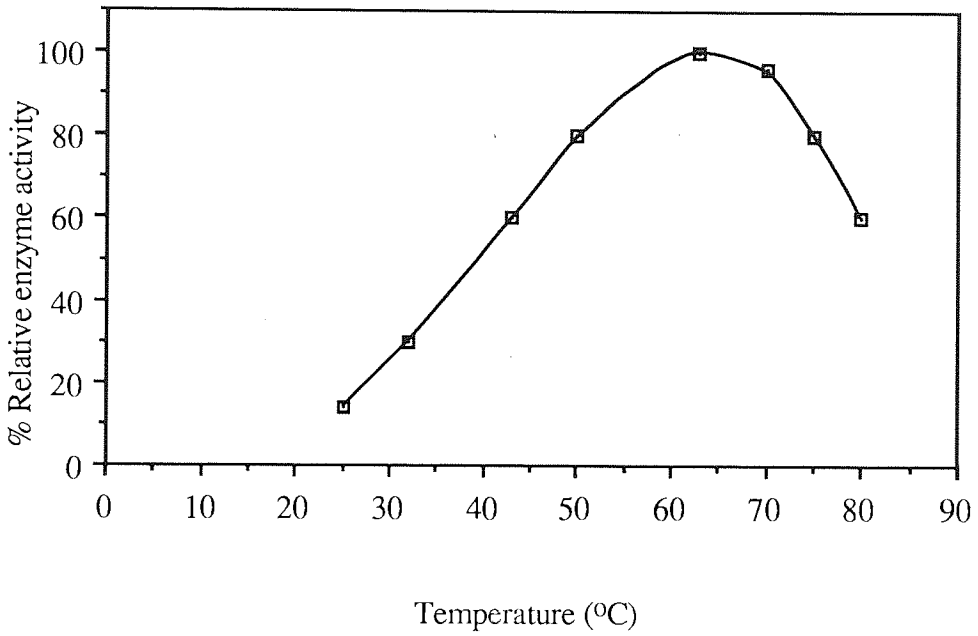


Figure 3.2: Effect of temperature on maltogenase enzyme activity

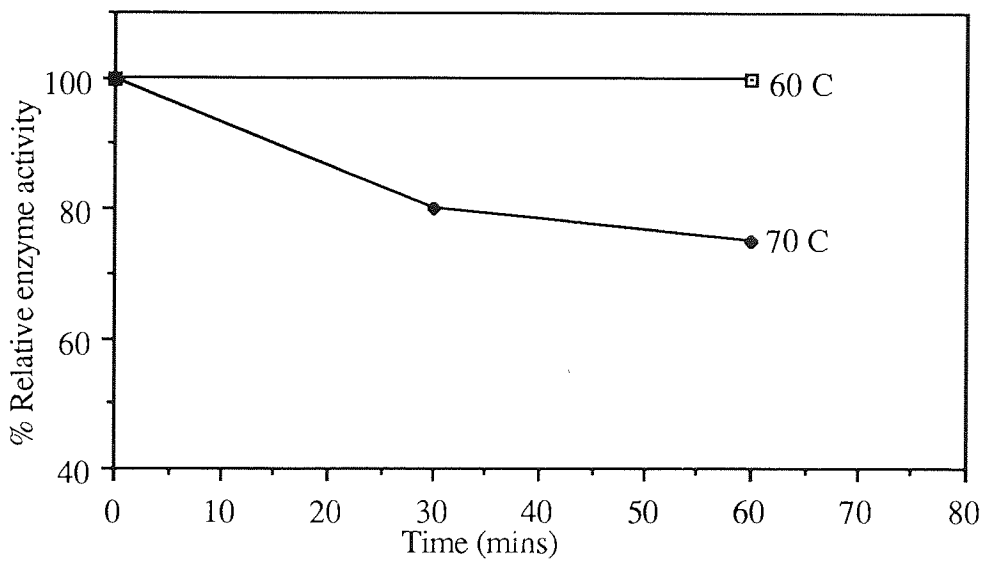


Figure 3.3: Effect of temperature on the stability of maltogenase

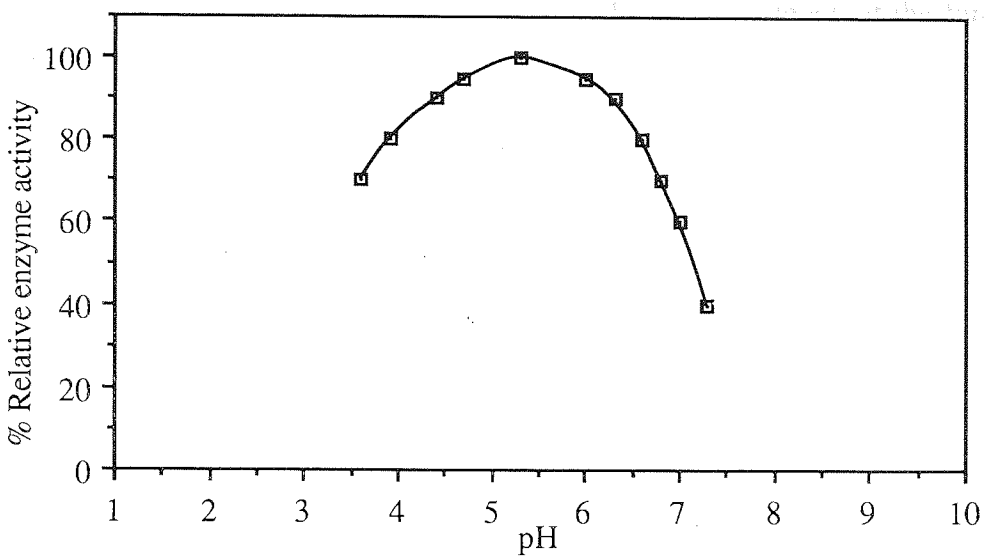


Figure 3.4: Effect of pH on maltogenase enzyme activity

3.1.8 Lactase

Lactase is a ß-galactosidase used for the hydrolysis of lactose in dairy products. This enzyme is produced by a number of different microorganisms including the yeast *Saccharomyces lactis*, the mold *Aspergillus niger*, the bacterium *Escherichia coli*[69] and fungal lactase from *Aspergillus oryzae*[70]. Lactases have been used in the soluble and immobilised forms for the hydrolysis of lactose[71-72]. The fungal lactase from *Aspergillus oryzae* was used during this project because of its availability and cost. This was supplied by Quest (Biocon), Ireland.

3.1.8.1 Properties of Lactase from *Aspergillus oryzae*

This lactase has an effective pH range of 2.5-7.0 with an optimum range at 4.5-5.5 [70-71] (Figure 3.5). At this pH range, the enzyme has an optimum temperature range at 55-60°C[70-71] (Figure 3.6).

Galactose which is a product of the hydrolysis of lactose is a competitive inhibitor of the *A. oryzae* lactase. Friend and Shahani [71] studied the effect of galactose on the *A. oryzae* lactase and showed that, at a pH of 6.5 with 200mM lactose as the substrate, the presence of only 5mM galactose can decrease the activity of the soluble enzyme by 50%. If

galactose is not removed from the system, the hydrolytic efficiency of the fungal lactase will decrease rapidly with time and severely limit its usefulness on a commercial process.

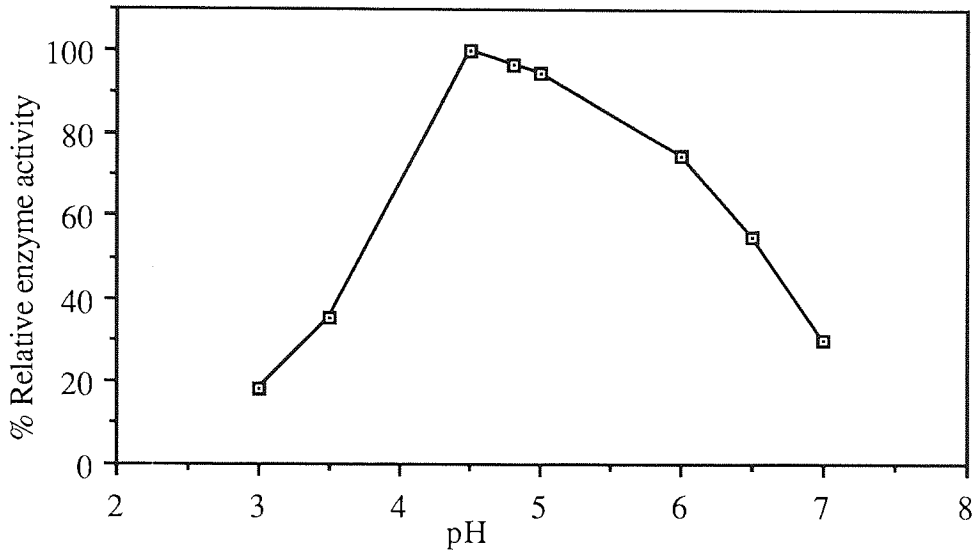


Figure 3.5: Effect of pH on A. oryzae lactase activity

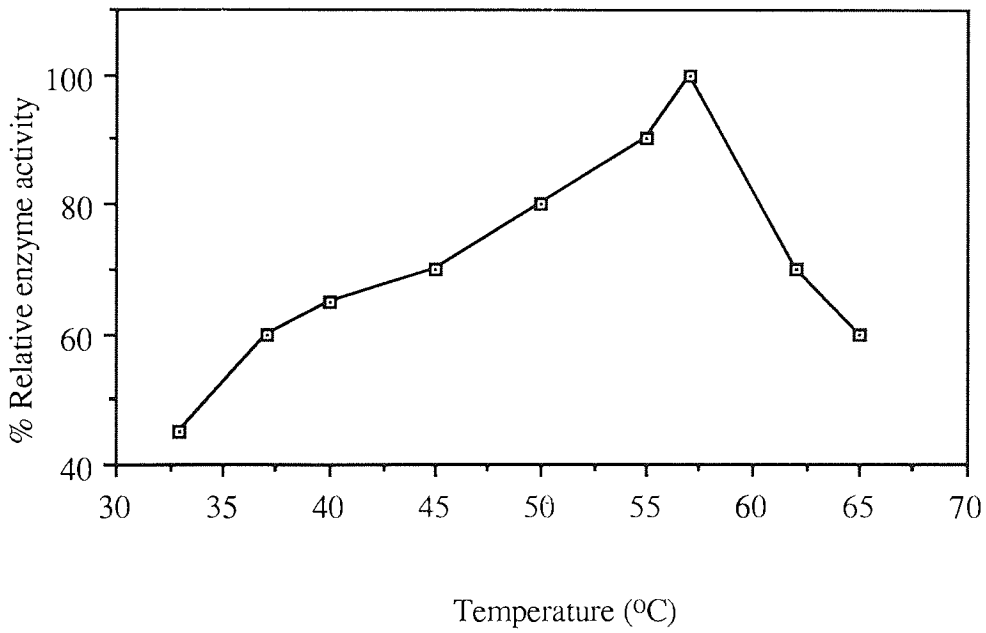


Figure 3.6 : Effect of temperature on A. oryzae Lactase activity

3.1.9 Dextransucrase

Dextransucrase (sucrose : 1,6-α-D-glucan 6-α-glucosyl transferase EC 2.4.1.5) is an extracellular inducible enzyme responsible for the conversion of sucrose to dextran and fructose. Dextran produced by *Leuconostoc mesenteroides* NRRL strain B-512F dextransucrase has been reported to be suitable for pharmaceutical use as a blood volume expander and blood flow improver[73].

3.1.9.1 Properties and Mechanism of Dextransucrase action

Dextransucrase has an optimum pH of 5.2 and a maximum activity at 25-30°C although at temperatures greater than 25°C rapid decay of activity occurs[74-75]. This enzyme is known to be very unstable even under the conditions specified above. As a result, it is very difficult to immobilise[76].

The mechanisms of action of dextransucrase was proposed by Robyt *et al.*[77]. They proposed the existence of two active groups (nucleophiles) at the active site of the enzyme which attack the sucrose by displacing fructose and forming a covalent complex with the glucosyl unit. Glucose units are inserted between the enzyme and the reducing end of the growing dextran chain. Each time a glucose unit is transferred, a new molecule of sucrose is hydrolysed to give the formation of a glucosyl-enzyme complex. The glucose or dextran can be displaced from the complex by acceptors thus terminating the dextran chain . Fructose which is formed during the reaction is an acceptor molecule. Other sugars such as maltose also act as acceptors. The reaction mechanism is represented diagramatically in Figure 3.7

3.1.9.2 Production and Purification of Dextransucrase

Dextransucrase is produced by secreting large amounts of the enzyme into the culture broth during the growth of the *L. mesenteroides*. A pH between 6.0-7.0 and temperatures ranging from 20-30°C have been used[78]. The production can be conducted either aerobically or unaerated. Ajongwen *et al.*[78-79] reported activities of up to 450

dextransucrase units (DSU) per cm^3 when fermentations were conducted under unaerated conditions. The need for sucrose as an inducer in the fermentation medium has always created the problem of the enzyme solutions contaminated with dextran.

The dextransucrase whole broth in addition to the cells contain unused nutrients and a number of soluble impurities such as fructose, dextran, leucrose, manitol, other oligosaccharides, small quantities of levansucrase and invertase together with other proteins and colouring substances[8,78]. Purifications of enzymes can represent half the cost of their production mainly incurred due to the sensitive nature of enzymes and problems associated with scaling up. All the purification techniques used involving dextransucrase have been a combination of any of the following; ultrafiltration, precipitation, phase partition, gel chromatography, ion exchange chromatography, centrifugation and microfiltration. Kaboli *et al.*[76], Monsan *et al.*[80] and Auriol *et al.*[81] working on very small scales reported enzyme recoveries of 54%, 96.4% and 95% respectively. Akintoye[16] working on a large scale, reported an overall enzyme recovery of up to 60%.

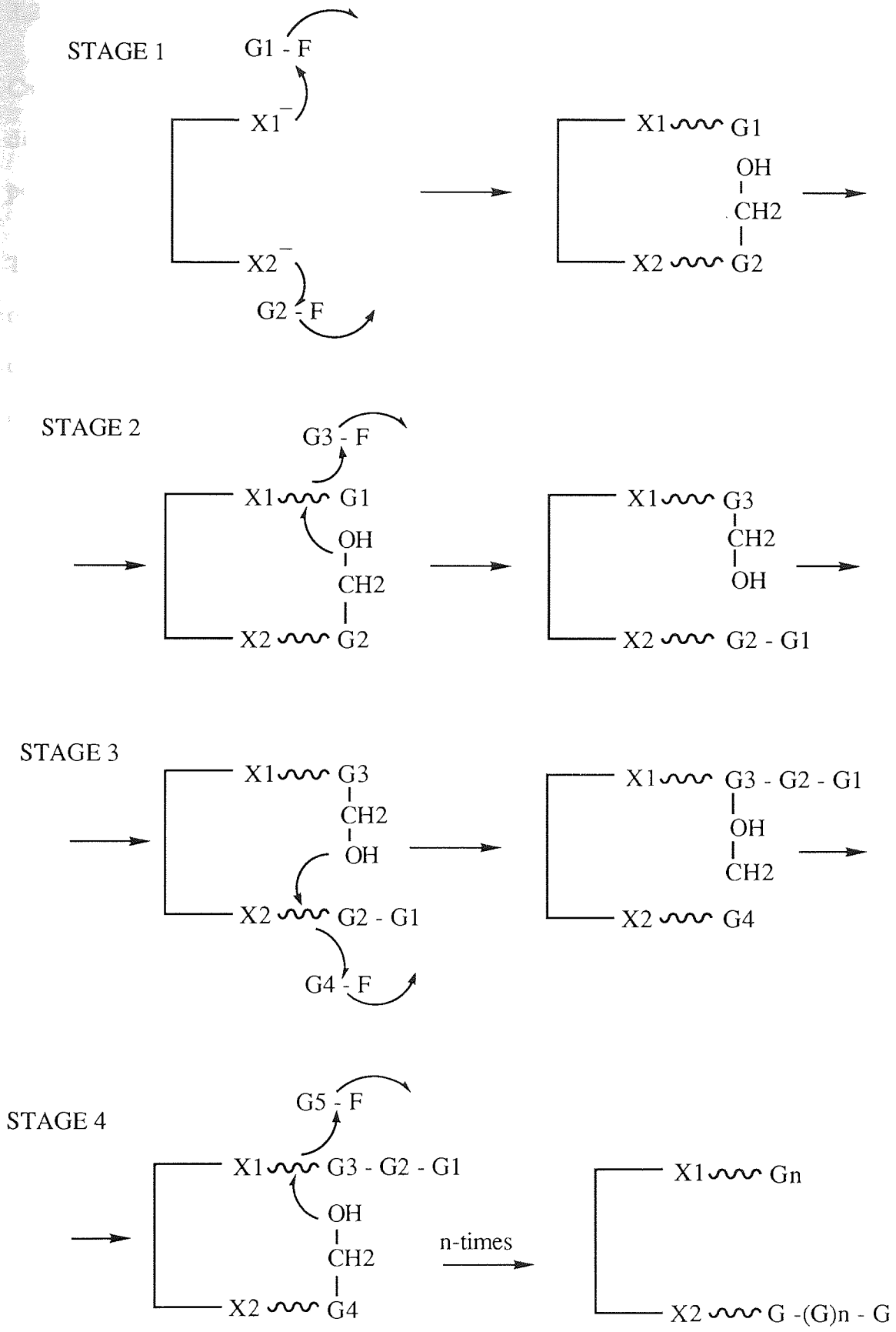


Figure 3.7: Schematic representation of the reaction mechanism of the enzyme dextransucrase

3.2 CARBOHYDRATES

Carbohydrates have the general emperical formula $(CH_2O)_n$, where $n \geq 3$. They are divided into three basic categories: monosaccharides, oligosaccharides and polysaccharides.

The monosaccharides usually have between three and nine carbon atoms and contain only one aldehyde or ketone functional group or are derivatives of these molecules. The oligosaccharides are generally two to ten monosaccharides linked together by the formation of glucosidic bonds. These are subdivided into disaccharides (e.g lactose, sucrose and maltose), tri-saccharides (e.g raffinose) etc, depending on the number of carbohydrate units they contain. Carbohydrate polymers containing more than ten monomeric units are called polysaccharides and can have molecular weights of many millions. Starch, dextran, glycogen and cellulose are examples of polysaccharides.

3.2.2 Dextran and Its Uses

Dextran is the collective name given to the family of extra-cellular bacterial polysaccharides with predominantly α-(1-6) glucosidic linkages. Beside the α-(1-6) glucosidic linkages, the different dextrans possess varying amounts of α-(1-2), (1-3) and (1-4) glucosidic linkages. The molecules also have varying degrees of branching. The properties of dextran are dependent on its molecular size as well as the detailed structure of the molecules. The structure of the molecule is governed by the type of bacterial strain used for production. There are a vast number of bacteria which produce dextrans, the most notable being *Leuconostoc mesenteroides* NRRL B512F. This strain has emerged as the bacterium of choice for the commercial production of dextran and is the only strain approved medically for the production of dextran for intravenous injections. This strain produces a water soluble dextran containing 95% α-(1-6) linear linkages and 5% α-(1-3) branch linkages. The chemical structure of dextran is shown in Figure 3.8.

The major uses of dextran is in the medical field where it is used as blood volume expanders (MW ≈ 70,000) and blood volume improvers (MW ≈ 40,000)[8]. The

molecular weight distribution (MWD) of these fractions is controlled by various pharmacopoeial and national specifications. Some of the advantages of using dextran in medicine are :

(1) Their stability to heat sterilisation.

(2) Long-term stability of dextran solutions.

(3) Freedom from transmissible disease, with very low incidence of adverse reactions.

(4) Independence of blood type recipients.

Dextran is also used for the production of Iron dextran which is used for the treatment of anaemia[82,83].

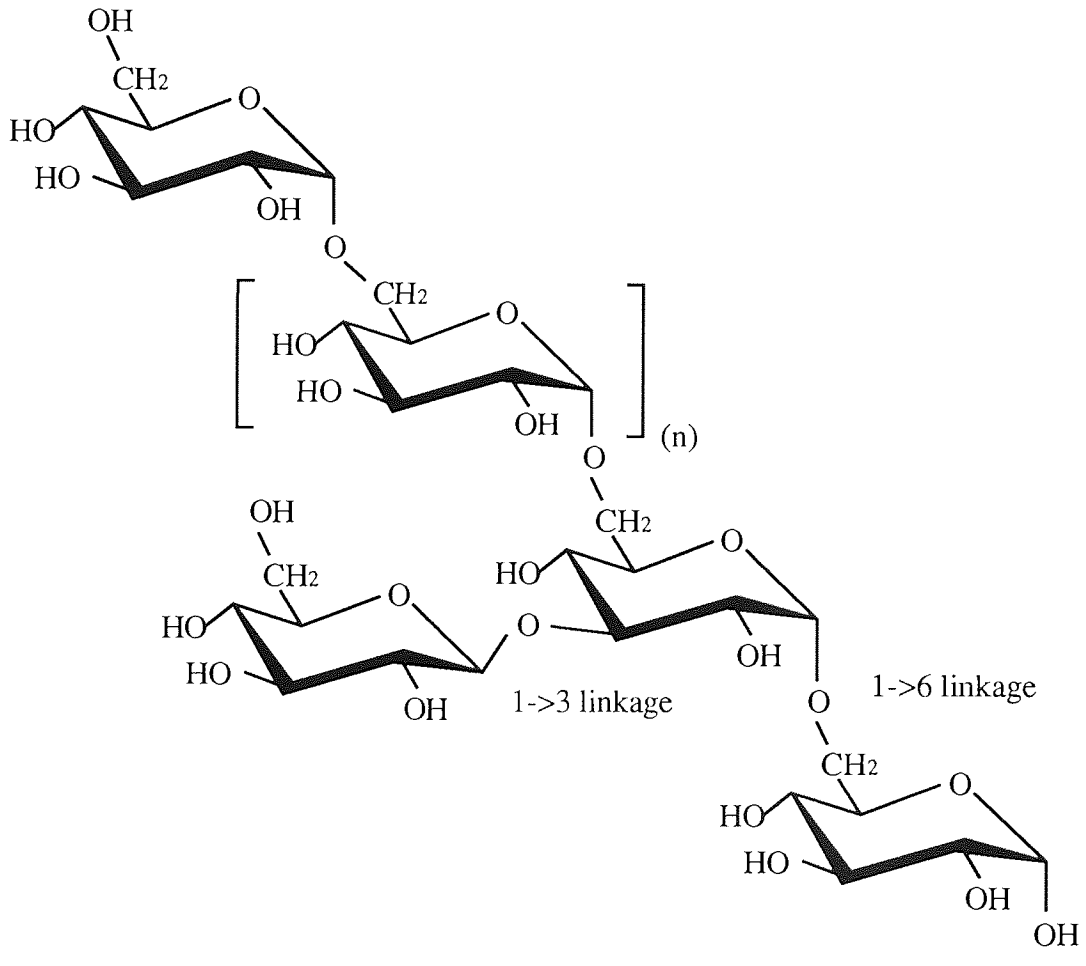


Figure 3.8: Chemical structure of Dextran

3.2.2.1 Industrial Production of Dextran

Commercial production of dextran uses the bacteria *L. mesenteroides* NRRL B512F. This strain is predominantly used as a source of clinical dextran because the dextran it produces has a minimum number of side chains. The dextransucrase enzyme is the primary product produced during fermentation. This enzyme then converts excess sucrose in the medium to dextran and fructose. Details of this process have been reported by Jeanes[84].

The dextran produced, called 'native' dextran has a broad molecular weight distribution (MWD). However the commercially important product is high molecular weight (HMW) dextran. The conventional process for producing dextran is shown in Figure 3.7.

This process has the disadvantage of cell, enzyme and dextran production under a set of conditions which is not constant throughout the fermentation. By maintaining a pH of 6.7 and a temperature of 23 ^{o}C throughout the fermentation, Tsuchiya *et al.*[85] were able to increase dextransucrase activity by a factor of six although the enzyme exhibited instability at this pH. Subsequent work by Barker and Ajongwen[79] further increased enzyme activities in fed-batch unaerated fermentations. Higher enzyme activities will result in improved product yields per unit time which is an important consideration in commercial processes. Hehre[86] reported that this process could be further optimised by removing cellular material from the fermentation broth, leaving a cell free enzyme solution which can then be used to synthesize dextran under controlled, optimum conditions.

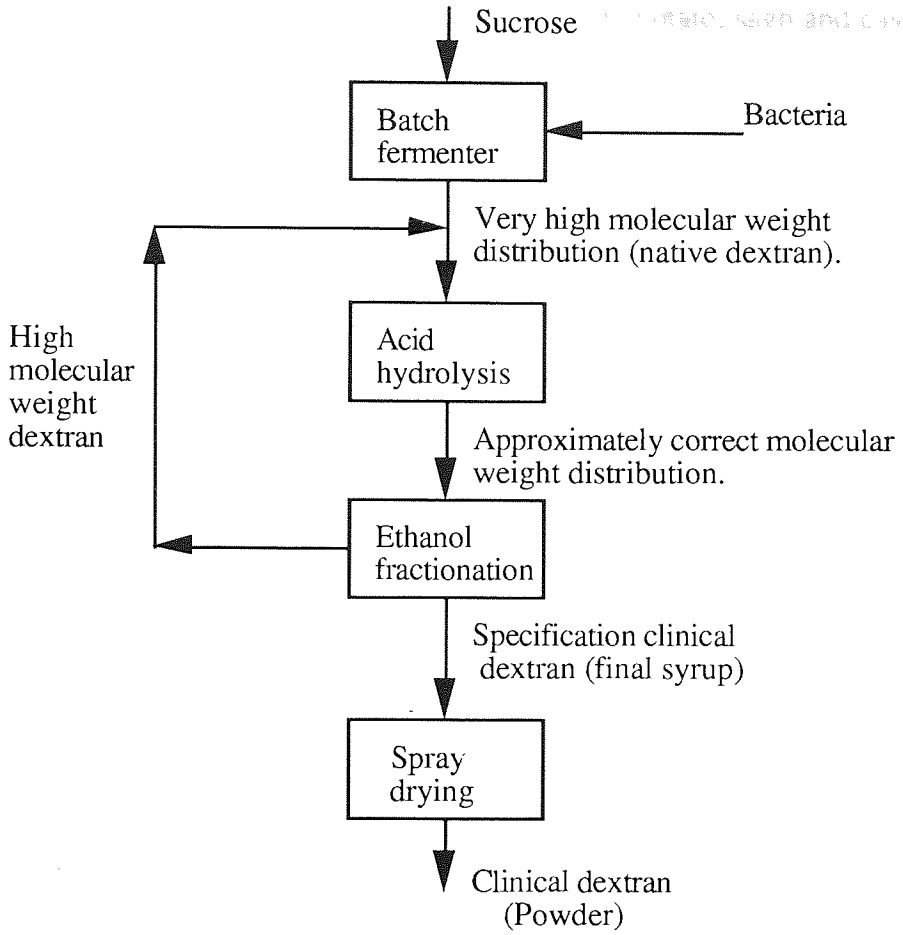


Figure 3.9: Industrial Production of Dextran

3.2.3 Starch

Starch is a glucose polymer which consists of amylose, a linear glucose polymer containing α-(1-4) glucosidic links and a branched polymer, amylopectin, in which linear chains of α-(1-4) glucose residues are interlinked by α-(1-6) glucosidic bonds. The glucosidic bonds can be hydrolysed to produce syrups containing glucose, maltose and other oligosaccharides. This can be achieved by acid hydrolysis but undesirable by-products are formed which are not permitted for human consumption.

The hydrolysis is therefore carried out using enzymes classified as endo-amylases, exo-amylases and debranching enzymes.

Commercial sources of starch include corn, wheat, barley, potato, sago and cassava. The utilisation of one or more of these sources is carried out according to the climatic conditions and agricultural output of a particular country.

An aqueous starch slurry is obtained as a result of physical and chemical treatment of the cereal or root crop. After the physical and chemical treatment, the starch is still intact and requires further treatment referred to as the liquefaction step before it is degraded to sugars (saccharification).

3.2.4 Sucrose and Lactose

Sucrose and lactose are disaccharides. Sucrose consists of one fructose and one glucose molecule and on hydrolysis produces an equimolar mixture of the two monosaccharides. The principal sources of commercial interest are sugar cane, sugar beets and the sap of maple trees[87].

Lactose consists of one galactose and one glucose molecule which on hydrolysis produces equal amounts of the monosaccharides. Lactose is commercially produced from whey which is obtained as a byproduct in the manufacture of cheese[88].

3.2.5 Glucose and Fructose

Glucose and fructose are the most common monosaccharides occurring free in fruits, honey and other living material. Glucose is dextrorotatory with a specific rotation of $(\alpha)^{20}D = 52.7^{o}$ in water, while fructose is strongly laevorotatory with $(\alpha)^{20}D = -132.2^{o}$[87]. Glucose is sometimes referred to as dextrose and fructose as laevoluse.

The formula of both sugars is $C_6H_{12}O_6$. Glucose is an aldoshexose, since it contains the aldehyde group (CHO), and fructose is a ketohexose, since it contains the carbonyl group (C = O). Glucose in solution exists with the isomeric ring structures shown in Figure 3.10 and an equilibrium exists between these forms which is only slightly dependent on temperature and concentration. The average equilibrium composition is 40% of α-D-glucopyranose and 60% of ß-D-glucopyranose[89-91].

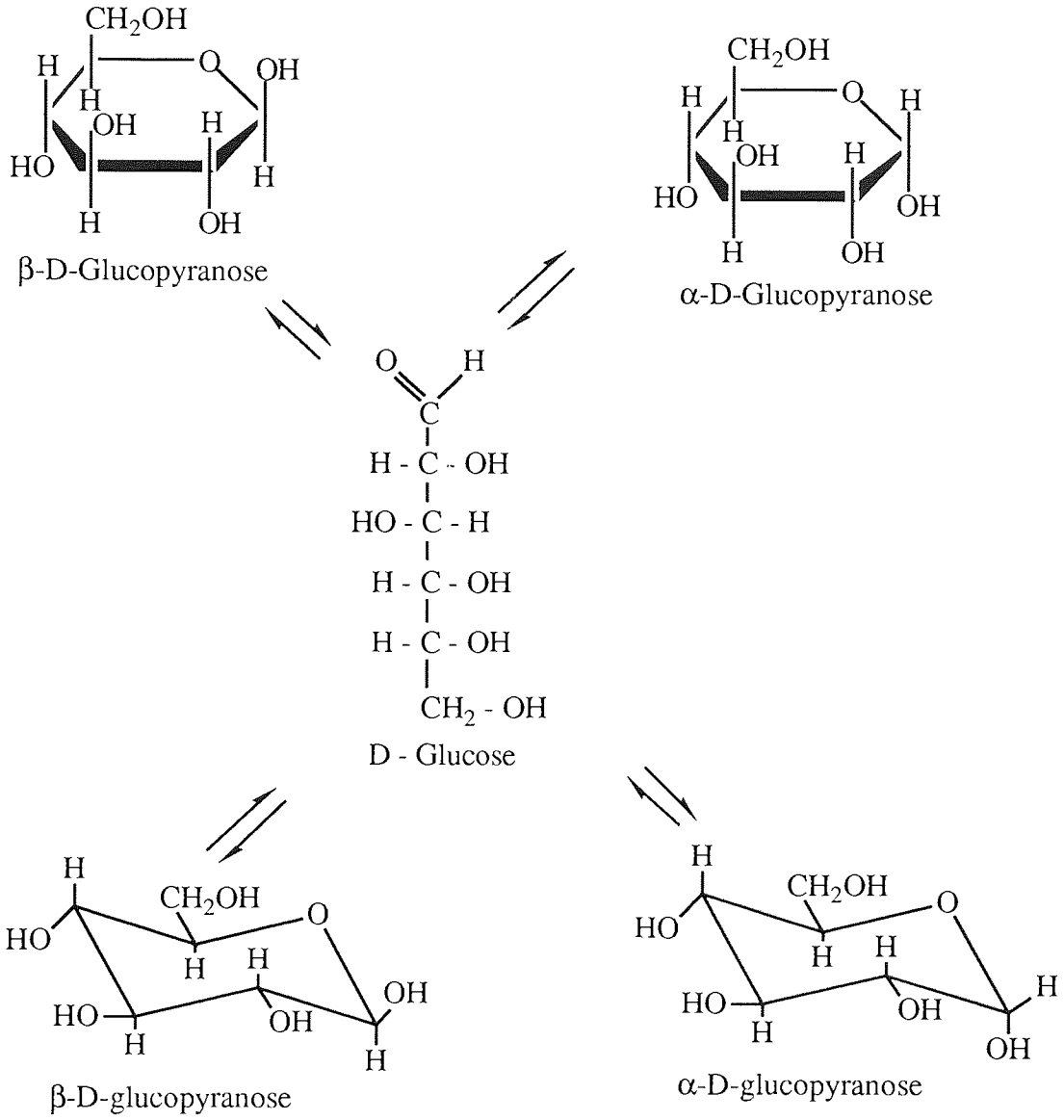


Figure 3.10 Isomeric forms of glucose in solution

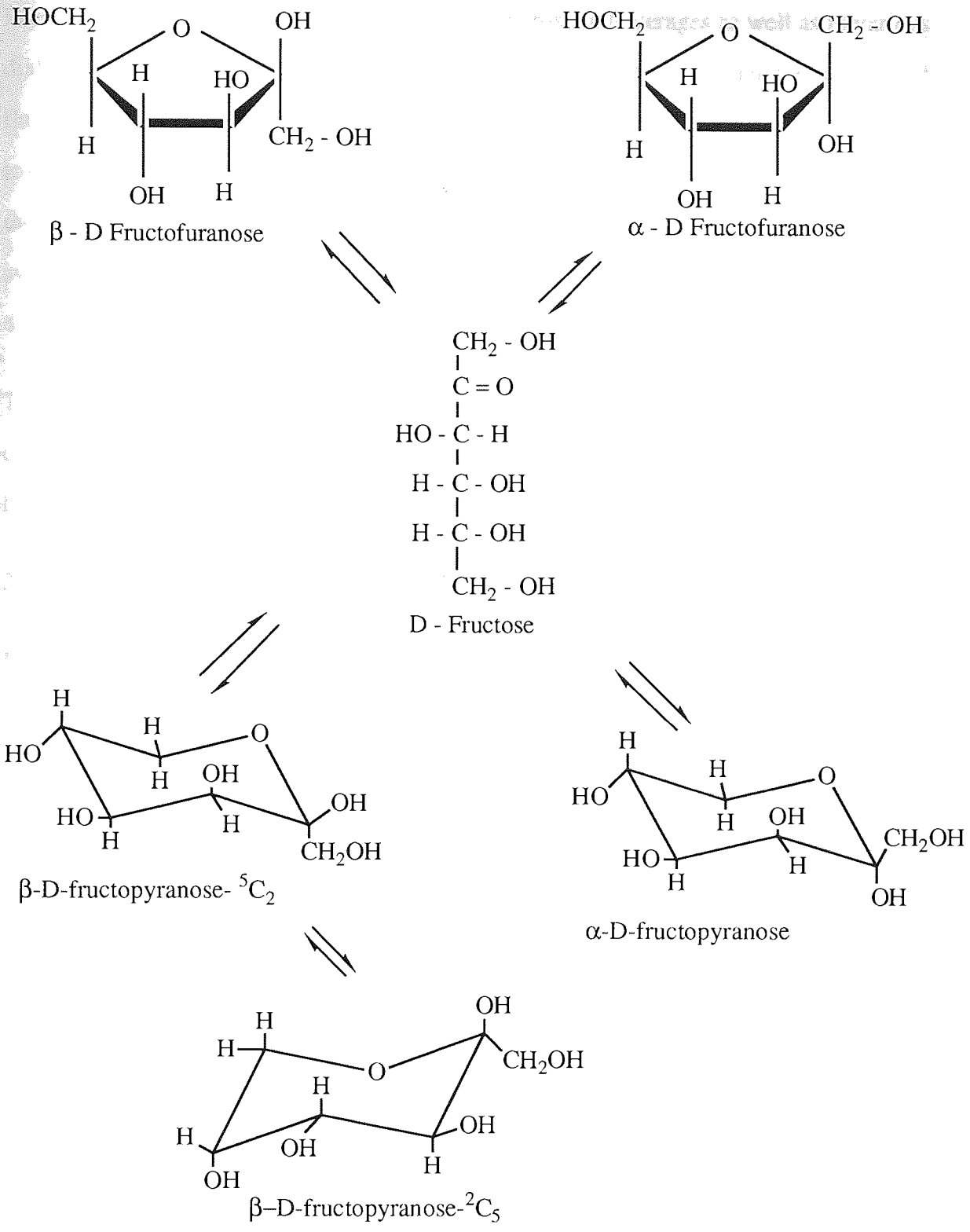


Figure 3.11 Isomeric forms of fructose in solution

In solution, fructose also exists in the isomeric forms shown in Figure 3.11. The equilibrium is affected by temperature, but the effect of solution concentration is minimal[89-91].

Fructose has been established as a substitute to sucrose in beverages as well as in various diabetic food products. It is also used as a raw material in the industrial manufacture of flavours and as a flavour enhancer in meat dishes. Fructose is a natural sugar without any toxic properties, and has traditionally been used as an alternative to glucose in infusion therapy for patients with low tolerance to intravenous glucose. Fructose is about 1.8 times sweeter and has a calorific value slightly lower than sucrose (3.7Kcal/Kg compared to 4.0Kcal/Kg for sucrose).

The usage of fructose is expanding rapidly and although it is unlikely to replace sucrose completely, it should become more favourable when new economical production methods have been developed and utilised.

3.2.6 Maltose

Maltose is a disaccharide consisting of two glucose molecules linked between carbons 1 and 4 by an oxygen bridge. Maltose has an optical rotation of $[\alpha]^{20}_{D}$= 130.4° and is commercially produced from starch by enzymic hydrolysis.

Various maltose-containing syrups are used in the brewing, baking, soft drink, canning, and confectionery industries. Among the important functional characteristics of high maltose syrups are low hygroscopicity, low viscosity in solution, resistance to crystallization, low sweetness, reduced browning capacity and good heat stability. Thus maltose-containing syrups can be used as moisture conditioners, crystallization inhibitors, stabilizers, carriers and bulking agents. Highly purified maltose may be used to replace glucose in intravenous feed and can be administered at higher concentrations without elevating the blood glucose level thus suitable for diabetics[92].

3.2.7 Galactose

Galactose is a frequent constituent of oligosaccharides, notably lactose, melibose and raffinose. Galactose is commercially produced by acid or enzymatic hydrolysis of lactose. The glucose is removed by fermentation with yeasts and crystallization of the galactose.

Two crystalline isomers of galactose are known. The α-form is the stable form, obtained under most conditions. The ß-isomer is prepared by crystallization from cold alcoholic solution. Galactose and glucose differ only in the configuration of carbon 4, and this difference results in a greater tendency for galactose to give furanose derivatives.

3.3 MECHANISM OF SEPARATION OF CARBOHYDRATES BY CHROMATOGRAPHIC SYSTEMS

The separation of carbohydrates in chromatographic columns packed with ion exchange resin in the calcium form is due to two main mechanisms, complex formation and size exclusion.

Complex formation between various metals and sugars have been known and studied as early as the nineteenth century. However, most of the chemical formulas suggested by the early investigators for compounds formed by the interaction of carbohydrates with metal bases are mere assumptions based on insufficient evidence. A very comprehensive account of the work achieved by the early researchers was compiled by Von Lippman[93], and Vogel and Georg[94].

Saltman and Charley[95] showed by dialysis experiments that calcium, magnesium, barium and strontium ions form soluble chelates in aqueous alkaline solutions with D-galactose, D-fructose, D-arabinose, D-ribose, maltose and lactose. Charley and Sarkar[95] showed that the absence of any precipitation of alkaline earth metal hydroxide in an aqueous solution containing D-fructose and an alkaline earth salt when made alkaline (pH 12) is an additional evidence for the existence of such complexes.

Angyal[96] reported that the coordination complexes of sugars and cations are formed by the displacement of water molecules in the solvation sphere of the cations by the hydroxyl groups of the sugar, which is polyalcohol. Since water solvates ions much better than does a monoalcohol, the latter will not displace water to any considerable extent. If two or more hydroxyl groups in a compound, namely sugar, are in a sterically favourable arrangement, they may displace two or more molecules, respectively, of water from the solvation sphere.

There are no cases known of di-alcohol complexing strongly with cations in aqueous solutions, it appears that at least three hydroxyl groups in favourable steric arrangement are required for complex formation. Angyal's work[96-100] over several years has established the fact that for complex formation with alkaline earth (Ca^{2+}, Ba^{2+}, Sr^{2+}) cations, sugars in their ring forms require either:

(1) an axial - equatorial - axial arrangement of three hydroxyl groups on successive carbon atoms of the tetrahydrofuran or

(2) a cis - cis relationship on successive carbon atoms of the tetrahydrofuran ring.

From the above hypothesis by Angyal, glucose will not form a complex with Ca^{2+} and only the ß-D form of fructose has an axial - equatorial - axial hydroxyl group required for complexing with Ca^{2+} (Figure 3.10 and 3.11). Reports on the subject are numerous and the application of the calcium-fructose complexing effect as a means for separating fructose from a mixture of sugars is already a proven success as in the production of high fructose syrups.

In size exclusion the retention is based on molecular sizes. Those solute molecules whose molecular sizes are too large to diffuse into the resin matrix move through the column faster than the smaller molecules. The smaller molecules such as the monosaccharides (fructose, galactose and glucose) diffuse in and out of the resin matrix resulting in a slower movement through the column. Diasaccharides such as sucrose and maltose, and higher saccharides are largely excluded from the resin matrix and move faster through the column, thus separation between mono, di, and higher saccharides occur.

CHAPTER 4

ANALYTICAL EQUIPMENT, ENZYME ASSAY TECHNIQUES AND KINETIC STUDIES

4.1 INTRODUCTION

This chapter describes the analytical equipments that were used during this project. Analysis was carried out quantitatively on samples collected from the SCCR-S system. In this chapter, the methods used for the enzyme assay and for the determination of the kinetics of the enzymes used during this project are reported.

The high pressure liquid chromatography (HPLC) and gel permeation chromatography (GPC) systems employed in this study had similar equipment arrangements as summarised in Figure 4.1.

4.2 HIGH PRESSURE LIQUID CHROMATOGRAPHY (HPLC)

4.2.1 HPLC System Description and Operation

The carbohydrate concentrations and compositions in the samples were determined using the HPLC system. This system shown in Figure 4.1 consisted of:

- A Bio-Rad 1330 pump (Bio-rad UK Ltd, Herts). This pump contains two pistons which operate 180^{o} out of phase thus minimising pulsations;
- A Bio-Rad differential refractometer type 1750;
- A Talbot ASI-3 autosampler (Talbot instrument Ltd, Cheshire, UK);
- A Spectra-Physics integrator type SP4270 (Spectra-Physics UK Ltd, Herts);
- An Anachem (Luton, Beds) debubbler was used to trap any air bubbles in the eluent delivery tubing;

- A 30cm long by 0.78cm diameter Aminex HPX-87C (Bio-Rad UK Ltd) ion-exchange chromatographic column. The Aminex column was packed with 8% divinylbenzene cross-linked resin in the calcium form (9 μm particle size). Separation within this column occurrs because of the interaction of the sample components with the resin.

The eluent used was distilled and de-ionised water. The eluent was degassed by heating to 80°C and prefiltered using a 0.45μm sintered metal filter before being pumped into the column which was protected using a LichroCART RP-18 precolumn (BDH Ltd, Atherstone, Warwicks, UK).

All samples were filtered using 0.45μm disposable filters (Gelman Sciences, Northampton, UK) to remove particulate matter and to prolong the life of the main and guard columns. Samples containing enzyme were heat treated in a boiling water bath for about 5 minutes to denature the enzyme.

Samples were injected automatically using the autosampler. The components in the sample were resolved in the Aminex column and detected at the column outlet by the refractometer. The Bio-Rad refractive index (RI) monitor measured the differential RI between a pure reference eluent and the eluent from the column. The RI monitor provided a 1volt full scale output to the integrator which processed the RI data and produced a chromatogram from which individual components were identified by their retention times and component concentrations were determined by the areas under the chromatograph peaks compared to those of known standard samples.

4.2.2 HPLC System Maintenance

The cationic resin used in the HPLC column was in the Ca^{2+} form. Some of the samples analysed contained small amounts of ions such as Na^{+} and K^{+}. These ions slowly replaced the Ca^{2+} on the resin with time and therefore caused reduction in the efficiency of the column. When a reduction in the performance of the column was noted by a reduction in sample resolution, the column was cleaned with a 30% acetonitrile solution and then

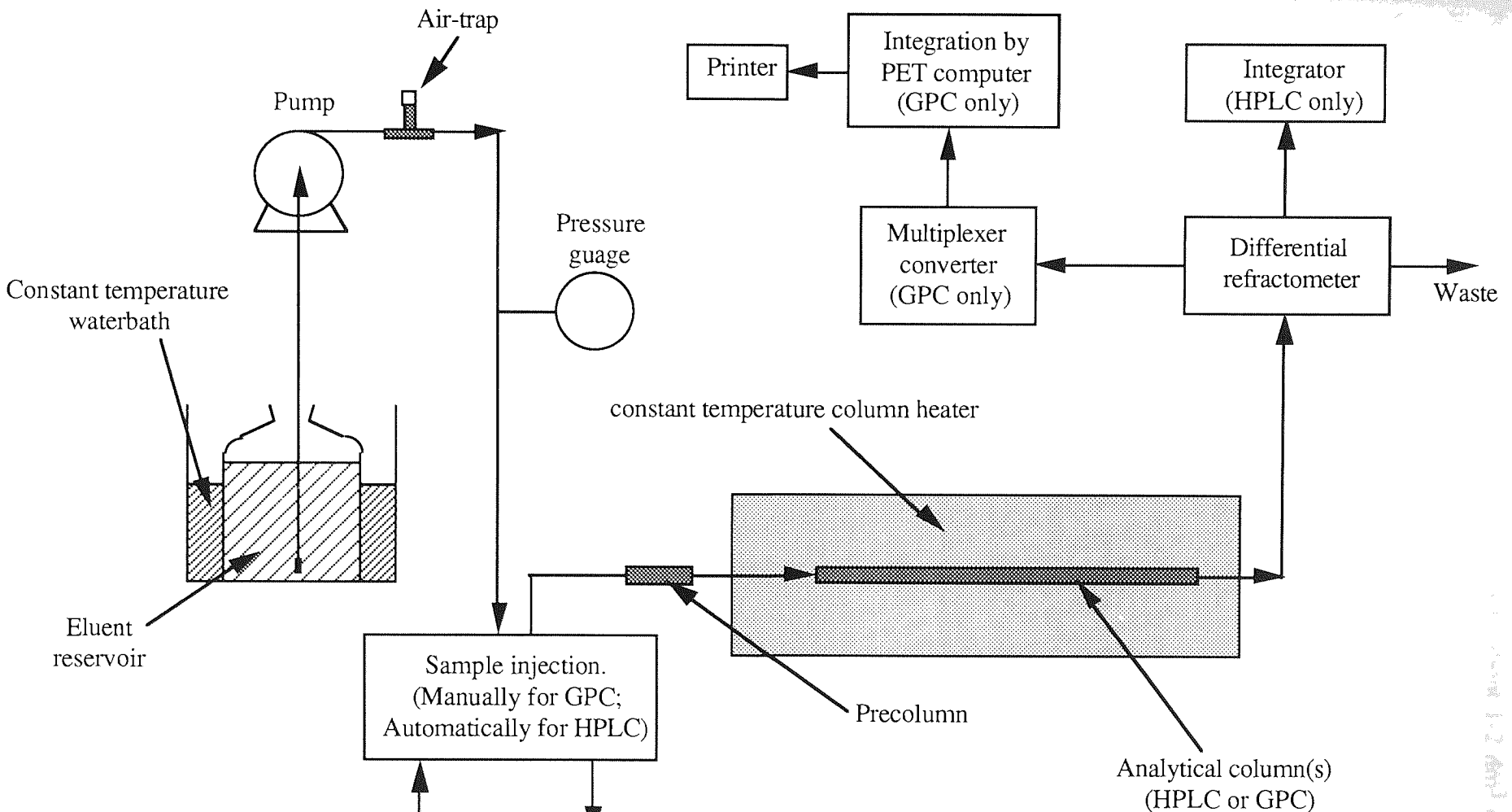


Figure 4.1 Schematic Diagram Of The GPC / HPLC Analytical System

regenerated using 0.1M calcium acetate and rinsed with about 1-2 dm^3 of distilled de-ionised water.

When the system was not used for a long period, it was flushed with a 0.02%w/v solution of sodium azide to prevent microbial growth.

4.3 ANALYTICAL GEL PERMEATION CHROMATOGRAPHY SYSTEM (GPC)

GPC is a chromatographic technique which separates components in a sample according to their size differences. This is achieved using columns packed with gels or some other porous materials of variable pore size. GPC was used to measure the molecular weight distribution of the dextran obtained from the biosynthesis reaction. The detailed description, operation, calibration and techniques of this system have been reported by previous workers[9,12,13,16].

4.3.1 Equipment Description and Analytical Techniques

The arrangement of the GPC system was similar to that of the HPLC analytical system (Figure 4.1). The eluent used was de-ionised distilled water which was deaerated by heating to 80°C. Hibar prepacked Lichrosopher Diol columns (BDH Ltd, UK) were used. These columns have been found suitable for the analysis of native dextran fractions containing some protein material[101]. The columns were packed with 10μm spherical hydrophilic support material coated with 1,2-dihydropropoxypropyl chains. Three columns joined in series to provide a wide molecular weight fractionating range were used in the GPC system. The properties of these columns are summarised in Table 4.1. These columns were protected using precolumns packed with the 1000 Diol material.

The fractionated dextran from the GPC columns passed through a differential refractometer (Model R401, Waters Associates Ltd, London), where it was compared with a standard reference of de-ionised water. The analogue output from the refractometer was registered by a multiplexer (model PCI 1001, CIL Electronics Ltd, Sussex) which converted it to a digital signal. This digital signal was then recorded by a PET computer (model CBM

4032, 32K bytes, Commodore, UK). The PET was programmed to store and print the heights such that they could be used to obtain the molecular weight distribution (MWD) of dextran.

Table 4.1: Properties of the GPC columns

Column	Packing	Fractionating Range (Mw)	Dimensions (cm x cm)
1st	1000 Diol	$1x10^4$ - $2x10^6$	25 x 0.4
2nd	500 Diol	$4x10^3$ - $7x10^5$	25 x 0.4
3rd	100 Diol	$2x10^2$ - $4x10^4$	25 x 0.4

4.3.2 Data Acquisition Using a PET Computer

A multiplexer, Commodore PET computer and a Commodore printer (model CBM 4022P) were used for the on-line acquisition of data. This system was used to obtain accurate values of heights at set time periods. These values were used to calculate the MWD of dextran using a computer program developed by Vlachogiannis[102]. This program has been successfully used by other researchers[10,12,13,16,101]. The listing of the program is given in Appendix A.

This program recorded and printed the heights in the following way :

- Immediately after injecting the sample, the program was initiated and readings from the refractometer were recorded via the multiplexer.

- Every two seconds the program read from the refractometer. This procedure was repeated until five readings were taken. Five successive readings were averaged to give an average height every 10 seconds of the chromatogram.

- The program entered a loop during the calculations of the heights. These heights were continually displayed on the VDU of the computer.

- The program came out of the loop when the time reached its maximum setting.

Once the sample elution had finished, the recorded heights along with the sample times were printed out. This printout was used to calculate the MWD using a calibration method programmed into the computer.

4.3.3 Calibration of the GPC columns

Calibration of the GPC columns was necessary in order to relate an elution volume (Vr), through the distribution coefficients (Kd), to the molecular weight (MW). Detailed investigation by Vlachogiannis [102] and Bhrambra [103] showed that the most convenient method of calibrating GPC columns was that devised by Nillson and Nillson [104]. The molecular weight (MW) of a component, i, was expressed as a five term polynomial as follows :

$$(MW)_i = b_5 + \exp [b_4 + b_1(Kd_i) + b_2(Kdi)^2 + b_3(Kdi)^3] \qquad 4.1$$

$$Kd = \frac{(V_r - V_o)}{V_p} \qquad 4.2$$

$$V_p = V_t - V_o \qquad 4.3$$

Where, V_p = the gel volume in the column

V_t = total volume of the column and

V_o = the void volume of the column

The values of the calibration constants b_1 to b_5 were obtained by the following procedure :

- The values of Vt and Vo were determined experimentally by using a glucose and a very high MW dextran standard respectively. The glucose molecules penetrate the pores easily and was used to give Vt. The high MW dextran is totally excluded, hence it gave the pore volume Vo.

- A series of several Pharmacia standard dextran T-fractions whose weight average MWs had been measured previously by light scattering techniques were chromatographed on the GPC system.

- Once the elution profiles from each T-fraction was obtained, the calibration constants b_1 to b_5 were found using a computer program originally written by Vlachogiannis [102]. The Hertley modification of the Gaussian-Newton method[105] was used in this program. It required inputs of the heights, elution volumes and light scattering MW values of the T-fraction chromatograms with initial guesses of the constants b_1 to b_5. Alsop and Vlachogiannis[106] reported that all the calibration standards should have individual average MWs obtained by GPC within 90-110% of those obtained by light scattering before proceeding with the analysis of samples by GPC.

The calibration was checked regularly by analysing standard T-fractions and comparing the weight average molecular weight values with the light scattering values. The GPC was recalibrated when necessary.

Calibration results are shown in Table 4.2 and a typical resultant calibration curve is shown in Figure 4.2. In an ideal situation the GPC columns should be calibrated and used within the linear Section Mx to My on the calibration curve shown in Figure 4.2. The GPC system was used to determine MWs in the range of Mx to My, which was between 180 and 2×10^6 MW.

4.3.4 Determination of Molecular Weight Distribution and the Molecular weight average

The peak heights which are directly proportional to sample concentration were taken at fixed intervals during the analysis of a sample from the time of injection. These peak heights were stored in the PET computer with other information such as, peak elution times of a high molecular weight dextran peak and a glucose peak, the start and finish times of the sample and the calibration constants b_1 to b_5. These inputs were used to calculate the MWD of the sample using the computer program listed in appendix A. This program

calculated the MW of each discrete fraction, i, for each 10 second time interval eluting from the column using Equations 4.1 and 4.2. This then gives a complete MWD profile for a given sample.

Due to the polydisperse nature of dextran, it is not normally possible to characterise it by a single MW. To overcome this problem various averages such as number-average ($\overline{M}_N$) and weight-average ($\overline{M}_W$) molecular weight are calculated. The program calculated these values from the chromatographic data using the following Equations 4.4, 4.5 and 4.6.

$$\text{Normalised height, } hi = \frac{\text{Chromatogram height } hi'}{\text{Total Chromatogram heights } \Sigma hi'} \qquad 4.4$$

$$\overline{M}_W = \Sigma hi \ MWi \ / \ \Sigma hi \qquad 4.5$$

$$\overline{M}_N = \Sigma hi \ / \ (\Sigma \ hi \ / \ MWi \ ) \qquad 4.6$$

For a polydisperse polymer $\overline{M}_W > \overline{M}_N$. The values are the same for a monodisperse polymer. Polydispersity (D) is often used to describe the breadth of a molecular weight distribution and can be calculated from the expression:

$$D = \overline{M}_W / \overline{M}_N \qquad 4.7$$

A detailed discussion of the above process and an extensive review of a number of other GPC calibration procedures have been reported by Bhambra[103].

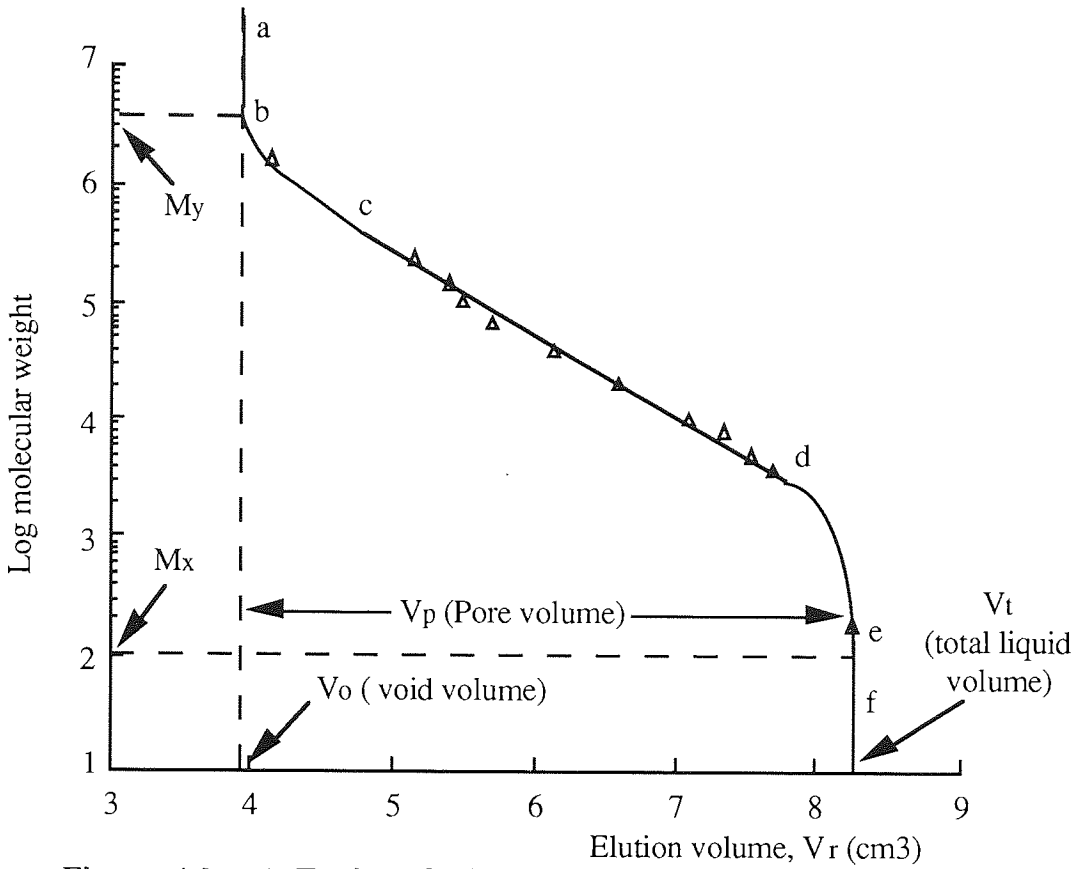


Figure 4.2 A Typical Calibration Curve for a GPC Column

Table 4.2 Typical Diol Column Calibration Data

Batch number	Weight average molecular weights		$\frac{GPC}{LS}$ x 100%
	Light Scattering	GPC	
PJ 3636	238 192	239 400	100.5
PH 1078	147 545	145 062	98.3
PG 7427	102 209	103 960	101.7
PF 1601	70 276	70 316	100.1
PB 5227	40 908	40 272	98.4
PE 5382	21 471	21 798	101.5
PA 0094	10 470	10 352	98.9
JD 2985	7 971	7 813	98.0
DK 8868	5 127	5 368	104.7
PD 2335	3 836	3 735	97.4
Glucose	180	180	100.0

Calibration Constants				
b_1	b_2	b_3	b_4	b_5
-8.802	4.745	-3.373	14.459	-948.641

4.4 DIALYSIS

The dextran produced using the SCCR-S system also contained some unwanted materials such as glucose, fructose and unreacted sucrose. These materials were removed by dialysis, a membrane-based process that separates material according to size.

Dialysis membranes with a quoted molecular weight cut off of 1000 (Medicell International Ltd, Liverpool Road, London) were used during the biosynthesis of dextran studies. About 5cm^3 fractions of sample were clamped within a piece of dialysis tubing and placed in a large volume of de-ionised water for 36-48 hours. The de-ionised water was changed regularly to aid the removal of any low MW contaminants.

4.5 OTHER ANALYTICAL TECHNIQUES USED

(1) C^{13} Nuclear Magnetic Resonance (NMR): This was used to analyse a sample of the dextran product from the SCCR-S. This was carried out at the Department of Chemistry in Birmingham University by Dr Baggett.

(2) Atomic Absorption (AA): The AA technique was used to analyse the amount of ions such as Na^+, K^+ and Ca^{2+} present in the dextransucrase solution used for the biosynthesis of dextran. The AA used was a Perkin Elmer Series 360 atomic absorption spectrophotometer.

4.6 ENZYME ASSAY TECHNIQUES

The activities of the enzymes maltogenase and lactase were determined using the HPLC method. The Hostettler's method[107] was used for the dextransucrase.

4.6.1 Maltogenase Assay

The activity of the enzyme maltogenase was measured in U/cm^3 (or μmol/min/cm^3) which is defined as the amount of enzyme required to produce 1 μmol of maltose in one minute at pH 5.3 and 60°C.

In the determination of the maltogenase activity, 2% soluble starch in an acetate buffer was incubated at 60°C with suitably diluted enzyme solution and mixed for 30 minutes. The reaction was then quenched by increasing the pH to about 10-12 using sodium hydroxide. The rate of maltose formation was calculated from the chromatographic data.

4.6.2 Lactase Assay

The determination of the lactase activity was carried out using 5%w/v lactose substrate in 0.1M sodium acetate buffer at pH 5.0 and temperature of 55°C. A suitably diluted amount of the enzyme was added to the lactose solution and incubated at 55 °C. The reaction was terminated after exactly 10 minutes by immersing the sample in a boiling bath of water. The amount of glucose produced was analysed on the HPLC and the lactase activity was expressed as μmol glucose produced/min/cm^3. One U of lactase activity is defined as the amount of enzyme required to produce 1 μmol of glucose in one minute at pH 5.0 and temperature 55°C.

4.6.3 Dextransucrase Assay

The Hostettler method[107] was used to determine the dextransucrase activity. This method has been used by previous workers[11,12,16,78,108] with dextransucrase. Although the HPLC method for dextransucrase activity analysis has the advantage that it allows both the sucrose and fructose concentrations to be followed, it suffers from the disadvantage that the time involved in carrying out this technique is considerable when compared to the Hostettler's method. Previous researchers have shown that there is more than 90% agreement between the Hostettler and HPLC methods.

The Hostettler's method is as follows:

1 cm^3 of enzyme containing 30-50 U/cm^3 is added to 4cm^3 of 6.25%w/v sucrose in 0.1M sodium acetate buffer at pH 5.2. Immediately, 0.5cm^3 of this mixture are taken out and the rest incubated in a water bath at 25°C. The 0.5cm^3 sample is added to 1cm^3 of Sumner reagent (10g 3-5 dinitrosalicylic acid, 300g potassium-sodium tartrate dissolved in 1 dm^3

of 0.4 μM NaOH), placed in boiling water for about 5 minutes, cooled and the volume made up to 12.5cm^3 with distilled water. This provides the unincubated sample.

Every 5 to 10 minutes, samples are taken from the reaction mixture and treated in a similar way for the next 20 minutes. The optical densities of the samples are read at 530nm against a blank solution on a spectrophotometer (Pye Unicam SP1800 Ultraviolet Spectrophotometer). The enzyme activity is then calculated from the following Equation:

$$\text{DSU cm}^{-3} = \frac{(OD_i - OD_u) \times d \times 60 \times 2}{OD_F \times 0.52 \times 0.2 \times t} \qquad 4.8$$

Where OD_i = Optical density at 530nm of incubated sample

OD_u = Optical density at 530nm of unincubated sample

d = Dilution factor of enzyme solution

t = Incubation time (minutes)

OD_F = Optical density of a 0.2%w/v fructose standard solution at 530nm (about 0.8±0.05)

One dextransucrase unit (DSU) is defined as the amount of enzyme which will convert 1 mg of sucrose to dextran and fructose in 1 hour at 25°C and pH 5.2.

4.7 DETERMINATION OF ENZYME KINETICS

The rate of action of the enzymes, maltogenase, lactase and dextransucrase on their specific substrates starch, lactose and sucrose respectively were studied by measuring the initial reaction rate for a range of initial substrate concentrations. These studies were carried out in a conventional batch reactor at a constant temperature and pH required for the reaction. Samples were removed from the reaction vessel at 5 minute intervals and placed into a test tube immersed in a boiling bath for about 5 minutes to quench the reaction. The samples were then analysed on the HPLC.

The data obtained from the HPLC were used to calculate the kinetic constants, maximum initial velocity (Vmax) and the reaction constant (Km) using the direct linear plot

method[67]. The substrate inhibition constant, Ki, was calculated from Equation 3.2 by differentiating the equation at maximum initial reaction velocity.

4.7.1 Maltogenase

The kinetic studies of the enzyme maltogenase were carried out using an enzyme activity of 60U/cm^3 at a temperature of 60°C and pH 5.3. The substrate was a modified starch (Crystal Gum, from National Starch and Chemical, Manchester, UK) with an average molecular weight of 5x10^5 daltons and dextrose equivalent (DE) of 3.0. The starch concentration was varied from 5-40% w/v.

Figure 4.3 shows that the maximum initial rate of reaction occurred at an initial substrate concentration of about 25% w/v. The plot shows that the initial stage of the reaction does not follow a zero-order kinetic since there was a reduction in the initial reaction velocity above a concentration of 25% w/v. The variation of the initial rate of the reaction is shown to follow the inhibited Michaelis-Menten model (Equation 3.2).

The reduction in the initial reaction velocity probably arises from substrate inhibition. The kinetic constants (Vmax and Km) were obtained from the direct linear plot (Figure 4.6). This gives a Vmax value of 1.15x10^{-2} g/cm^3/min and Km value of 0.125g/cm^3. These values are in close agreement with the values obtained by Sarmidi[50]. The substrate inhibition constant value was 0.52g/cm^3.

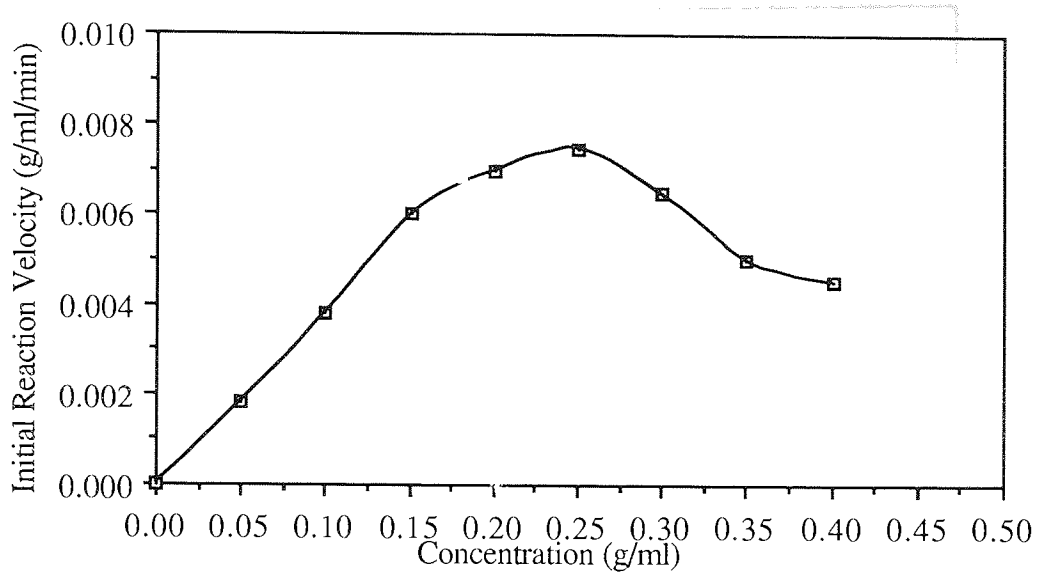


Figure 4.3:Variation of Initial Reaction Velocity with Starch Concentration Using the Enzyme Maltogenase

4.7.2 Dextransucrase

The kinetics of dextransucrase action in the biosynthesis of dextran were studied by measuring the initial reaction rate for a sucrose concentration range of 1.25-15% w/v. This study was carried out in a batch mode at constant temperature (25°C) and pH 5.2. Enzyme of activity 25DSU/cm^3 was used.

From this study, it was found that for sucrose concentrations of less than 8.5% w/v, the initial velocity increased linearly with sucrose concentration. This corresponds to that of a first order reaction. At sucrose concentrations greater than 10% w/v a decrease in the initial reaction velocity was observed (Figure 4.4). This is due to substrate inhibition. These results fit well with the modified Michaelis-Menten model (Equation 3.2).

The key parameters of the Michaelis-Menten model were calculated using the same procedure as in Section 4.7.1. The maximum velocity (Vmax) was calculated to be 0.089g/dm^3/min and Km was 6.1g/dm^3. These values also agree with those obtained by previous workers[16,109].

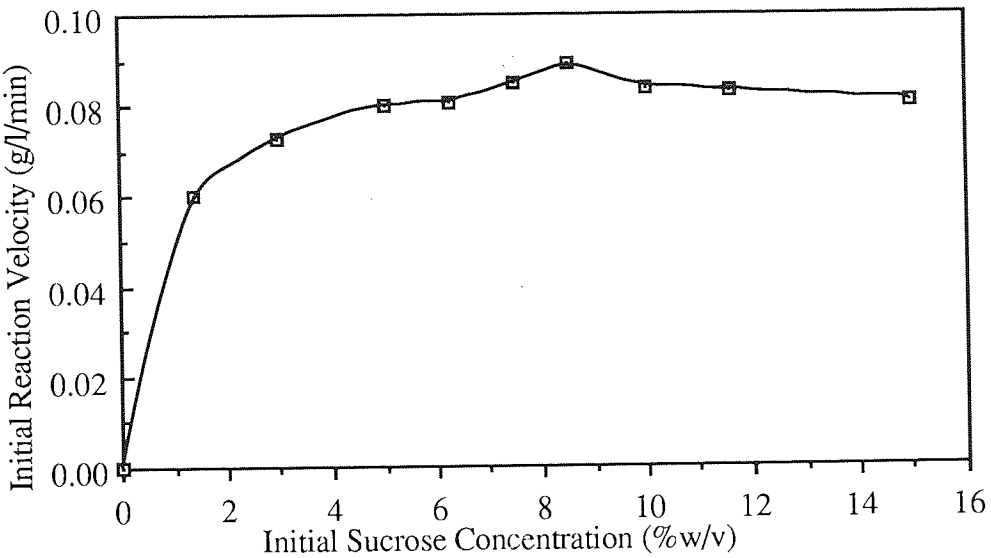


Figure 4.4: Variation of initial velocity with sucrose concentration

4.7.3 Lactase

Kinetic studies of the enzyme lactase with lactose as the substrate were carried out under initial reaction velocity conditions in a batch process at a pH of 5.0 and a temperature of 55°C. The lactose concentration varied from 1.0-15%w/v. The enzyme activity used was 60U/cm^3.

The results (Figure 4.5) show that the kinetics of the enzyme, lactase, fit well with the modified Michaelis-Menten model (Equation 3.2). The initial reaction velocity was found to increase linearly at lactose concentrations below 10%w/v and decrease at higher concentrations. This is due to inhibition by the substrate and one of the reaction products, galactose. The kinetic constants (Vmax and Km) were calculated using the same procedure as in Section 4.7.1.

The Km and Vmax values were 0.050g/cm^3 and 6.3x10^{-3}g/cm^3/min respectively. The value of Km is close to that obtained by Friend and Shanani[71]. Friend and Shanani[71] carried out extensive investigations to find the value of the inhibition constant, Ki, for the lactase enzyme. They found the value of Ki to be 1.7x10^{-3}g/cm^3.

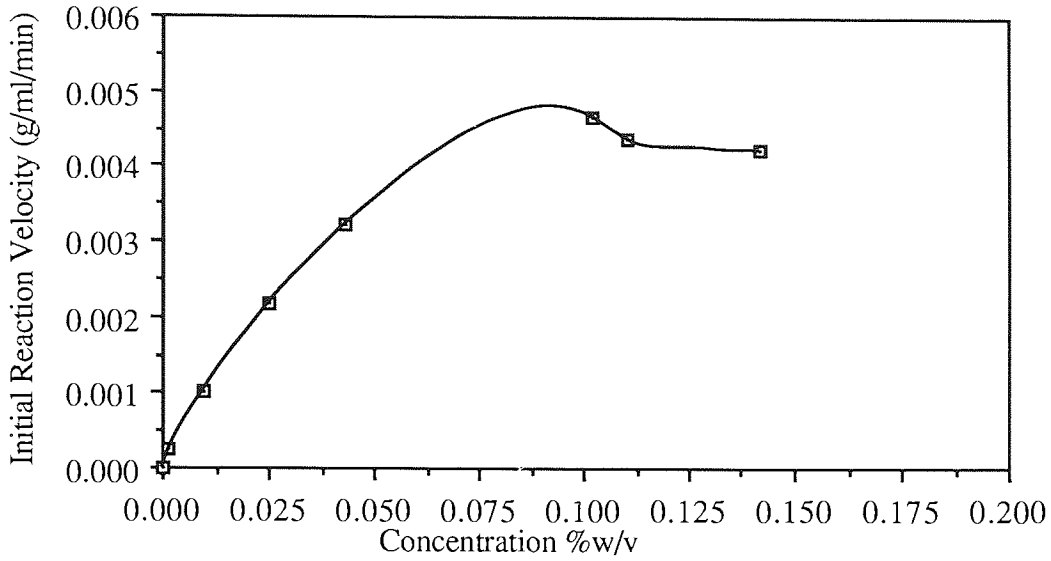


Figure 4.5: Variation of reaction velocity of the enzyme, lactase, with lactose concentration

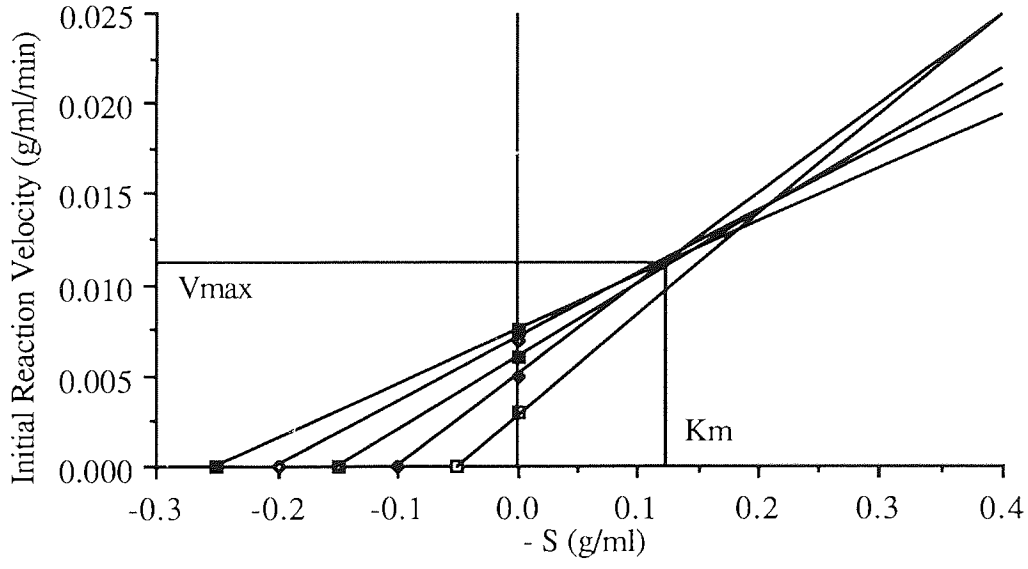


Figure 4.6: Direct Plot for the Enzyme Maltogenase

CHAPTER 5

DESCRIPTION, OPERATION AND CHARACTERISATION OF A SIMULATED COUNTER-CURRENT CHROMATOGRAPHIC BIOREACTOR-SEPARATOR SYSTEM (SCCR-S)

5.1 INTRODUCTION

The advantages of continuous chromatographic processes over batch processes, led Barker and co-workers [110,111] into developing the simulated counter-current chromatographic refiners (SCCR) which were successfully used in gas and liquid separations. These systems have been scaled up to column diameters of 10.8cm[112].

The SCCR-S system used in this work was originally constructed by Abusabah[113] for the separation of carbohydrate compounds. This system was modified and used successfully by Ganetsos[13] for his research work in the separation of carbohydrates.

Akintoye[16] modified the SCCR-S system to allow for simultaneous bioreaction and separation to take place. He then used this system to simultaneously invert sucrose and separate the products glucose and fructose[49].

The description of the SCCR-S is summarised in this section. The detailed description and design of this system has been reported by Barker and co-workers[110,112]. The principles of operation and characterisation of the SCCR-S system are presented in this chapter.

5.2 DESCRIPTION OF THE SCCR-S SYSTEM

5.2.1 Overview

The SCCR-S system consists of 12 stainless steel columns, 75 cm long and 5.4 cm internal diameter. Different types of packing material were used during the study. These included:

(1) Calcium charged 7% cross-linked polysterene resin (KORELA VO7C) supplied by Finn-Sugar Engineering (Finland) with a mean particle size of 270μm. This was used during the saccharification of modified starch studies.

(2) Calcium charged 4% cross-linked polysterene resin (DOWEX 50W-X4) supplied by British sugar. The mean particle size of this resin was 150μm. This was used for the hydrolysis of lactose studies.

(3) Calcium charged 5.5% cross-linked polysterene resin (PUROLITE PCR563) with a mean particle size of 450μm was used for the biosynthesis of dextran studies.

Figure 5.1 is a photograph of the SCCR-S used in this work, while the equipment layout is shown in Figure 5.2. The columns are mounted on a mobile frame and are surrounded by a constant temperature enclosure. The inlet and outlet lines are arranged in a ring distribution network connected to the supply pumps and product collection devices. Six pneumatic poppet valves were associated with each column namely the feed, eluent and purge inlet valves, the two product valves and the transfer valve to the next column. The sequential functioning of the poppet valves was governed by a pneumatic controller. A description of the individual units of the equipment is given in the following Sections.

5.2.2 The Columns and Fittings

The twelve columns were fabricated from type 321 seamless stainless steel tube and designed according to BS5500 : 1976 for pressure vessels. Each column was 75 cm long and 5.4 cm internal diameter and was fitted with ten 20 mm x 1/8 BSP sampling points. These sampling points were 2 cm long stainless steel tubes welded to the column. The tube ends were plugged with silicone rubber septa which were held in position by simplifix nuts. Samples were taken by inserting hypodermic needles through the septa into the columns.

The column inlet assembly consisted of a 65 x 40mm polypropylene inlet head and a compression plunger made of 130 x 19mm stainless steel rod and a 54 x 50μm

polypropylene piston. A polypropylene ring was fitted at the end of the piston and it retained a 100μm polypropylene mesh and a polypropylene distributor used to ensure an even velocity profile at the column inlet. Two Dowty O-rings, No. 20-830-4470 were fitted around the polypropylene piston to ensure perfect sealing. The first 10cm of the inlet of the column was accurately machined to obtain a good seal with the Dowty O-rings. Four 12.7 mm x 1/4 BSP ports were tapped on the inlet head to accomodate CK-1/4 PK4-KU type Festo plastic connectors for feed, eluent, purge and transfer lines. Inside these ports, 12mm x 2mm thick neoprene gaskets of 6mm diameter central hole were placed inside these ports to prevent leakages. Liquid channels of 3mm diameter were drilled through the head, the plunger and the piston.

The outlet assembly consisted of two parts, a stainless steel packing support and a 70 x 38mm polypropylene head machined to a T- shape to form a bottom 70 x 10mm flange. The packing support consisted of two meshes of different sizes sandwiched together. The top mesh was a 50μm mesh used to prevent fine resin particles falling through and blocking the liquid ducts and the bottom was a 100μm mesh which was used for supporting the bed. Three 12.7mm x 1/4 BSP ports were tapped on the outlet head and were fitted with the same Festo fittings as the inlet head. The ports were connected to the product lines and the transfer line. A 3^{o} cone was turned on the inside face of the outlet block to ensure uniform flow of liquid out of the column. A 2mm thick neoprene gasket was placed on each side of the support mesh to prevent leakages.

5.2.3 Poppet Valves and Pneumatic Controller

The control of liquid flow into and out of the columns and hence the counter-current operation of the SCCR-S was achieved by the pneumatic operation of 72 acting poppet valves. These valves were constructed by Aston Technical Services Ltd Birmingham. Some of the advantages of this type of valves are their high degree of reliability, smooth operation and application for a wide range of operations. These valves have a minimum internal volume, thus minimising the system dead volume. The switching sequence of these valves during a cycle has been illustrated by Ganetsos[13].

The valves were operated by compressed air supplied by the Department's main air compressor. A pressure relief valve, set at 700 KNm^{-2} was fitted on the main air supply line. The air supply was divided into bias and actuating streams whose pressures were controlled to 240 KNm^{-2} and 550 KNm^{-2} respectively by a pressure regulator. The bias supply line was branched and piped directly to the poppet valves via a ring main while the actuating lines were individually piped to the pneumatic controller. All the pneumatic lines were made from Festo PP3 polyamide tubing with 3 mm internal diameter. Figure 5.3 is a photograph of one of the poppet valves with its parts. In a closed position, the bias pressure was applied to the lower side and in the open position, the actuating pressure was applied to the upperside of the diaphram.

The operation of the poppet valves was governed by a pneumatic controller. This consisted of a number of solenoid valves activated individually, by a rotating Festo type PN-20B camshaft mechanism. Twelve of these solenoid valves were used for the twelve columns and each valve directly activated the appropriate transfer valve to isolate the corresponding column. The solenoid valves were each linked to double return valves which activated the appropriate feed, eluent, purge inlets and the product outlets. A digital timer programmable down to 10 seconds was used to control the rotating mechanism which in turn opened and closed the solenoid valves at a fixed time period. Any change to the sequence of operation of a poppet valve can be achieved by altering the relative positions of the coloured " program carriers " on the rotating mechanism. The timer was set at a preselected fixed time period which corresponded to the switch time, and at the end of that period an electrical signal was triggered, activating the electric motor to rotate the camshaft unit to the next position.

5.2.4 Fluid Delivery

An Elgastat B224 deioniser was used to provide the deionised water for the eluent and purging streams for the SCCR-S. The deionised water was stored in two large stainless steel tanks with a combined capacity of 750dm^3. The deionised water from the tanks was passed through a Millipore filter to remove any impurities present, then finally stored prior

to use in an elevated $0.12m^3$ polypropylene tank. The water in this tank enabled good control of the pH and minimised any possible hazard during overnight operation. The deionised water was fed from the tank by gravity through 4mm i.d Festo tubing and a solenoid valve into a $75dm^3$ heated glass container. The temperature and water level in the glass container was controlled by a CAL9000 temperature/level controller supplied by RS component Ltd., UK.

The purge was pumped through a flow measuring device into the SCCR-S system. When the eluent was a dilute enzyme solution, it was prepared by mixing deionised water and enzyme to the required strength. The eluent was then store in a $50dm^3$ polypropylene container at 4 ^oC to minimize the loss of enzyme activity. The eluent was then pumped into the SCCR-S system via a flow measuring device. When the eluent was deionised water with no enzyme, it was pumped directly from the container *via* the flow measuring device into the SCCR-S system.

The feed once prepared was stored inside an elevated $25dm^3$ polypropylene container at the required temperature and then fed through a flow measuring device into the SCCR-S system. For the experiments where the enzyme was fed into the SCCR-S system with the feed, the enzyme was prepared to the required strength and stored in a $25dm^3$ polypropylene container at 4 ^oC from which it was fed into the SCCR-S system *via* a flow measuring device. The enzyme and feed are then combined just before entering the system.

The set of pumps used during this research were the E-range metering pump series supplied by MPL Pumps Ltd. Middlesex, UK. Loading valves were fitted on all pump lines to control the flow of liquid through the pump and to prevent flowrate fluctuations due to pressure differences in the columns.

All the liquid supply lines were made of Festo PP4 and PP6 ployamide tubing and were colour coded. Alternate columns were inverted so that the outlet of one column can be connected to the inlet of an adjacent column with minimum tube lengths. This arrangement

reduced the system dead volume which was estimated to be about 4% of the total system volume. The products streams were collected in plastic containers.

5.2.5 Pressure Control

Four Beta pressure sensors (Loba Ltd, Reigate, Surrey) were fitted to the SCCR-S system, two on the eluent line, one on the purge inlet and one on the feed line inlet stream. The high pressure sensor settings were 345 KN/m^2 on the purge stream and 483 KN/m^2 on the eluent stream. The low pressure settings on the feed and eluent lines were 28 KN/m^2 each. These sensors were connected to the SCCR-S power supply board. In the case of an emergency such as valve blockage or a tube bursting, the system pressure will increase or decrease respectively. This would be detected by the pressure sensors and the electric supply to the SCCR-S system will be broken to switch the system off.

Smooth liquid flow was ensured by incorporating a pulsation damper on each of the eluent and feed lines. The dampers were made of stainless steel with nitrile rubber diaphrams.

5.2.6 Heating Facilities and Controls

The twelve columns, the valves and the fluid inlet and outlet networks were enclosed in a heated enclosure constructed from galvanised steel sheets lagged with 50mm thick glass fibre pads and covered with aluminium foil. A 5KW U-shaped finned air heater supplied by Elton Ltd. was used to heat the enclosure. The heater was controlled by a diamond DH82 type temperature controller. An electrical fan placed in the centre of the enclosure was used to circulate the hot air in the enclosure.

The temperature in the enclosure, on the column surface and in the liquid lines were monitored using a set of Nickel-Chrome thermocouples connected to a 12-way type K selector switch box supplied by RS components Ltd. The selector digitally displayed the temperature readings.

5.2.7 Product Splitting and Quenching Units

Two 3-way solenoid valves supplied by AJ Foster Ltd., Manchester, were installed in the two product lines of the SCCR-S system. These solenoid valves were controlled by a RS346-390 digital timer and used to separate the dilute product fractions from the concentrated fractions coming out from the SCCR-S system. After a preselected time the solenoid valves were switched from one position to the other allowing the splitting of the corresponding product into two separate fractions.

To quench the reaction as the products exit the SCCR-S system, a quenching unit was installed at the product lines to denature the enzyme during product collection. The quenching unit consisted of two glass heat exchangers, one for each product line connected to a heater/circulator unit supplied by Techne Ltd.

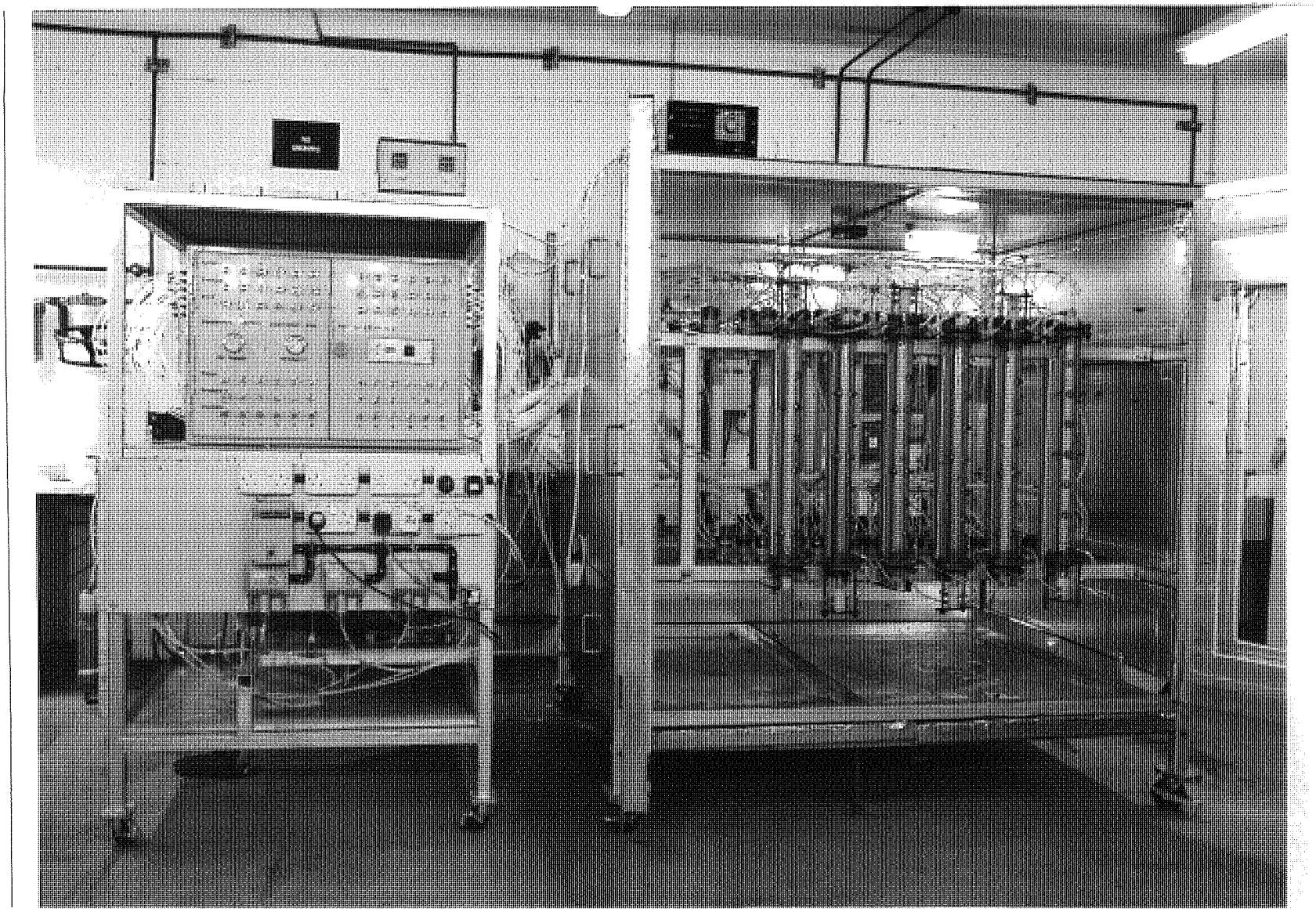


Figure 5.1: Photograph of the SCCR-S Equipment

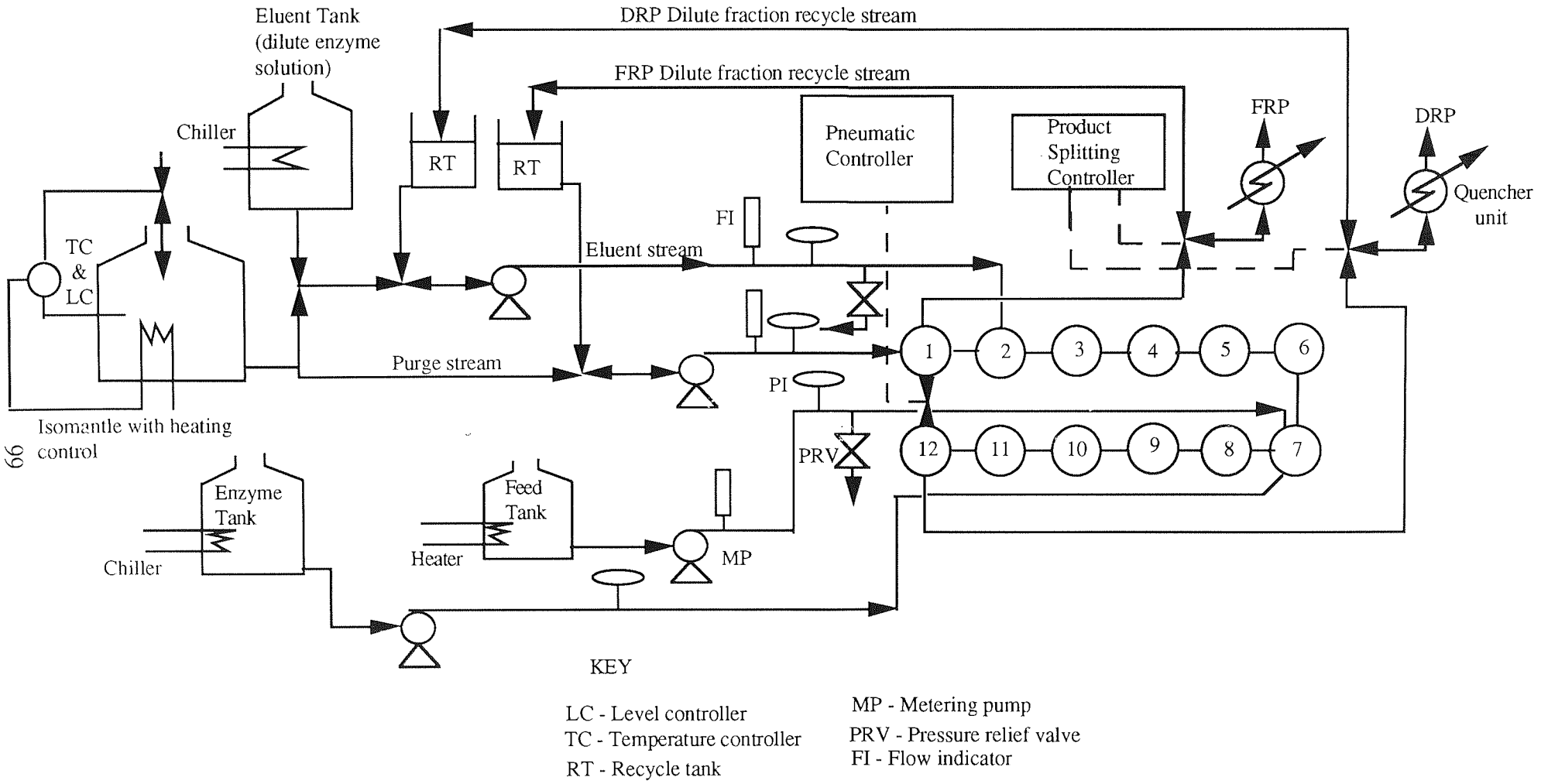


Figure 5.2: The SCCR-S Equipment Layout

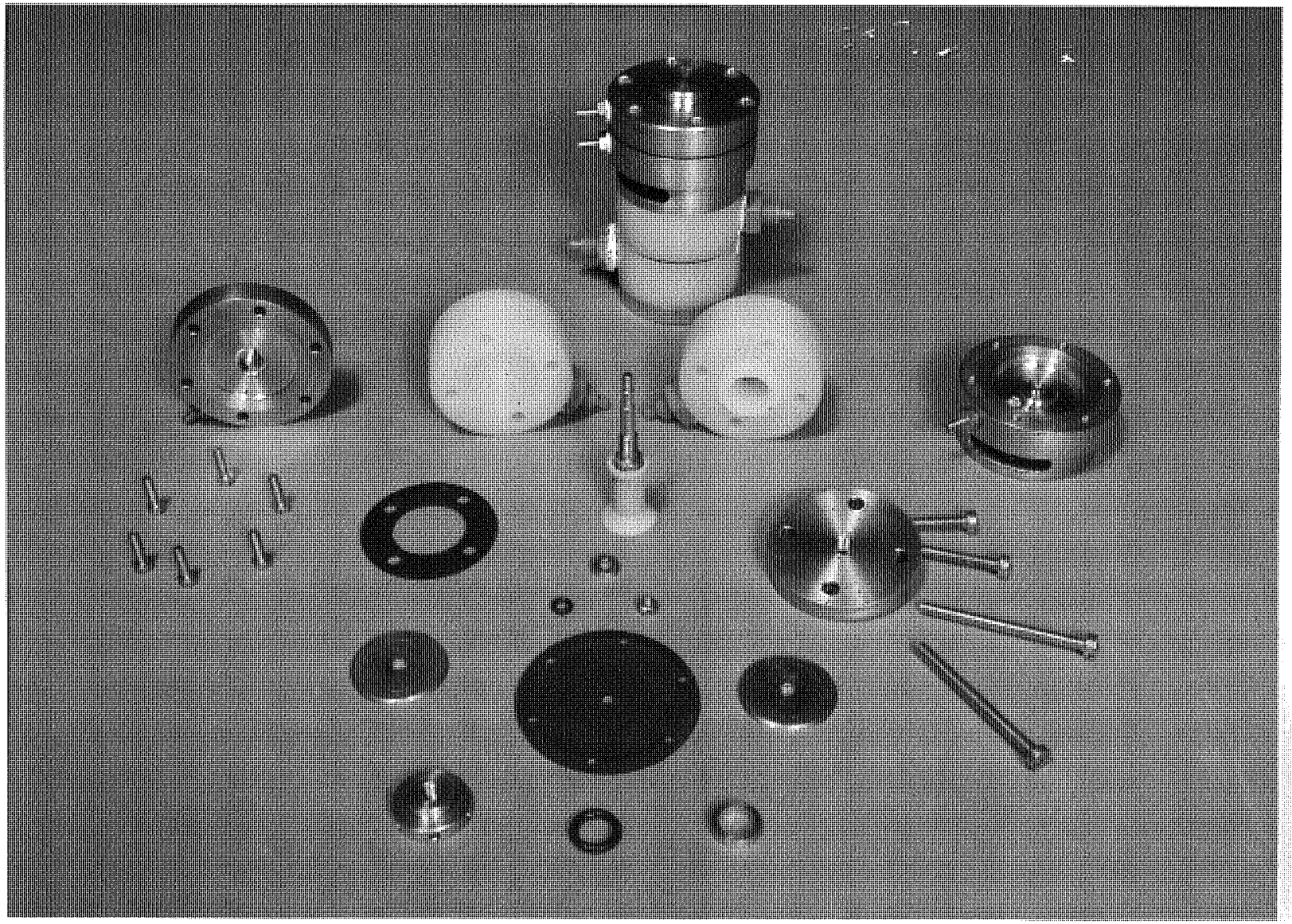


Figure 5.3: Photograph of the Pneumatic Poppet Valve and its Parts

5.3 PRINCIPLE OF OPERATION OF THE SCCR-S SYSTEM

The principle of operation of the SCCR-S system can be illustrated using the biosynthesis of dextran as a model bioreaction. The bioreaction can be represented by:

$$\underset{\text{Sucrose}}{n(C_{12}H_{12}O_{11})} \xrightarrow{\text{Dextransucrase}} \underset{\text{Dextran}}{(C_6H_{10}O_5)_n} + \underset{\text{Fructose}}{n(C_6H_{12}O_6)} \qquad 5.1$$

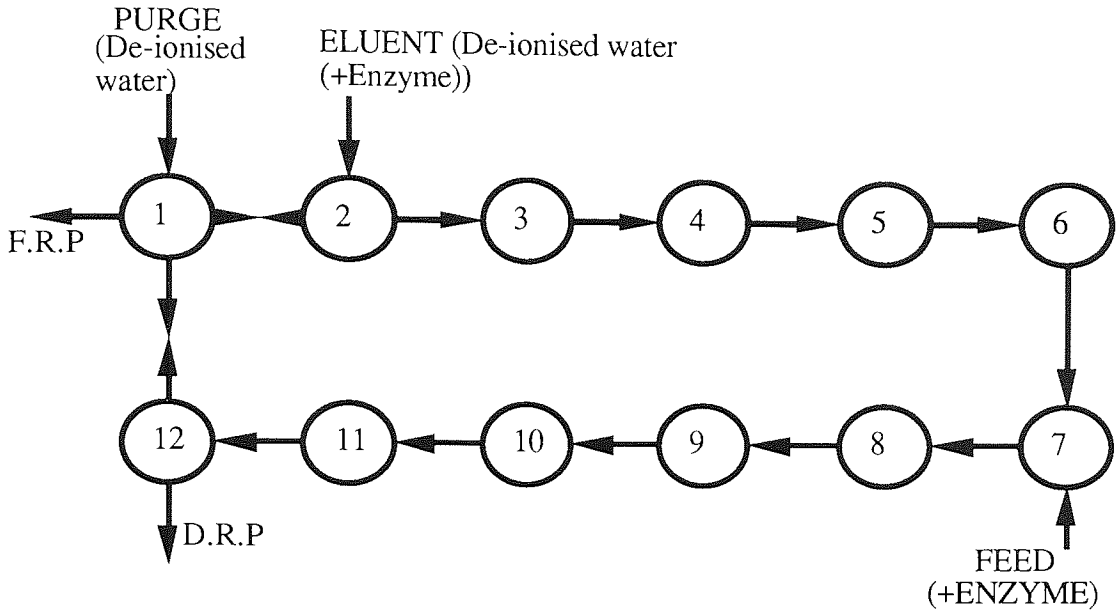
The principle of operation is shown schematically in Figure 5.5 where the whole system is illustrated as a closed loop.

The eluent/enzyme (mobile phase) enters the system at port M. In the case where the enzyme is not added to the eluent, the enzyme enters the system at port F. The feed is also fed at port F. On contact with the enzyme, the bioreaction takes place produces dextran and fructose. The dextran and unreacted sucrose move preferentially with the mobile phase towards the dextran rich product (DRP) take off. The fructose forms a complex with the calcium ions on the resin and is retarded. A Section of the loop is isolated at anytime by two valves V1 and V2, and an independent purge stream (deionised water) enters at port N, removing the adsorbed fructose and exits from the fructose rich product (FRP) end of the system.

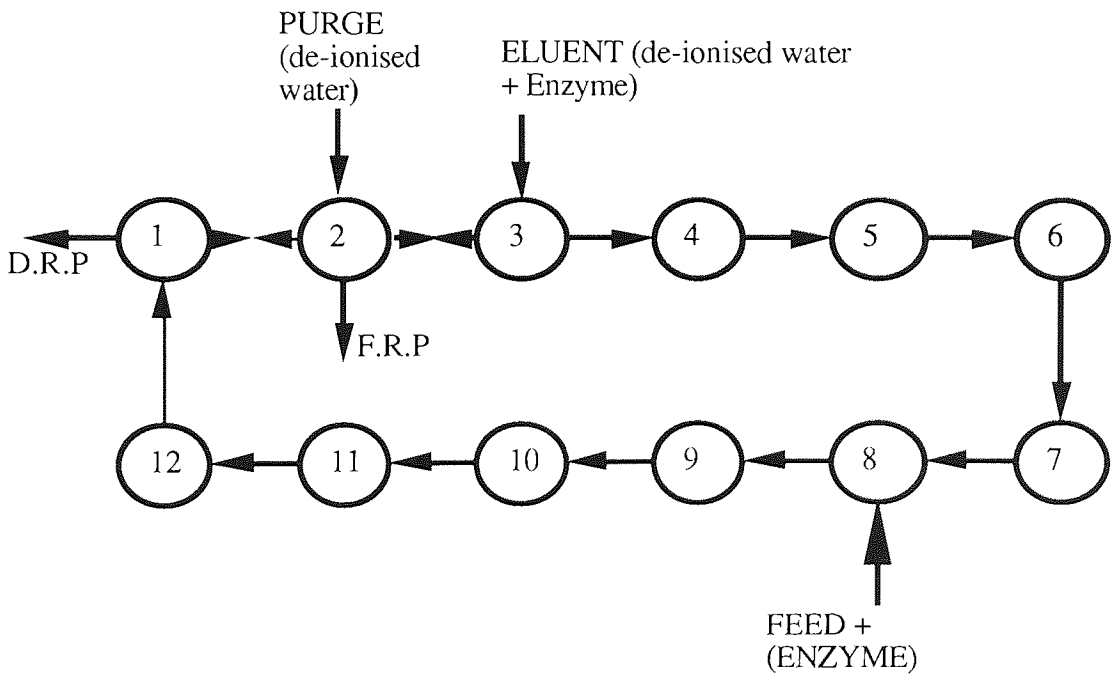
The distribution of the components, sucrose, dextran and fructose within the system soon after "start up " is shown in Figure 5.5(a). In Figure 5.5(b), all the port functions have been advanced by one position in the direction of the mobile phase flow. This port advancement results in a simulated movement of the stationary phase. To achieve separation of the reaction products and hence two enriched product streams, the rate of port advancement must be greater than the more strongly adsorbed fructose migration velocity through the bed and lower than the migration velocities of the dextran and unreacted sucrose, Figure 5.5(c). The frequency with which this port advancement occurs is known as the "switch time ".

Figure 5.4 shows the counter-current operation mode of the SCCR-S system. Figure 5.4(a) represents the first switch period where column 1 is isolated and purged to give the fructose rich product (FRP). The feed / enzyme and eluent / enzyme enter columns 7 and 2 respectively and the dextran rich product (DRP) is eluted from column 12. In the second switch period, Figure 5.4(b), all ports are advanced by one position, now column 2 is purged, feed / enzyme and eluent / enzyme enter columns 8 and 3 and DRP exits from column 1. After twelve such advancement (12 columns), a "CYCLE" is completed. Previous workers [13,16] have shown that it takes about 6 cycles for the SCCR-S to reach steady state.

(A) SWITCH ONE

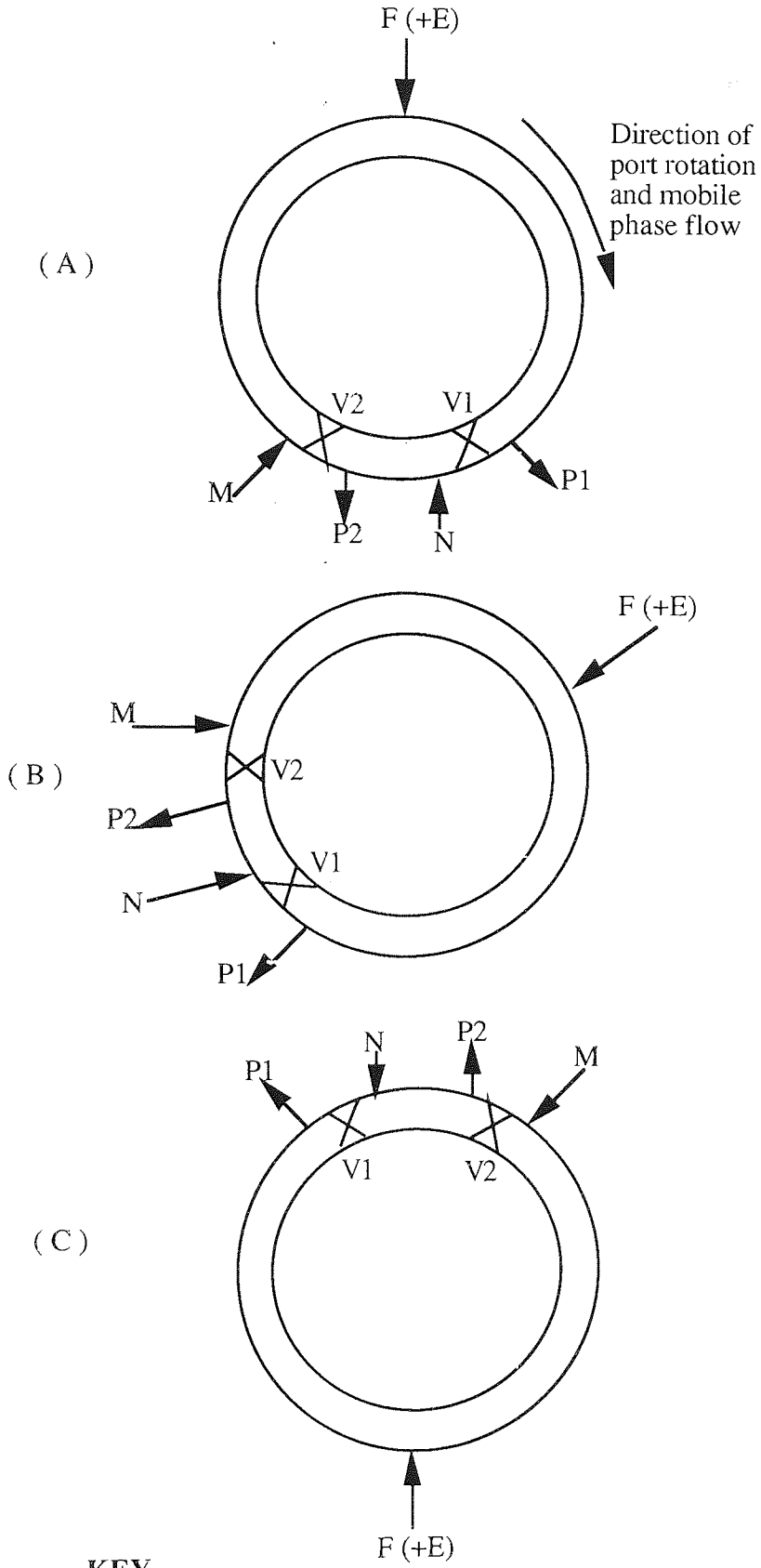


(B) SWITCH TWO



DRP - DEXTRAN RICH PRODUCT
FRP - FRUCTOSE RICH PRODUCT

Figure 5.4: Sequential Operation of the SCCR-S System



KEY

M = Mobile phase inlet
N = Purge inlet
F = Feed (+ Enzyme) inlet
P1 = Dextran rich product
P2 = Fructose rich product
V1, V2 = Transfer valves

Figure 5.5: Principle of Operation of the SCCR-S System

5.3.1 Idealised Operating Conditions

When the biosynthesis of dextran is performed on the SCCR-S system, the dextran produced and the unrecated sucrose move with the mobile phase while the fructose complexes with Ca^{2+} ions on the resin and is retarded thereby moving with the stationary phase. An idealised model can be constructed relating the mobile phase and stationary phase flowrates, reaction rate and component separation. Assuming that the reaction takes place only in the mobile phase, a material balance on the dextran product about the feed point (Figure 5.6) gives :

$$Le.Y_d + P.X_d + V_l.r = 0 \qquad 5.2$$

Where, V_l = mobile phase volume (cm^3)

Le = mobile phase flowrate (cm^3/min)

P = stationary phase effective flowrate (cm^3/min)

= (Total stationary phase volume) / (Total number of columns x switch time)

r =Reaction rate (g/cm^3/min)

Y_d = dextran concentration in mobile phase (g/cm^3)

X_d = dextran concentration in the stationary phase (g/cm^3)

For dextran to move preferentially with the mobile phase and where the reaction is taking place in the mobile phase,

$$(Le.Y_d + V_l.r) > P.Xd \qquad 5.3$$

Dividing by P and Y_d and rearranging gives :

$$\frac{Le}{P} + \frac{V_l.r}{P.Y_d} > \frac{X_d}{Y_d} \qquad 5.4$$

By defination $Kd_D = \frac{X_d}{Y_d}$ 5.5

Then $$\left(\frac{Le}{P} + \frac{V_l.r}{P.Y_d}\right) > Kd_D \qquad 5.6$$

For sucrose to move with the mobile phase,

$$\left(\frac{Le}{P} - \frac{V_l.r}{P.Y_s}\right) > Kd_S \qquad 5.7$$

Similarly for fructose to move with the stationary phase,

$$\left(\frac{Le}{P} + \frac{V_1.r}{P.Y_f}\right) < Kd_F \qquad 5.8$$

The theoretical limits of the mobile and stationary phase to obtain good separation of dextran and fructose by combinning Equations 5.6 and 5.8 is,

$$Kd_D < \left(\frac{Le}{P} + \frac{V_1.r}{P.}\left(\frac{1}{Yd} + \frac{1}{Yf}\right)\right) < Kd_F \qquad 5.9$$

As each column contains eluent phase in the void volume, Vo, the effective mobile phase flowrate is reduced to,

$$Le = L_1 - \frac{Vo}{S} \qquad 5.10$$

where L_1 = mobile phase inlet flowrate (cm^3/min)

S = switch time (min.)

Because of the feed flowrate, L_2, the effective mobile phase flowrate is different before and after the feed point. Thus the effective mobile phase rate in the post feed Section Le', becomes

$$Le' = Le + L_2 = (L_1 + L_2) - \frac{Vo}{S} \qquad 5.11$$

In the case where the feed and enzyme enter the system at the same port,

$$L_2 = \text{Feed flowrate + enzyme flowrate } (cm^3/min) \qquad 5.12$$

Therefore Equation 5.9 becomes:

$$Kd_D < \left(\frac{Le}{P} + \frac{V_1.r}{P.}\left(\frac{1}{Yd} + \frac{1}{Yf}\right)\right) < \left(\frac{Le'}{P} + \frac{V_1.r}{P.}\left(\frac{1}{Yd} + \frac{1}{Yf}\right)\right) < Kd_F \qquad 5.13$$

This Equation gives the true theoretical limits.

The purging flowrate L_3 in the isolated column is also governed by:

$$\frac{L3}{P} >> Kd_F \qquad 5.14$$

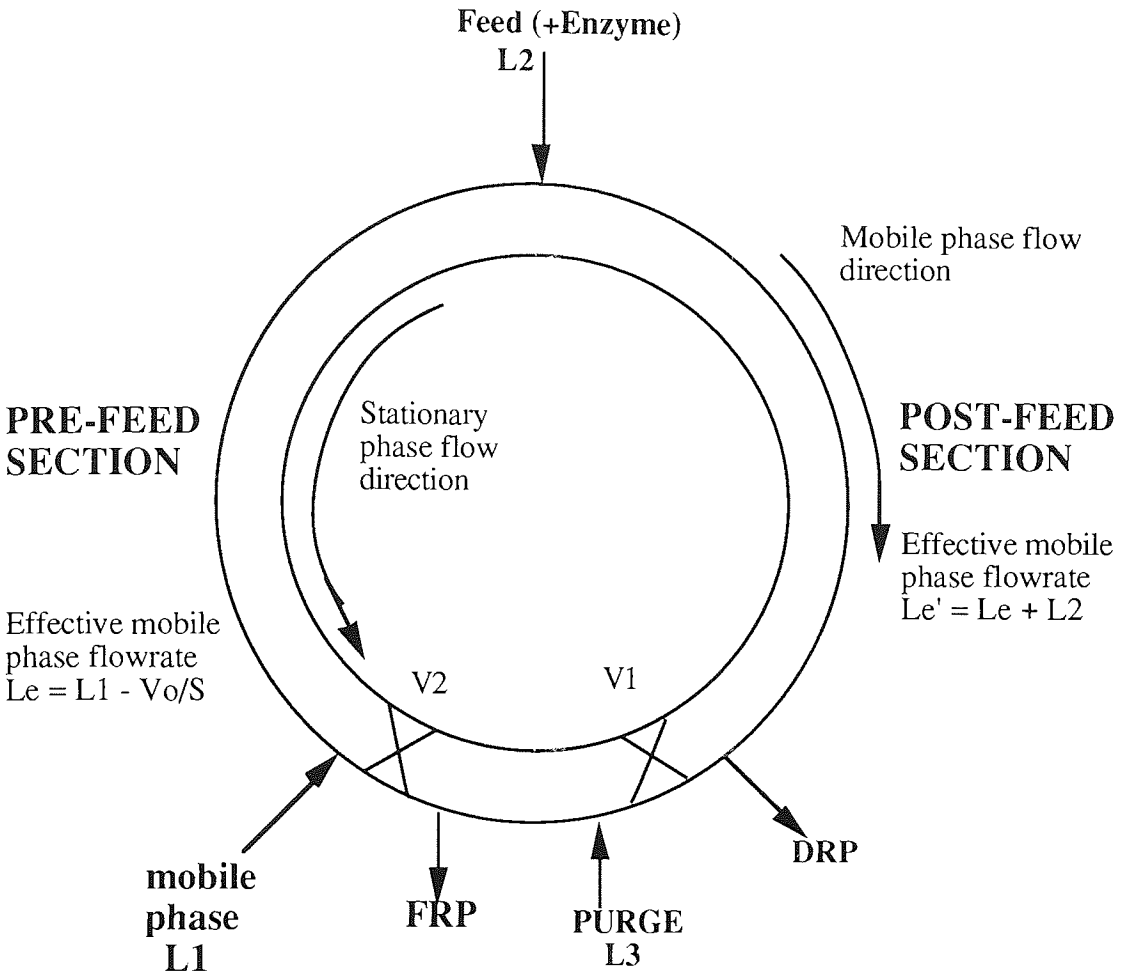


Figure 5.6: Diagramatic Representation of the SCCR-S Principle of Operation

5.4 COLUMN CHARACTERISATION

Characterisation of the SCCR-S system was achieved by individually characterising the 12 columns of the system. Characterising a chromatographic column enables one to investigate the separating capacity of the packing material. This was carried out on a batch mode and at infinite dilution conditions, i.e at very low sugar concentrations. Although, in practice, high sugar concentrations are encountered, correlations have been developed[13] to accomodate for this effect. The methods used for the packing and characterisation of the columns were similar to those employed by previous workers[13,16]. This allowed direct comparison with previous work.

5.4.1 Ion Exchange Resins

Ion exchange resins are particles consisting of porous matrices with electrically charged functional groups which are covalently bonded. Four main types exist:

(1) Synthetic resins;

(2) Cellulose ion exchangers;

(3) Ion exchange polydextran (Sephadex);

(4) Inorganic exchangers based on aluminium silicone.

The synthetic resins most commonly used in chromatographic processes are solid insoluble high molecular weight polyelectrolytes. They consist of a three dimensional matrix with a large number of attached ionizable groups. These matrices are produced by polymerization of styrene cross-linked with itself and divinyl-benzene (DVB).

The process of cross-linking is easily controlled. The degree of cross-linking is very important in chromatography. It defines the average porosity of the exchangers. The more the cross-linking, the less the exchanger swells. Swelling is known to disturb the chromatographic operation due to increase in pressure especially when resins of low cross-linking are used. Lower cross-linked resins exchange ions more rapidly but are less selective.

In this research work, synthetic cation exchangers were used and the mechanism of operation is as follows:

$$R{-}X^+ + Y^+ \rightleftharpoons R{-}Y^+ + X^+$$

The forward process is called adsorption and the reverse desorption. Equilibrium is gorverned by the concentration of the solute ions (Y^+) and the relative affinities of the ions for the exchangers.

5.4.2 Column Packing Technique

Before packing the columns with the resin, a settling technique was used to remove the fines present in the resin which could cause blockages in the valves, column inlet and outlet ports.

The resin slurry contained an equal volume of deionised water and resin and was added to the column at the same rate as water was removed by a vacuum suction. This slurry packing method was used to minimise the segregation of particles apparent in gravitational settling. During packing, deionised water was added continuously and the column tapped at random. When the packing reached a height of about 66cm, the procedure was stopped. The inlet plunger was then fitted and the column reassembled. Deionised water was pumped through the packed column for an hour to remove any fines remaining.

5.4.3 Experimental Technique Used for Column Characterisation

The columns in the SCCR-S system were characterised everytime the columns were repacked either with the same resin or a different one and after prolonged usage to check the performance.

Each packed column in the system was individually linked to the eluent delivery pump and a sample detection system as shown in Figure 5.7. A T-piece was connected as close as possible to the column inlet with a silicon rubber septum fitted to one of the T-piece inlets. The sample was injected into the column by inserting a needle through the septum. A portion of the stream from the outlet was passed through the detection system which consisted of, a peristaltic pump, a refractometer and a chart recorder. An eluent flowrate of 25cm^3/min was used for the characterisation of the columns.

The columns were characterised for :

(1) The saccharification of modified starch. These columns were packed with the KORELA VO7C resin with mean particle size of 270μm. Separate 1.0% w/v solutions of dextran T70 and maltose were made and 10 cm^3 of each of these solutions were injected

through the inlet rubber septum. A peak was produced on the chart recorder as the samples were eluted (Figure 5.8). The results obtained are shown in Table 5.1.

(2) The hydrolysis of lactose : The columns were packed with the DOWEX 50W-4X resin with mean particle size of 150 μm. The same procedure was used as for the saccharification. The samples injected were dextran T70, lactose, glucose and galactose solutions. The results obtained are presented in Table 5.2.

(3) The biosynthesis of dextran : For these studies, the columns were packed with PUROLITE PCR563 resin with mean particle size of 450μm. The samples used for the characterisation were dextran T70, sucrose and fructose solutions of 1.0%w/v. The results obtained are shown in Table 5.3.

All the characterisation experiments were carried out at 25°C with eluent flowrates of 25 cm^3/min.

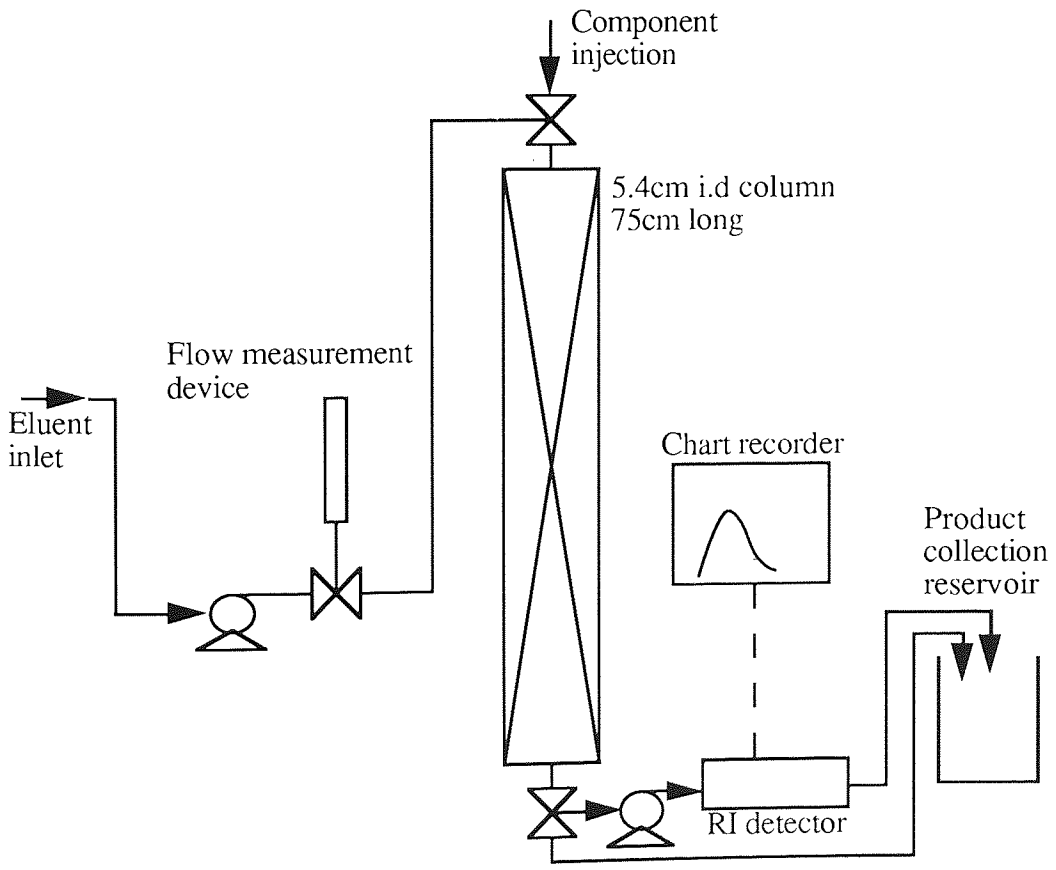


Figure 5.7: Equipment Set-up for Column Characterisation

5.4.4 Calculation of Column Parameters

The column parameters were calculated from the elution profiles of the samples injected into the columns (Figure 5.8). The elution volume, Vi, of the individual components were calculated by multiplying their respective retention times, T_{Ri}, by the eluent flowrate.

Dextran has a very large and wide molecular weight range. Due to their molecular size, the dextran molecules only travel through the void volume and do not diffuse into the pores of the resin. The elution volume of dextran T70 was therefore used to measure the void volume Vo, of the column. A measure of the separation potential of the column is the separation factor, α, and for two components say sucrose and fructose mixture :

$$\alpha = \frac{Kd_F^{\infty}}{Kd_S^{\infty}} \qquad 5.15$$

where,

Kd_F^{∞} = distribution coefficient of fructose at infinite dilution

Kd_S^{∞} = distribution coefficient of fructose at infinite dilution

$$Kd_i^{\infty} = \frac{V_i - V_o}{V_T - V_o} \qquad 5.16$$

where V_T = total volume of the column

The apparent number of theoretical plates, (N*), with respect to the individual components was calculated from the corresponding chromatogram using the Gluekauf Equation[26] shown below.

$$8\left(\frac{T_{Ri}}{W^{h/e}}\right)^2 \qquad 5.17$$

where, $W^{h/e}$ is the band width measured at a height, h, divided by the base of the natural logarithm, e, (Figure 5.8).

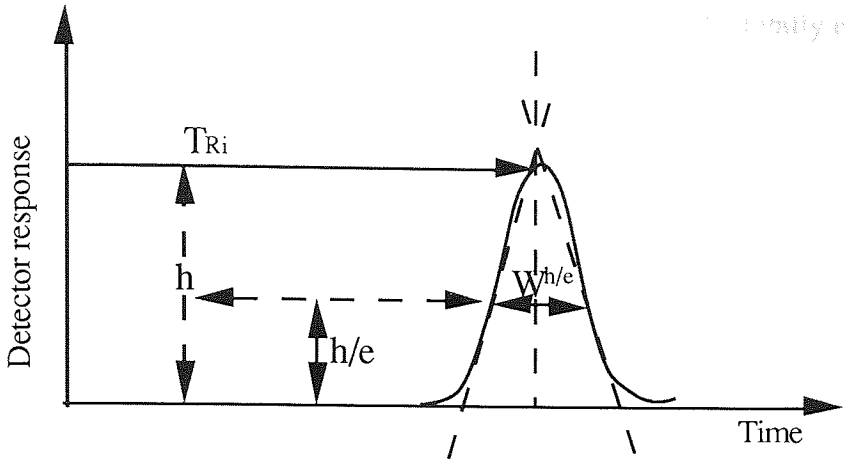


Figure 5.8: Elution Profile of a Component Injected into a Column during Characterisation of the SCCR-S System

5.4.4.1 Results and Discussion

The results for each column and the average values obtained during characterisation of the columns in the SCCR-S system are shown in Tables 5.1, 5.2 and 5.3. The average column voidage when the columns were packed with KORELA VO7C resin, DOWEX50W-X4 resin and PUROLITE PCR563 were 0.35, 0.34 and 0.32 respectively. These values are lower than the theoretical voidage of a bed of spherical particle reported to be 0.4[114]. The different packing geometry and the non-uniformity of the packing material is believed to be the cause of these low voidage values. The smaller particles were despersed and filled the empty space between the large ones and was enchanced by the compression exerted by the plunger.

The packing of the SCCR-S columns was reasonably consistent since most of the voidages were close to the average with variations of up to ± 8.5%, ± 8.8% and ± 12.5% for the columns packed with KORELA VO7C, DOWEX50W-X4 and PUROLITE PCR563 respectively.

During the characterisation of the columns for the saccharification of modified starch, the dextran molecules were totally excluded and were not delayed. The maltose molecules were retarded due to size exclusion and chemical complex formation with the calcium ions on the resin thus giving a higher elution volume than the dextran. The unreacted starch,

dextrins, which also have very high molecular weights would also be totally excluded, and were assumed to have the same elution volume to dextran (Table 5.1).

When the columns were characterised for the hydrolysis of lactose experiments, the lactose molecules diffused slightly into and out of the pores and hence were delayed slightly with respect to dextran. The glucose molecules, due to their small sizes compared to the lactose molecules, diffuse more into the pores and hence were delayed more than the lactose molecules. The result of this was a larger elution volume for glucose than lactose. The galactose molecules were retarded due to a complex formation with the calcium ions on the resin, thus the elution volume of galactose was higher than that of glucose.

The separation factor for a lactose and galactose mixture was found to be 1.86 and for a glucose and galactose mixture it was 1.37 (Table 5.2). Comparing this to the glucose and fructose mixture studied by Akintoye[16] which had a separation factor of 2.27 means the glucose and galactose mixture is more difficult to separate even with the smaller particle size resin used.

The colums for the biosynthesis of dextran were packed with PUROLITE PCR563 resin with a mean particle size of 450μm. The large particle size resin was used because high pressure was expected due to the viscosity of dextran and because the separation between dextran and fructose is relatively easier. The dextran molecules are totally excluded while the fructose molecules due to their smaller size and complex formation with the calcium ions on the resin were retained thus giving higher elution volumes. The separation factor for a sucrose/fructose mixture was found to be 9.17.

Variation in voidage, distribution coefficient and HETP values from column to column are due to the non-uniformity of packing and the different degree of calcium charging of the individual columns. Ideally all these column parameters should be constant in order to ensure similar migration rates of the individual components through successive columns. However, these variations can be tolerated in the eleven serially linked columns of the

Table 5.1: SCCR-S Columns Packed with KORELA VO7C Resin (270μm mean) Characterisation Result

Column No.	Packed Height (cm)	Elution Volume cm^3		Infinite Dil. Distribution Coefficient w.r.t	Number of Theoretical Plates w.r.t	HETP (cm)	Voidage "e"
		Dextran (Vo)	Maltose (Vm)	Maltose	Maltose		
1	65.0	500.0	635.0	0.14	34.0	1.91	0.34
2	66.0	505.0	650.0	0.14	41.0	1.61	0.33
3	65.5	520.0	640.0	0.12	36.4	1.80	0.35
4	65.0	470.0	645.0	0.17	37.0	1.76	0.32
5	66.0	535.0	660.0	0.13	37.5	1.76	0.35
6	66.0	550.0	660.0	0.12	40.0	1.65	0.36
7	65.0	535.0	635.0	0.11	32.5	2.00	0.36
8	65.0	515.0	670.0	0.16	34.0	1.91	0.35
9	65.5	570.0	675.0	0.11	34.5	1.90	0.38
10	65.5	550.0	655.0	0.11	32.5	2.02	0.37
11	66.0	530.0	635.0	0.11	33.0	2.00	0.35
12	65.0	515.0	650.0	0.14	34.0	1.91	0.35
Average	**65.5**	**524.6**	**650.8**	**0.13**	**35.5**	**1.85**	**0.35**

Table 5.2: The SCCR-S columns packed with DOWEX 50W-X4 (150μm mean) characterisation results

Column No.	Bed height (cm)	Infinite Dilution Distribution Coefficient			Number of theoretical plates w.r.t			HETP/cm w.r.t			Voidage "e"
		Lac.	Gluc	Gal.	Lac.	Gluc.	Gal.	Lac.	Gluc	Gal.	
1	65.0	0.30	0.38	0.51	65.0	109.0	96.0	1.0	0.60	0.68	0.34
2	65.5	0.27	0.39	0.53	78.0	125.0	100.0	0.84	0.53	0.66	0.35
3	65.5	0.27	0.40	0.55	66.0	101.0	95.0	0.99	0.65	0.69	0.36
4	65.0	0.30	0.42	0.55	69.0	114.0	105.0	0.94	0.57	0.62	0.35
5	65.0	0.29	0.37	0.52	81.0	100.0	102.0	0.80	0.65	0.64	0.32
6	66.0	0.30	0.41	0.54	65.0	102.0	96.0	1.02	0.64	0.69	0.34
7	65.5	0.30	0.41	0.53	61.0	100.0	92.0	1.07	0.66	0.71	0.33
8	65.5	0.25	0.30	0.48	62.0	100.0	95.0	1.06	0.66	0.69	0.31
9	65.0	0.24	0.34	0.48	62.0	104.0	104.0	1.05	0.63	0.63	0.33
10	65.5	0.26	0.36	0.49	65.0	95.0	93.0	1.01	0.69	0.70	0.32
11	65.5	0.23	0.33	0.47	69.0	90.0	90.0	0.95	0.73	0.73	0.33
12	65.5	0.28	0.37	0.51	66.0	93.0	95.0	0.99	0.70	0.69	0.35
Average	**65.4**	**0.28**	**0.38**	**0.52**	**67.4**	**100.7**	**96.9**	**0.97**	**0.65**	**0.68**	**0.34**

Table 5.3: SCCR-S Columns Packed with PUROLITE PCR563 Resin (450μm mean) Characterisation Results

Column No.	Packed Height (cm)	Infinite Dil. Distribution Coefficient w.r.t	Number of Theoretical Plates w.r.t	Infinite Dil. Distribution Coefficient w.r.t	Number of Theoretical Plates w.r.t	HETP (cm) w.r.t		Voidage "e"
		Sucrose	Sucrose	Fructose	Fructose	Sucrose	Fructose	
1	67.0	0.10	57.9	0.58	8.60	1.16	7.79	0.28
2	65.5	0.05	35.7	0.52	8.83	1.84	7.44	0.31
3	65.5	0.04	44.3	0.5	10.0	1.48	6.55	0.32
4	65.0	0.07	30.5	0.46	8.5	2.12	7.65	0.34
5	66.0	0.06	45.4	0.60	9.0	1.44	7.33	0.33
6	65.0	0.03	50.0	0.61	8.4	1.32	7.74	0.33
7	65.0	0.06	52.7	0.56	9.0	1.24	7.22	0.31
8	65.7	0.07	48.8	0.52	10.4	1.36	6.32	0.31
9	65.7	0.04	55.0	0.51	9.2	1.20	7.14	0.32
10	65.0	0.05	36.6	0.54	10.1	1.76	6.44	0.32
11	65.0	0.07	38.9	0.51	8.9	1.68	7.30	0.31
12	66.5	0.07	41.0	0.65	9.0	1.64	7.39	0.3
Average	**65.6**	**0.06**	**44.7**	**0.55**	**9.16**	**1.52**	**7.19**	**0.32**

SCCR-S system and the continuous cyclic operation ensures essentially constant component migration rates through the system.

5.4.5 Effects of Eluent Flowrate and background Concentration on Kd.

Thawait[115] and Ganetsos[13] in their research work have shown that operating conditions such as eluent rate and solute background concentration affect the distribution coefficient of sugars in a chromatographic column. Ganetsos[116] developed linear correlations linking the eluent flowrate expressed as linear velocity (flowrate over column cross Sectional area) and expressing the variation in distribution coefficients in percentage terms for glucose, fructose and dextran mixtures.

During this work, it was intended to also computer simulate the hydrolysis of lactose on the SCCR-S unit. It was therefore necessary to develop similar correlations for lactose, glucose and galactose for incorporation in the model. These experiments were carried out using the analytical HPLC system described in Chapter 4. The analytical column in the system was repacked with the KORELA VO7C resin (270μm mean). For the effect of eluent flowrate (deionised water), the flowrate was varied from 0.2 to 1.0 cm^3/min. For the effect of background concentration, the concentration of the sugar in the eluent was varied from 0%w/v to 30%w/v. All the experiments were carried out at 55°C. The results obtained are shown in the following tables :-

Table 5.4: Effects of Flowrate on the Distribution Coefficients

Linear Vel. (LV) (cm/min)	Lactose $K^{\infty}d_L$	% Change in $K^{\infty}d_L$	Glucose $K^{\infty}d_G$	% Change in $K^{\infty}d_G$	Galactose in $K^{\infty}d_F$	% Change in $K^{\infty}d_F$
0.52	0.167	126.5	0.251	110.9	0.326	107.6
1.04	0.132	100.0	0.228	100.0	0.303	100.0
1.56	0.108	81.8	0.207	90.8	0.278	91.8
2.08	0.106	80.0	0.190	83.3	0.239	78.9
2.60	0.089	67.4	0.181	79.4	0.233	76.9

From the above results, Equations 5.18, 5.19 and 5.20 were developed.

For lactose,

$$Kd_L = (116.79 - 15.327xLV) \times K^{\infty}d_L/100 \qquad 5.18$$

For glucose,

$$Kd_G = (132.6 - 26.577xLV) \times K^{\infty}d_G/100 \qquad 5.19$$

For galactose,

$$Kd_F = (114.20 - 14.327xLV) \times K^{\infty}d_F/100 \qquad 5.20$$

Table 5.5: Effect of Glucose Background Concentration on the Distribution Coefficients

Background Conc.(Cg) (%w/v)	Lactose Kd_L	% Change in Kd_L	Glucose Kd_G	% Change in Kd_G	Galactose in Kd_F	% Change in Kd_F
0.0	0.140	100.0	0.230	100.0	0.304	100.0
10.0	0.150	107.1	-	-	0.333	109.5
15.0	-	-	0.248	107.8	0.348	114.5
20.0	0.152	108.6	0.309	134.4	0.362	119.1
30.0	0.164	117.2	0.376	163.5	0.391	128.6

The linear correlations for the effect of background concentration of glucose were shown to be:-

For Lactose,

$$K^{G}d_L = (100.0 + 57.0xCg) \times K^{\infty}d_L/100 \qquad 5.21$$

For glucose,

$$K^{G}d_G = (92.017 + 211.67xCg) \times K^{\infty}d_G/100 \qquad 5.22$$

and for galactose,

$$K^{G}d_F = (100.0 + 95.333xCg) \times K^{\infty}d_F/100 \qquad 5.23$$

Table 5.6: Effect of Galactose Background Concentration on the Distribution Coefficients

Background Conc.(Cg) (%w/v)	Glucose Kd_G	% Change in Kd_G	Galactose in Kd_F	% Change in Kd_F
0.0	0.231	100.0	0.308	100.0
10.0	-	-	0.329	106.8
15.0	0.241	104.3	0.347	112.7
20.0	0.256	110.8	-	-
30.0	0.275	119.1	0.353	114.6

The linear correlations for the effect of galactose background concentration from the above results were:-

For glucose,

$$K^F d_G = (101.8 + 48.667 \times Cg) \times K^\infty d_G/100 \qquad 5.24$$

and for galactose,

$$K^F d_F = (98.25 + 63.667 \times Cg) \times K^\infty d_F/100 \qquad 5.25$$

These results are in good agreement with those obtained by Thawait [115] and Ganetsos [116]. They found that the distribution coefficients of sugars in a chromatographic column decreased with increasing flowrate and increased with an increase in the background concentration.

An increase in the background concentration creates a concentration gradient between the stationary and mobile phases. This effect forces the sugars to be retained more by the stationary phase either by diffusion or by osmosis thus increasing the distribution coefficients. Increasing the background concentration also increases the viscosity of the solution in the chromatographic column resulting in larger elution volumes. This causes all distribution coefficients to increase. This explains the rapid increase of distribution coefficients in increasing dextran background concentrations observed by Ganetsos[116].

5.5 PRELIMINARY CHECKS AND START-UP PROCEDURES FOR THE SCCR-S SYSTEM

Before start-up, the feed and the enzyme was prepared as described in Chapters 6, 7 and 8 and put in their respective tanks which were connected to the system. These tanks held enough capacity for about 10 cycles. Safe operation was possible by operating in accordance with the following procedure :

- The desired temperature settings were selected and the column oven heater and deionised water heater tank switched on. The air circulating fan in the oven was also switched on.
- The air compressor supplying the pneumatic valves was turned on and the pneumatic controller set to the required switch time for the run.
- All inlet and outlet valves where opened and product collecting containers were positioned. When automatic product splitting was required, the appropriate timers were set to the proper valves.
- The eluent, feed, enzyme and purge pumps were switched on and set to the required flow rates.
- When the correct operating pressure was reached, the automatic shut off mechanism was activated and controllers were checked.

5.6 PROCEDURE DURING AN EXPERIMENTAL RUN

5.6.1 Data Collection

The eluent, feed, enzyme and purge flow rates were checked frequently to ensure constant operating conditions. The pressure drop in the streams and the temperature inside the enclosure were monitored.

Before starting product collection, the quencher unit installed on the product lines was switched on and set to 80°C to deactivate the enzyme in the product streams.

Half way through a switch a sample was withdrawn from the same sample point on the same column and placed in boiling water for about 5minutes to denature the enzyme. This sample was analysed by HPLC to produce the on-column concentration profile for each cycle. This was possible because each column served a different function every switch i.e. as a feed, eluent entry, purge column or any other column of the separating length. At the end of each cycle, the products were weighed and analysed to carry out a mass balance.

5.6.2 Establishing " Pseudo-Equilibrium "

Due to the semi-continuous nature of the SCCR-S system, only a "pseudo-equilibrium" state can be achieved and not a "true" equilibrium like in any other continuous counter-current mass transfer process e.g. distillation. When the on-column concentration profiles of two consecutive cycles were identical, within experimental accuracy, the "pseudo-equilibrium" state was deduced to have been reached. During this research work, the "pseudo-equilibrium" state was reached after 5 or 6 cycles. When any operating parameter such as the eluent flowrate, switch time or feed concentration was changed during an experimental run, the system was left running for another 4 or 5 cycles before collecting samples. This ensured that the "pseudo-equilibrium" state had been reached.

5.7 SHUT - DOWN PROCEDURE

At the end of each experimental run, the following procedure was followed to shut down the system :

- All pumps were switched off.
- The heaters and fan were switched off.
- The product lines were blocked to prevent the packing from drying out.
- The timers were switched off.
- The main valve in the deionised water supply line was closed.
- The compressed air supply was closed.

When the SCCR-S system was not to be used for long periods, a 0.02% w/v Sodium azide was pumped through to prevent any bacterial growth.

CHAPTER 6

A STUDY OF THE SCCR-S SYSTEM AS A COMBINED BIOREACTOR-SEPARATOR FOR THE PRODUCTION OF MALTOSE FROM MODIFIED STARCH

6.1 INTRODUCTION

This chapter presents the results of a study of the SCCR-S as a combined bioreactor-separator for the saccharification of modified starch to produce maltose using the enzyme maltogenase. The objectives of the experiments were to study the effects of the main operating parameters on the performance of the SCCR-S as a combined bioreactor-separator.

The operating parameters considered in this study were the eluent flow rate, switch time, feed concentration and enzyme activity.

The SCCR-S system has not been used before for the separation of maltose and dextrin. It was desirable to carry out the separation of maltose and dextrin so as to provide a basis for the reaction-separation work.

6.2 SELECTING THE OPERATING PARAMETERS

The factors that affect the separating potential of the SCCR-S system have been identified by previous workers[13,16]. These factors include :

(1) The effect of solute concentration;

(2) The variation of solute migration velocity due to variations in temperature, pressure gradients and solute concentration;

(3) System characteristics, namely semi-continuous operation mode and limited length of the separation section.

By taking these factors into consideration, the basic design equation (Equation 5.13) can be modified for a separation process to give:

$$(K_G^{\infty}+\delta 1_G+\delta 2_G) < (\frac{Le+Lf}{P}) < (K_F^{\infty}-\delta 1_F-\delta 2_F) \qquad 6.1$$

or

$$(K_G^{\infty}+\delta 1_G+\delta 2_G) < (\frac{Lm}{P}) < (K_F^{\infty}-\delta 1_F-\delta 2_F) \qquad 6.2$$

where,

$$\frac{Lm}{P} = \frac{\frac{Le}{P} + \frac{Le + Lf}{P}}{2} \qquad 6.3$$

and $\delta 1_G$ and $\delta 1_F$ are wall effects changes due to the effect of concentration on the distribution coefficients and $\delta 2_G$ and $\delta 2_F$ are wall effects due to the effects of the system characteristics on distribution coefficients[116].

Equation 6.2 represents the true theoretical limits of the mobile and stationary phase rates of movement required to achieve effective separation of two components on the SCCR-S system. This equation was used to estimate the initial operating parameters for the separation of maltose from dextrin.

6.3 PREPARATION OF THE MODIFIED STARCH FEED

Two types of modified starch, namely, Crystal Gum UK, a tapioca starch of dextrose equivalent (DE) of 3 (Starch A) and NADEX 8755, a potato starch of DE=7 (Starch B) supplied by National Starch and Chemical, Old Trafford, Manchester were used for this study.

A feed solution of the required concentration was prepared by adding the starch to cold de-ionised water to form a slurry which was then boiled in a 50dm^3 fermenter with adequate temperature and pressure controls. The pressure regulator was required to reduce frothing in the fermenter. After boiling, the solution was cooled down to 60^{o}C, the volume adjusted to give the required concentration and the pH adjusted to 5.3. The feed solution

was then filtered (sintered glass filter, Whatman) to remove any solid particles and transferred to the feed tank.

6.4 PREPARATION OF THE ENZYME MALTOGENASE

The enzyme maltogenase contained maltose as the stabiliser. The maltose had to be removed from the enzyme solution before it was used.

The maltose was removed using a pilot scale ultrafiltration membrane system constructed by Akintoye[16]. The system consisted of a cold storage compartment, a membrane module fitted with a 30,000 molecular weight cut-off membrane cassette of 0.464m^2 area (Millipore) and a peristaltic pump (701 S/R, Watson Marlow, Plymouth). The maltose was completely removed after 8 diafiltrations. Sodium azide (0.02% w/v) was added to the purified enzyme solution to prevent microbial attack. The enzyme was then stored at 4^oC.

6.5 SEPARATION OF MALTOSE, GLUCOSE AND DEXTRIN ON THE SCCR-S SYSTEM

The maltose, glucose and dextrin used for this study were obtained by carrying out a batch reaction outside the SCCR-S system. The modified starch used for the batch reactions was the Crystal Gum UK (Starch A) and was prepared as in Section 6.2 and the enzyme maltogenase added to it. The enzyme activity used for the batch reactions was 60 U/cm^3 at pH 5.2-5.4 and temperature of 60^oC. The reaction time was between 12-15 hours. After this time the reaction was quenched using sodium hydroxide (pH 9-10) and the mixture analysed by HPLC. The mixture was then used as the feed to the SCCR-S system.

During the separation studies, two operating parameters, eluent flowrate and switch time which affect the performance of the SCCR-S system were investigated.

Each experimental run in the separation studies is represented in a codified form. For example 9.5-9-26-21 corresponds to the feed concentration (%w/v), feed flowrate (cm^3/min), eluent flowrate (cm^3/min) and the switch time (mins.) respectively.

6.5.1 Experimental Results and Discussion

6.5.1.1 Effect of Switch Time

Tables 6.1 and 6.2 show the operating conditions and results obtained studying the effect of switch time on the separation of maltose, glucose and dextrin obtained from the batch reaction.

From Table 6.2 comparing runs 15.1-9-26-20 and 15.1-9-26-21, it was observed that an increase in operating switch time improved the purity of the maltose rich product (MRP) while the purity of the dextrin rich product (DRP) was reduced. These effects were due to the broader maltose concentration profile in the system as shown by Figure 6.2.

It was also observed that by increasing the switch time, the MRP concentration was reduced while the DRP concentration was increased. Increasing the switch time increases the residence time in the system and also reduces the relative motion of the stationary phase (P) compared to the mobile phase thus allowing more of the solutes to be pushed forward with the mobile phase reducing the concentration of the MRP and increasing the concentration of the DRP.

The "cross-over" point, which is the point of intersection of the maltose and dextrin profiles (Figures 6.1 and 6.2) did not change significantly by varying the switch time as observed by previous workers[13] for the separation of glucose and fructose. This was due to the very high molecular weight of the dextrin which was eluted with the mobile phase. This was also observed by Akintoye[16] separating fructose and dextran on the SCCR-S system.

Table 6.1: Effects of Switch Time on Product Purities: Operating Conditions

Experimental Run	Average Flowrates cm^3min^{-1}			Feed Concentration %w/v				Switch Time (min)	Lm/P	Cycle No	Feed Throughput ($Kg/hr/m^3$ resin)
	Feed	Eluent	Purge	M	D	G	Total				
9.5-9-26-21	9.0	26.0	76.0	4.30	4.60	0.60	9.50	21.0	0.118	7	2.85
9.5-9-26-19	9.0	26.0	76.0	4.30	4.60	0.60	9.50	19.0	0.056	13	2.85
15.1-9-26-20	9.0	26.0	76.0	6.65	6.93	1.49	15.1	20.0	0.087	8	4.53
15.1-9-26-21	9.0	26.0	76.0	6.65	6.93	1.49	15.1	21.0	0.118	16	4.53

Table 6.2: Effects of Switch Time Experimental Results

Experimental Run	MALTOSE RICH PRODUCT				DEXTRIN RICH PRODUCT				Mass Balance
	Maltose purity %	Impurities %		Total product conc. %w/v	Dextrin purity %	Impurities %		Total product conc. % w/v	Output / Input
		D	G			M	G		
9.5-9-26-21	88.5	0.0	11.5	0.61	95.2	4.8	0.0	1.00	95.2
9.5-9-26-19	89.1	0.0	10.9	0.67	99.9	0.0	0.0	0.95	101.1
15.1-9-26-20	79.4	6.0	14.6	1.26	99.9	0.0	0.0	1.22	101.1
15.1-9-26-21	82.0	0.0	18.0	0.89	96.5	3.5	0.0	1.73	94.3

D = Dextrin, G = Glucose, M = Maltose

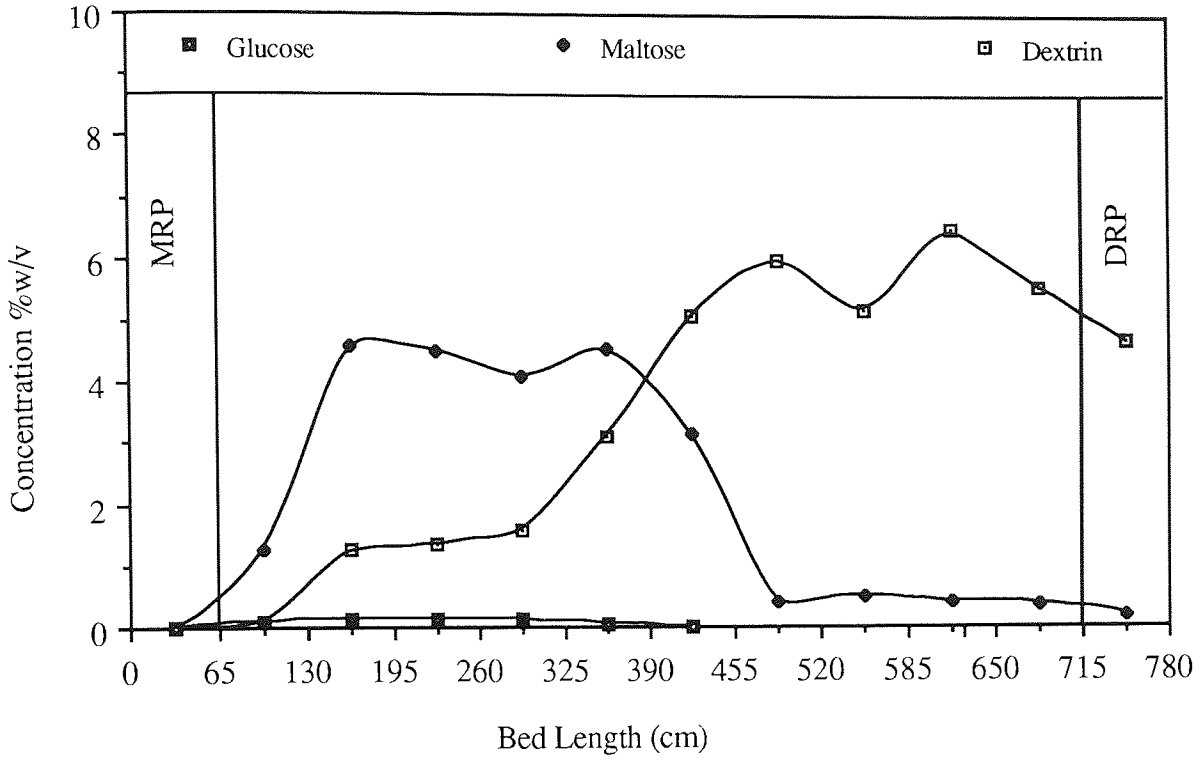


Figure 6.1: On-Column Concentration Profile for Run 15.1-9-26-20 Cycle 8

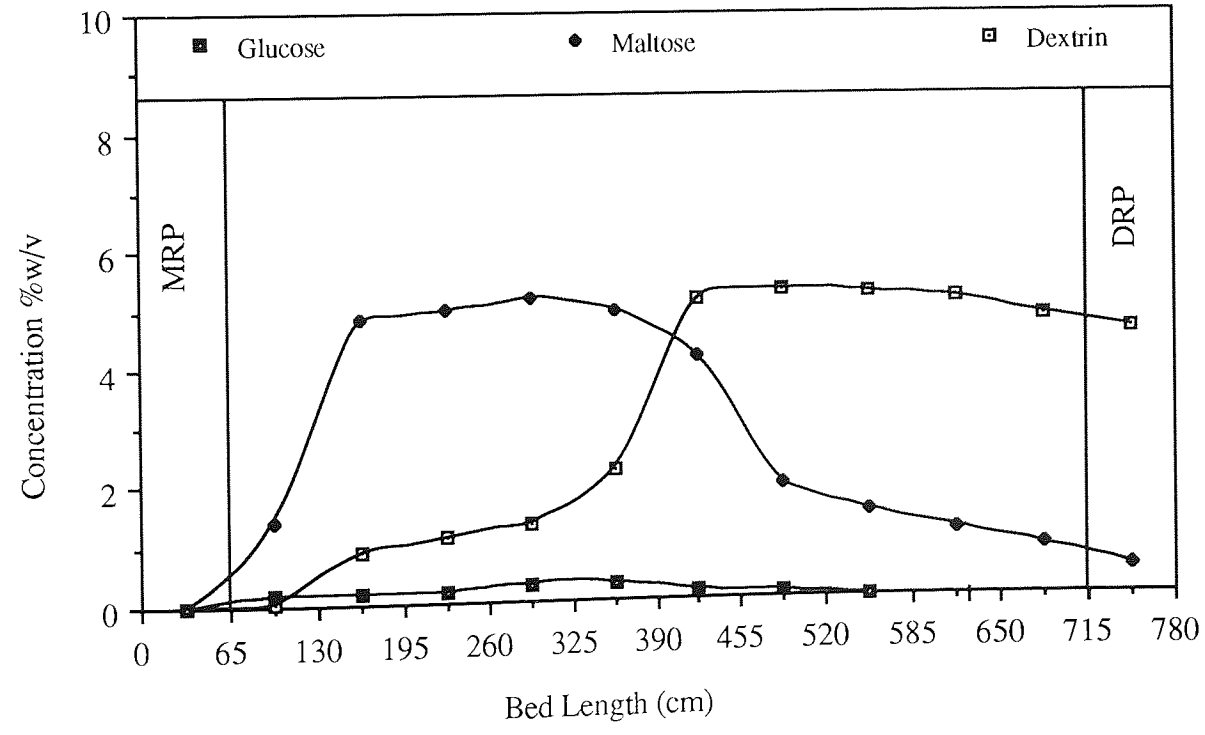


Figure 6.2: On-Column Concentration Profile for Run 15.1-9-26-21 Cycle 16

6.5.1.2 Effect of Eluent Flowrate

The flowrates and switch time are directly related to maintain the Lm/P ratio constant. Two sets of experiments were carried out to investigate the effects of the eluent flowrate while all the other operating conditions were kept constant. Tables 6.3 and 6.4 show the operating conditions and the results obtained during these experiments.

It was found that by increasing the eluent flowrate (thus increasing the eluent to feed rate ratio), the MRP purity was improved at the expense of the DRP purity which was reduced. By increasing the eluent flowrate, the residence time in the system was reduced. This was brought about by the increased movement of the mobile phase relative to the stationary phase. At high eluent flowrates, the amount of maltose that complexes with the Ca^{2+} on the resin is reduced and therefore more of the solutes are pushed forward with the mobile phase. This therefore caused the MRP concentration to reduce and the purity improved while the DRP concentration increased with reduced purity.

It was also observed that the maltose concentration profile (Figures 6.3 and 6.4) was broader with the "cross-over" point shifting towards the DRP end when the eluent flowrate was increased. This again was probably due to the stripping of some of the maltose that had complexed with the calcium ions on the resin. This maltose is recomplexed along the system but is moved forward.

Table 6.3: Effect of Eluent Flowrate: Operating Conditions

Experimental Run	Average Flowrates cm^3min^{-1}			Feed Concentration %w/v				Switch Time (min)	Lm/P	Cycle No	Feed Throughput ($Kg/h/m^3$ resin)
	Feed	Eluent	Purge	M	D	G	Total				
29.35-9-26-21	9.0	26.0	76.0	13.05	14.79	1.51	29.35	21.0	0.118	7	8.81
29.35-9-28-21	9.0	26.0	76.0	13.05	14.79	1.51	29.35	21.0	0.162	13	8.81

Table 6.4: Effect of Eluent Flowrate : Experimental Results

Experimental Run	MALTOSE RICH PRODUCT				DEXTRIN RICH PRODUCT				Mass Balance Output / Input
	Maltose purity %	Impurities %		Total product conc. %w/v	Dextrin purity %	Impurities %		Total product conc. % w/v	
		D	G			M	G		
29.35-9-26-21	86.7	0.0	13.3	1.73	94.6	5.4	0.0	3.31	93.7
29.35-9-28-21	88.6	0.0	11.4	1.58	91.5	8.5	0.0	4.23	95.6

D = Dextrin, G = Glucose, M = Maltose

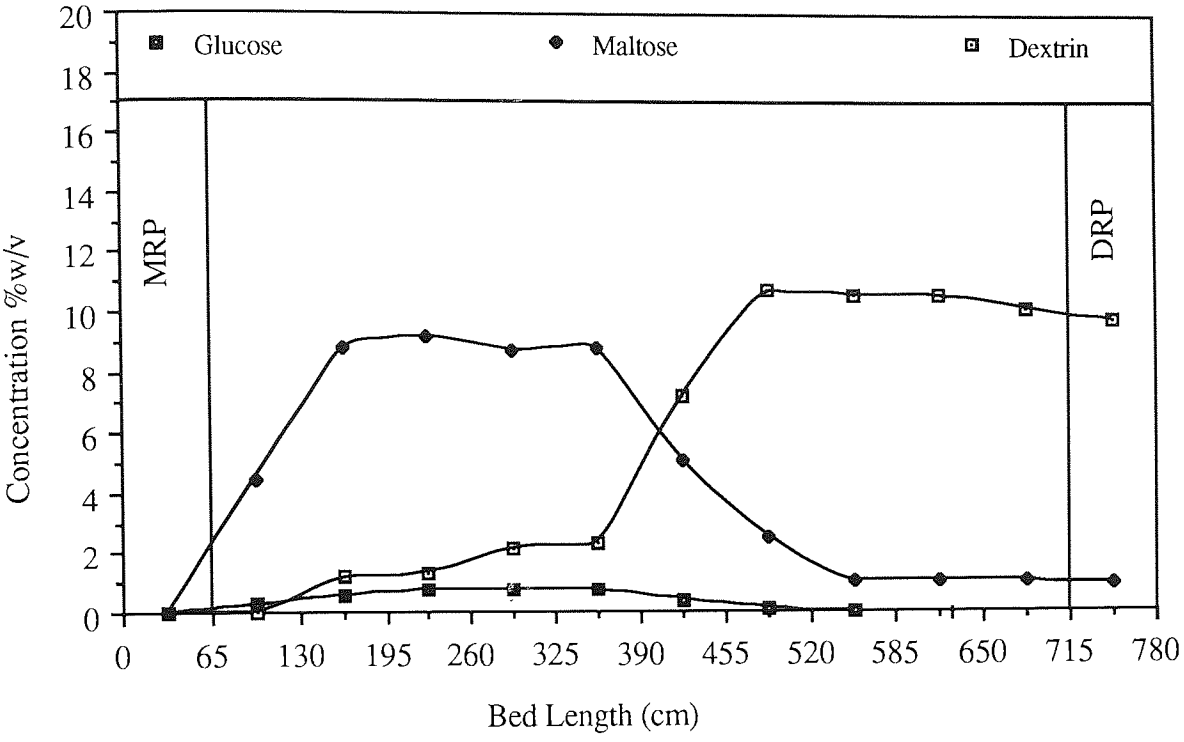


Figure 6.3: On-Column Concentration Profile for Run 29.35-9-26-21 Cycle 7

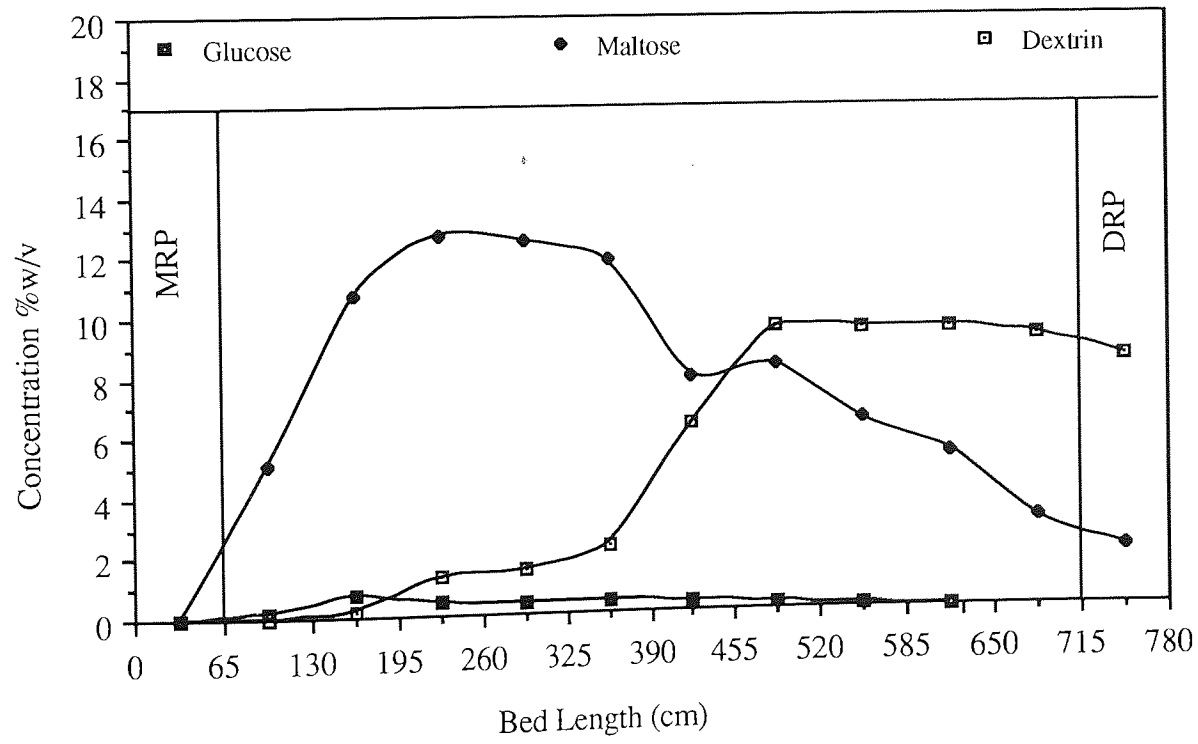


Figure 6.4: On-Column Concentration Profile For Run 29.35-9-28-21 Cycle 13

6.5.2 Conclusions

It has been shown that it is possible to separate maltose, glucose and dextrin on the SCCR-S system. The maltose and glucose moved with the stationary phase and were recovered as the MRP while the dextrin, due to its high molecular weight, moved with the stationary phase and was recovered as the DRP.

A basis for the simultaneous bioreaction and separation work has been established. It was observed that an eluent to feed flow rate ratio of about 3:1 and switch times in the range of 19-21 minutes were required to achieve separation between maltose and dextrin on the SCCR-S.

6.6 CONTINUOUS BIOREACTION AND SEPARATION ON THE SCCR-S SYSTEM FOR THE PRODUCTION OF MALTOSE FROM MODIFIED STARCH

Selecting the operating conditions required for simultaneous bioreaction and separation in the SCCR-S system requires an understanding of the reaction conditions and the separation conditions. The operating conditions required for separation have been discussed in the previous section of this chapter.

An understanding of the properties of the biocatalyst such as the optimum pH and temperature, stability and the reaction kinetics of the process gives some idea of the limitation of the reaction. Working within these limits the product yield can be maximised. The properties and kinetics of the enzyme, maltogenase used for this study have been discussed in Chapters 3 and 4 respectively.

The optimum temperature of 60°C and pH of 5.3 required for the enzyme reaction were used during all the experiments reported in this study. The enzyme maltogenase was very stable under cold conditions (4°C). It was therefore possible for the enzyme to be used for the duration of each experimental run (one week) without any significant change in activity.

The starch feed used for all the experiments was the Crystal Gum UK of DE=3 unless indicated otherwise.

6.6.1 Commissioning Run for the Production of Maltose

After using the SCCR-S system as a separator, it was regenerated with 10%w/v calcium nitrate and cleaned with de-ionised water.

Akintoye[16] in his work on the continuous inversion of sucrose used an eluent made up of the enzyme invertase and de-ionised water. He found that the optimum enzyme activity required by the SCCR-S system was 60U/cm^3.

The experimental conditions and results for the commissioning run are shown in Tables 6.6 and 6.7. Table 6.5 shows the product purities of both DRP and MRP streams collected after each cycle. From Table 6.5 it was observed that steady state was established after six cycles of continuous operation. Figures 6.5 and 6.6 show the on-column concentration profiles for cycles 6 and 7 which are very similar thereby confirming a pseudo-steady state had been reached.

The results obtained from the commissioning run looked very promising. It was observed that only 94.3 % of the theoretical amount of enzyme required by a conventional batch reactor was required to achieve a conversion of 63.7% at a feed throughput of 2.91Kg/hr/m^3 resin producing maltose at 99.99% purity.

It has been shown that the system establishes pseudo-steady state after six cycles of operation and therefore during the subsequent studies of the behaviour of the SCCR-S system operating as a bioreactor-separator, samples were collected after cycle six. When a parameter was changed the operation was continued for at least another four cycles to regain its steady state before samples were collected.

Table 6.5: Product Purities at the end of each cycle

Cycle	MRP Purity	DRP Purity
3	98.0	99.0
4	92.0	99.2
5	91.1	98.7
6	90.5	98.9
7	90.7	99.0
8	90.3	98.8
9	90.4	99.1

Table 6.6: Commissioning Run For Bioreaction-Separation: Experimental Conditions

Experimental Run	Average Flowrates cm^3min^{-1}			Starch Feed Conc.	Enzyme Activity in Eluent	Switch Time	Lm/P	Cycle No	Feed Throughput
	Feed	Eluent	Purge	%w/v	(U/cm^3)	(min)			($Kg/h/m^3$ resin)
9.7-9-26-60-19	9.0	26	76.0	9.7	60	19.0	0.057	6	2.91

Table 6.7 Commissioning Run For Bioreaction-Separation : Experimental Results

Experimental Run	MALTOSE RICH PRODUCT				DEXTRIN RICH PRODUCT				Conver-sion	Relative Enzyme usage Actual / Theoretical	Mass Balance Output / Input
	Maltose purity %	Impurities % D	G	Total product conc. %w/v	Dextrin purity %	Impurities % M	G	Total product conc. % w/v	%		%
9.7-9-26-60-19	99.9	0.0	0.0	0.74	98.9	1.1	0.0	0.92	63.7	0.943	101.3

M = Maltose, D = Dextrin, G = Glucose

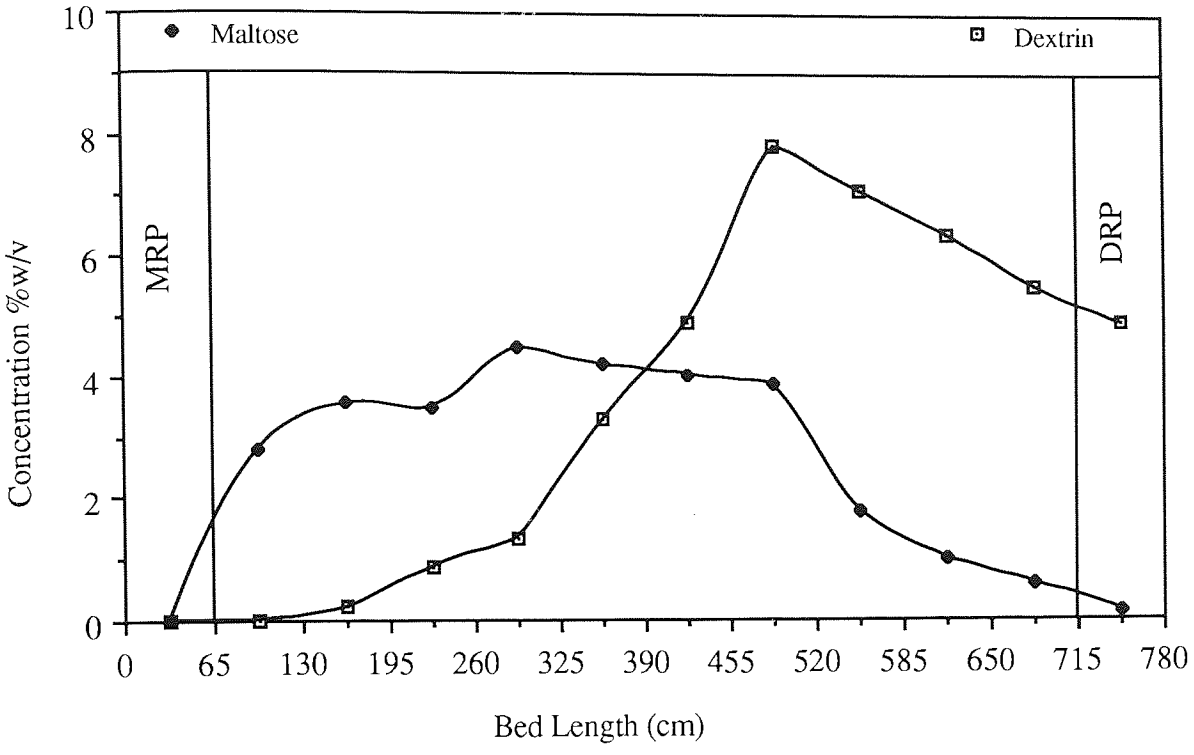


Figure 6.5: On-Column Concentration Profile for Run 9.7-9-26-60-19 Cycle 6

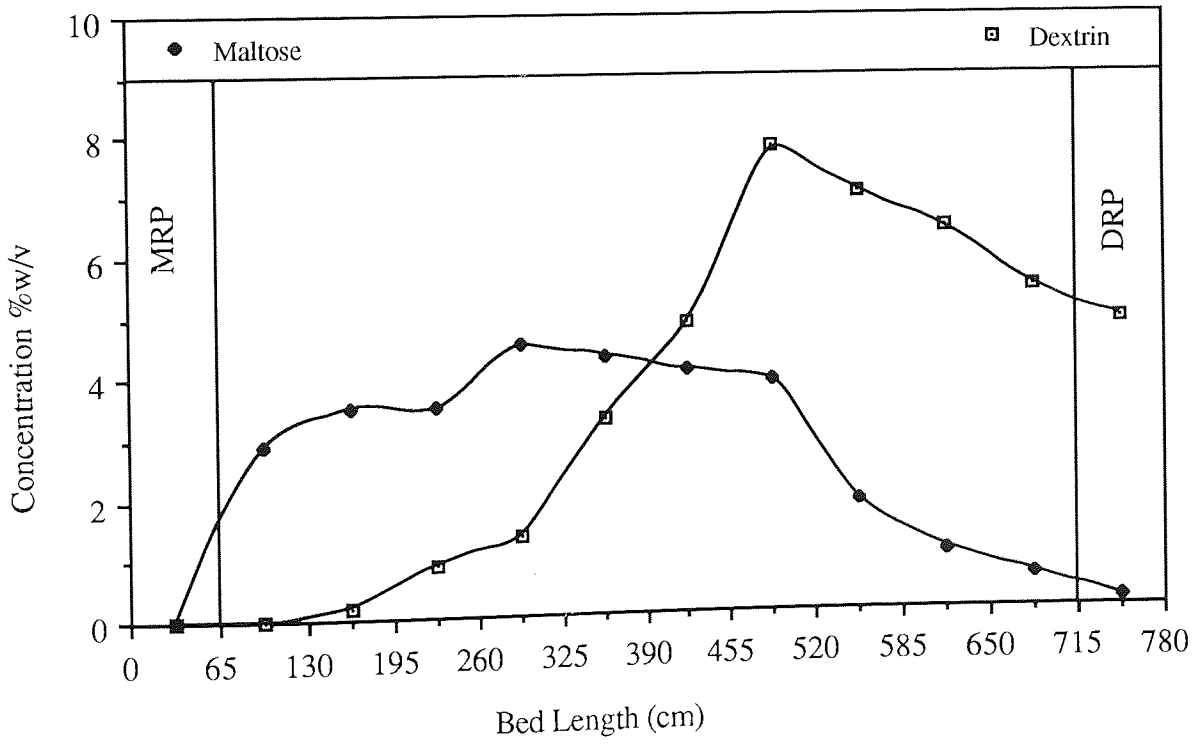


Figure 6.6: On-Column Concentration Profile for Run 9.7-9-26-60-19 Cycle 7

6.6.2 Effect of Eluent Flowrate

The effect of the eluent flowrate on the product purities and concentrations was investigated at a constant feedrate of 9cm^3/min keeping all other parameters constant. Two different feed concentrations were used during the experiments.

Tables 6.8 and 6.9 show the experimental conditions and results. By increasing the eluent flowrate, the reaction time in the system was reduced and the movement of the mobile phase relative to the stationary phase was increased. This resulted in a slight reduction in product formation despite an increase in the amount of enzyme used. Increasing the eluent flowrate also led to more maltose being pushed forward and removed with the mobile phase thus increasing the DRP concentration with lower purity. The MRP purity was improved with a decrease in product concentration.

At a higher feed concentration (run 20.9-9-24-60-19), up to 7.4% of the MRP was made of glucose. This was expected as the enzyme maltogenase can produce small amounts of glucose.

From the on-column concentration profile, Figures 6.7 and 6.8, it was observed that the unreacted starch (i.e dextrin), profile became less broader and moved towards the DRP Section while the maltose on-column concentration increased. The movement of the dextrin profile towards the DRP Section reflects the faster movement of the mobile phase as the eluent rate was increased.

Sarmidi[50] carried out a batch reaction using a feed concentration of 10% w/v of crystal Gum UK (starch A) and enzyme activity of 60U/cm^3 and achieved a maximum conversion of 58.1% after 12 hours. From Table 6.9 it can be seen that conversions of up to 59.4% were achieved at feed concentration of 9.5%w/v in the SCCR-S system even though only 46% of the theoretical amount of enzyme required by a conventional batch reactor was used in the SCCR-S. This was due to the ability of the bioreactor-separator to minimise the effect of substrate or product inhibition.

Table 6.8: Effect of Eluent Flowrate: Experimental Conditions

Experimental Run	Average Flowrates cm^3min^{-1}			Starch Feed Conc.	Enzyme Activity in Eluent (U/cm^3)	Switch Time	Lm/P	Cycle No	Feed Throughput ($Kg/h/m^3$ resin)
	Feed	Eluent	Purge	%w/v		(min)			
9.5-9-24-30-19.5	9.0	24.0	76.0	9.50	30	19.5	0.032	21	2.85
9.5-9-27-30-19.5	9.0	27.0	76.0	9.50	30	19.5	0.092	16	2.85
20.9-9-24-60-19	9.0	24.0	76.0	20.90	60	19.0	0.017	8	6.27
20.9-9-25.5-60-19	9.0	25.5	76.0	20.90	60	19.0	0.046	13	6.27

Table 6.9: Effect of Eluent Flowrate: Experimental Results

Experimental Run	MALTOSE RICH PRODUCT				DEXTRIN RICH PRODUCT				Conversion	Relative Enzyme usage Actual / Theoretical	Mass Balance Output / Input
	Maltose purity %	Impurities % D	G	Total product conc. %w/v	Dextrin purity %	Impurities % M	G	Total product conc. % w/v	%		%
9.5-9-24-30-19.5	99.9	0.0	0.0	0.70	98.8	1.2	0.0	1.10	59.4	0.460	104.7
9.5-9-27-30-19.5	99.9	0.0	0.0	0.57	84.6	15.4	0.0	1.17	58.3	0.556	99.9
20.9-9-24-60-19	70.5	22.1	7.4	1.90	94.2	5.8	0.0	1.38	60.6	0.428	101.0
20.9-9-25.5-60-19	79.3	17.2	4.5	1.74	93.4	6.6	0.0	1.58	60.1	0.466	99.3

M = Maltose, D = Dextrin, G = Glucose

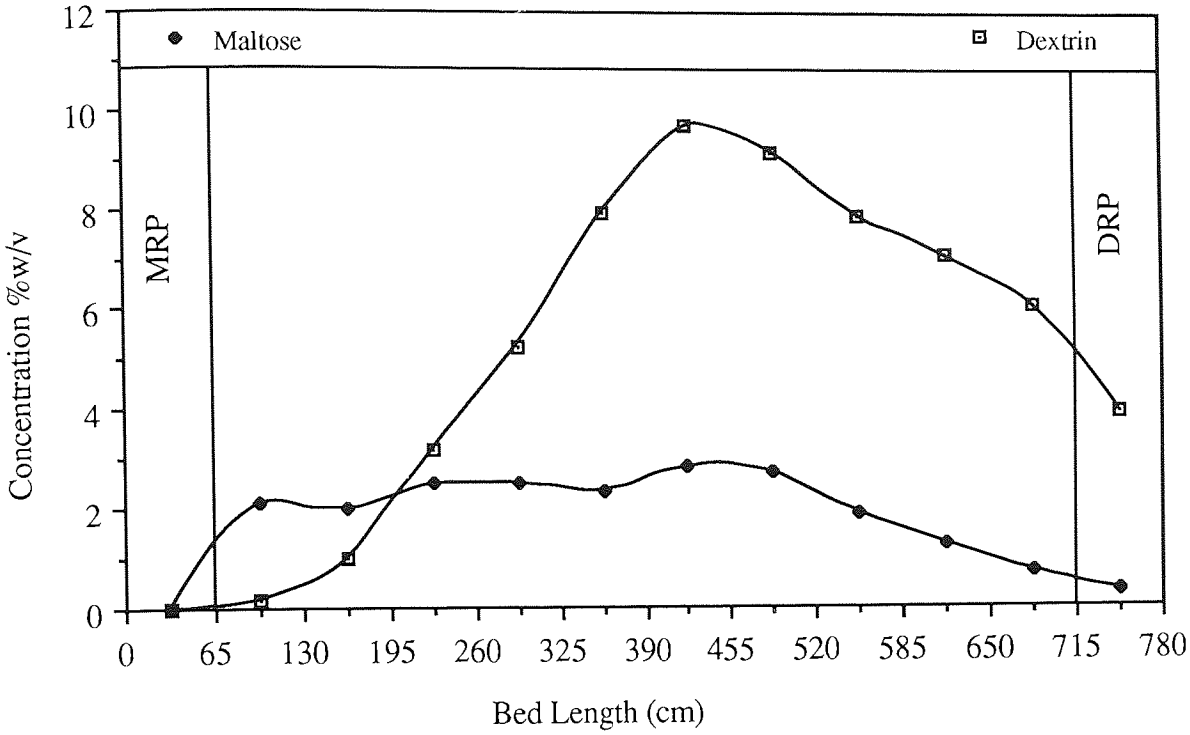


Figure 6.7: On-Column Concentration Profile for Run 9.5-9-24-30-19.5 Cycle 21

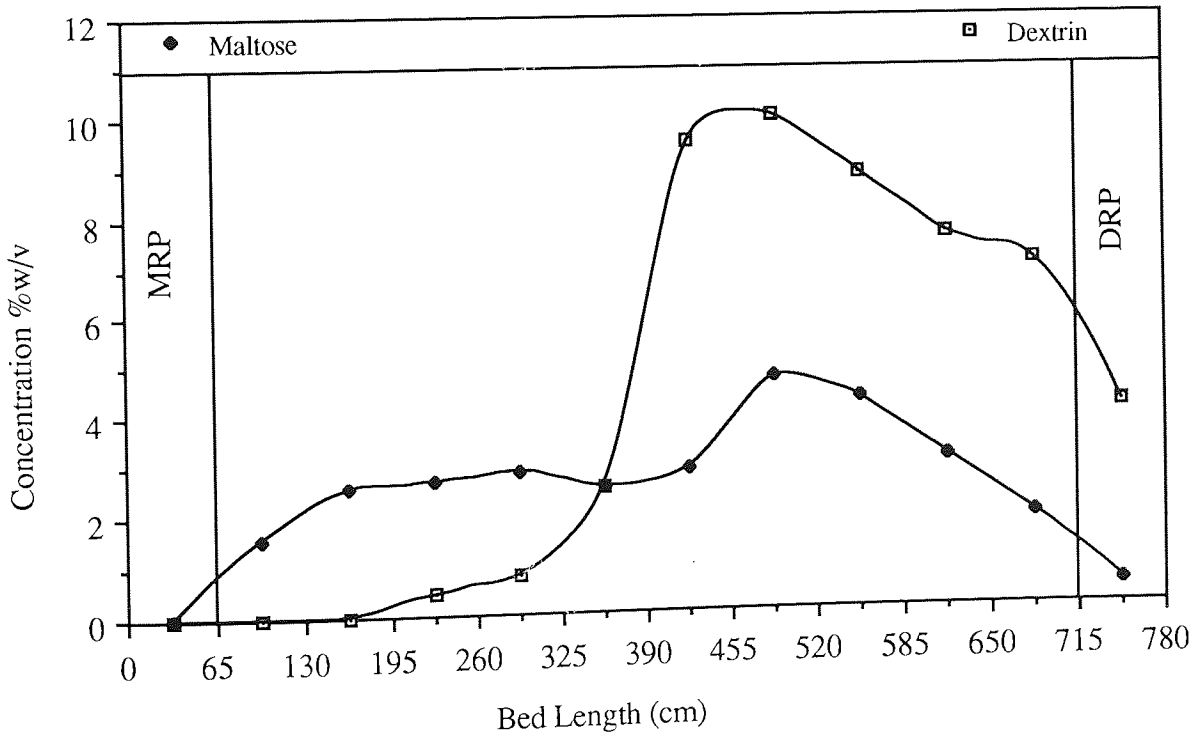


Figure 6.8: On-Column Concentration Profile for Run 9.5-9-27-30-19.5 Cycle 16

6.6.3 Effect of Switch Time

The switch time has been found to be a controlling parameter in the operation of the SCCR-S system[16]. It controls the residence time in the system and is related to the eluent flowrate by the Lm/P ratio. Tables 6.10 and 6.11 show the experimental conditions and the results obtained varying the switch time.

As the ratio of the mobile phase to the stationary phase flowrate (Lm/P) increases due to increase in the switch time, the conversion of the feed starch to maltose was found to decrease. The starch because of its high molecular weight does not diffuse in and out of the resin and therefore travels with the mobile phase. By increasing the switch time, the starch is eluted out of the system faster and therefore has less time to react thus resulting in lower conversion.

From Table 6.11 it can be observed that by increasing the switch time, the purities of the maltose rich products improved with reduced concentration while the dextrin rich products purities reduced with increased concentration. This was due to the longer time spent by the eluent in a column at higher switch times thereby causing less complexing and more stripping of the maltose along the system. At higher switch times, the "cross over" point advanced towards the DRP product end when comparing Figure 6.8 to 6.9 and Figure 6.10 to 6.11. This resulted in better MRP product purities. It was also observed that at the post feed Section of the system, the maltose profile became more prominent at higher switch times. The possible cause again is due to less complexing, more stripping of the complexed maltose and the subsequent hydrolysis of small amounts of maltotriose and maltotetraose formed in the system to maltose and very small amounts of glucose. The amount of glucose produced was greater at higher feed concentrations. Glucose formation was also observed by Sarmidi[50] who studied the saccharification of starch to maltose in a continuous annular chromatographic system.

Table 6.10: Effect of Switch Time: Experimental Conditions

Experimental Run	Average Flowrates cm^3min^{-1}			Starch Feed Conc.	Enzyme Activity in Eluent	Switch Time	Lm/P	Cycle No	Feed Throughput
	Feed	Eluent	Purge	%w/v	(U/cm^3)	(min)			($Kg/h/m^3$ resin)
9.5-9-27-30-19.5	9.0	27.0	76.0	9.50	30	19.5	0.092	16	2.85
9.5-9-27-30-20.5	9.0	27.0	76.0	9.50	30	20.5	0.124	9	2.85
14.78-9-25.5-60-20	9.0	25.5	76.0	14.78	60	20.0	0.077	7	4.43
14.78-9-25.5-60-21	9.0	25.5	76.0	14.78	60	21.0	0.108	14	4.43
23.3-9-25.5-60-20	9.0	25.5	76.0	23.30	60	20.0	0.077	7	6.99
23.3-9-25.5-60-21.5	9.0	25.5	76.0	23.30	60	21.5	0.123	14	6.99

Table 6.11: Effect of Switch Time: Experimental Results

Experimental Run	MALTOSE RICH PRODUCT				DEXTRIN RICH PRODUCT				Conversion	Relative Enzyme usage Actual/Theoretical	Mass Balance Output/Input
	Maltose purity %	Impurities % D	Impurities % G	Total product conc. %w/v	Dextrin purity %	Impurities % M	Impurities % G	Total product conc. % w/v	%		%
9.5-9-27-30-19.5	99.9	0.0	0.0	0.57	84.6	15.4	0.0	1.17	58.3	0.556	99.9
9.5-9-27-30-20.5	99.9	0.0	0.0	0.37	82.6	17.4	0.0	1.72	40.2	0.806	99.9
14.78-9-25.5-60-20	86.0	3.2	10.8	0.93	99.4	0.6	0.0	1.57	54.9	0.765	98.6
14.78-9-25.5-60-21	91.4	0.0	8.6	0.88	94.6	5.4	0.0	1.69	54.5	0.798	93.8
23.3-9-25.5-60-20	52.7	39.8	7.5	2.26	92.6	7.4	0.0	1.08	50.7	0.493	99.7
23.3-9-25.5-60-21.5	60.2	33.1	6.7	1.81	91.4	8.6	0.0	2.09	46.8	0.533	99.9

M = Maltose, D = Dextrin, G = Glucose

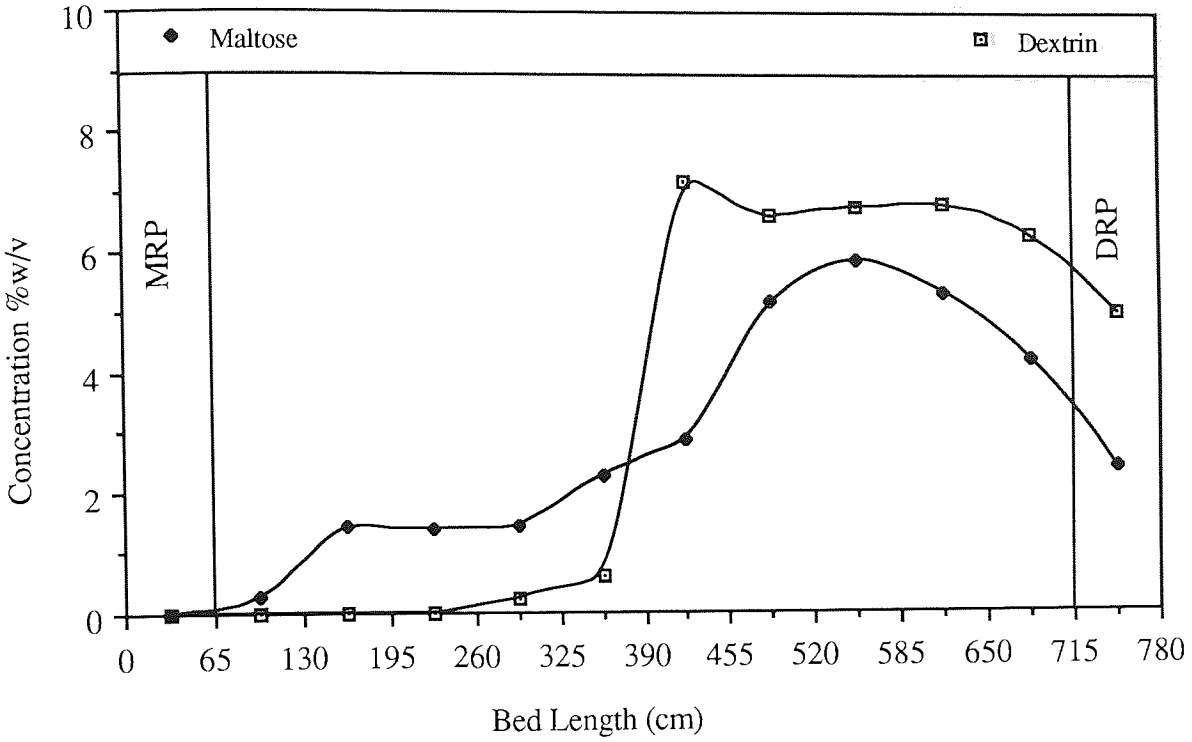


Figure 6.9: On-Column Concentration Profile for Run 9.5-9-27-30-20.5 Cycle 9

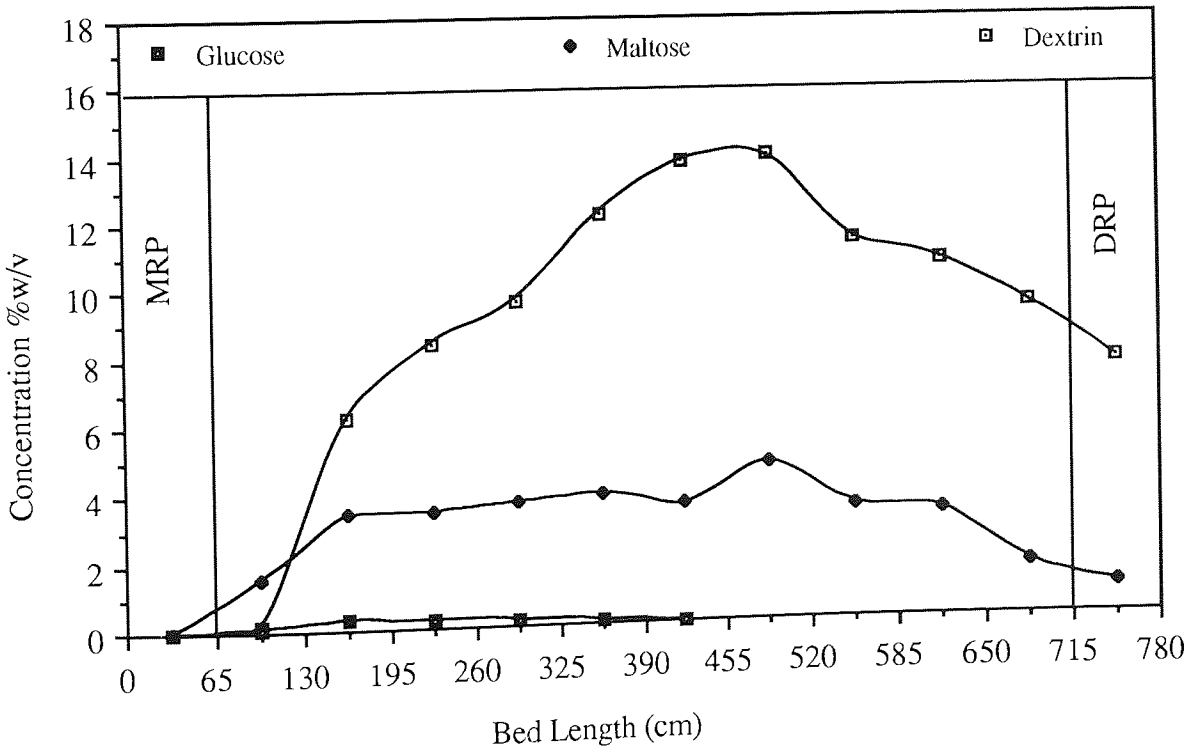


Figure 6.10: On-Column Concentration Profile for Run 14.78-9-25.5-60-20 Cycle 7

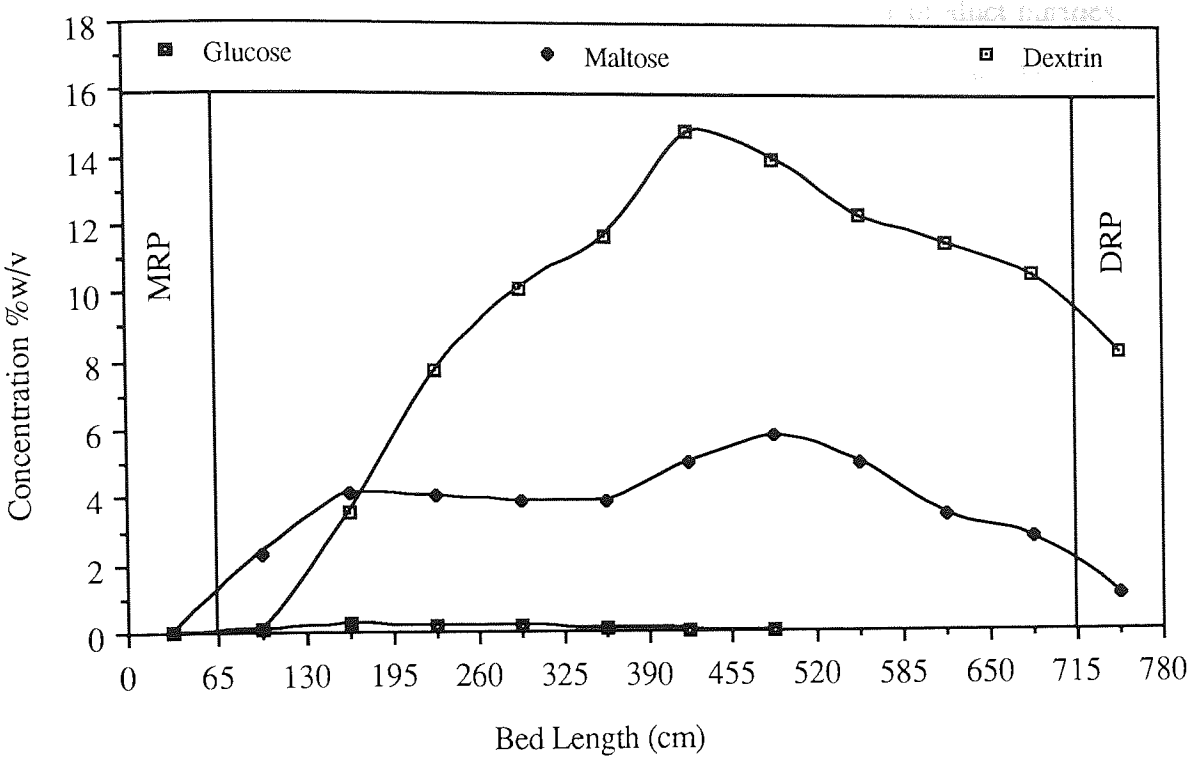


Figure 6.11: On-Column Concentration Profile for Run 14.78-9-25.5-60-21 Cycle 14

From these results, it was also observed that maltose products were contaminated with glucose at higher feed concentrations. The SCCR-S required less than the theoretical amounts of enzyme required by a conventional batch reactor to achieve the same conversion within a given time. This demonstrates the ability of the SCCR-S in minimising product or substrate inhibition thus using less enzyme.

6.6.4 Effect of Feed Concentration

The effect of feed concentration on the performance of the SCCR-S system was also studied. The experimental conditions and results are shown in Tables 6.12 and 6.13.

By increasing the feed concentration, the enzyme-to-feed ratio was reduced. Increasing the feed concentration also increased the viscosity in the system resulting in poor mixing of the enzyme and the starch substrate. This caused a reduction in the amount of starch converted to maltose. The substrate inhibition effect at high starch concentrations also contributed to reduction in the conversion.

It was observed that increasing the feed concentration resulted in poor product purities, with the MRP affected to the greater extent. By increasing the concentration from 9.6%w/v (run 9.6-9-25.5-60-19) to 20.9%w/v (run 20.9-9-25.5-60-19) the MRP purity reduced from 99.9 to 79.3% while the DRP purity reduced from 98.6 to 94.9%. This was due to the increased viscosity in the system which caused more dextrin to be retained in the pre-feed Section of the system thus reducing the MRP purity. Because of the poor mixing and reduced enzyme-to-feed ratio at higher concentrations, a longer length of the system was required for the enzyme to hydrolyse the starch closer to the α-(1-6) glucosidic linkages. This caused the higher proportion of maltose observed in the DRP product. The increase in viscosity was reflected in the increased operating pressure at higher feed concentrations. For a feed concentration of 9.6%w/v (run 9.6-9-25.5-60-19) the operating pressure was 181KN/m^2 while for a feed concentration of 20.9%w/v (run 20.9-9-25.5-60-19) the operating pressure was 448KN/m^2.

Table 6.12: Effect of Feed Concentration: Experimental Conditions

Experimental Run	Average Flowrates cm^3min^{-1}			Starch Feed Conc.	Enzyme Activity in Eluent	Switch Time	Lm/P	Cycle No	Feed Throughput
	Feed	Eluent	Purge	%w/v	(U/cm^3)	(min)			($Kg/h/m^3$ resin)
9.6-9-25.5-60-19	9.0	25.5	76.0	9.6	60	19.0	0.046	8	2.88
20.9-9-25.5-60-19	9.0	25.5	76.0	20.9	60	19.0	0.046	13	6.27
14.8-9-25.5-60-20	9.0	25.5	76.0	14.8	60	20.0	0.077	7	4.44
23.3-9-25.5-60-20	9.0	25.5	76.0	23.3	60	20.0	0.077	7	6.99

Table 6.13: Effect of Feed Concentration: Experimental Results

Experimental Run	MALTOSE RICH PRODUCT				DEXTRIN RICH PRODUCT				Conversion	Relative Enzyme usage	Mass Balance
	Maltose purity %	Impurities % D	Impurities % G	Total product conc. %w/v	Dextrin purity %	Impurities % M	Impurities % G	Total product conc. % w/v	%	Actual / Theoretical	Output / Input %
9.6-9-25.5-60-19	99.9	0.0	0.0	0.70	98.6	1.4	0.0	1.01	60.8	0.970	102.7
20.9-9-25.5-60-19	79.3	17.2	4.5	1.74	94.9	5.1	0.0	1.58	60.1	0.466	99.3
14.8-9-25.5-60-20	86.0	3.2	10.8	0.93	97.4	0.6	0.0	1.58	54.9	0.765	98.6
23.3-9-25.5-60-20	52.7	39.8	7.5	2.26	92.6	7.4	0.0	1.08	50.7	0.493	99.7

M = Maltose, D = Dextrin, G = Glucose

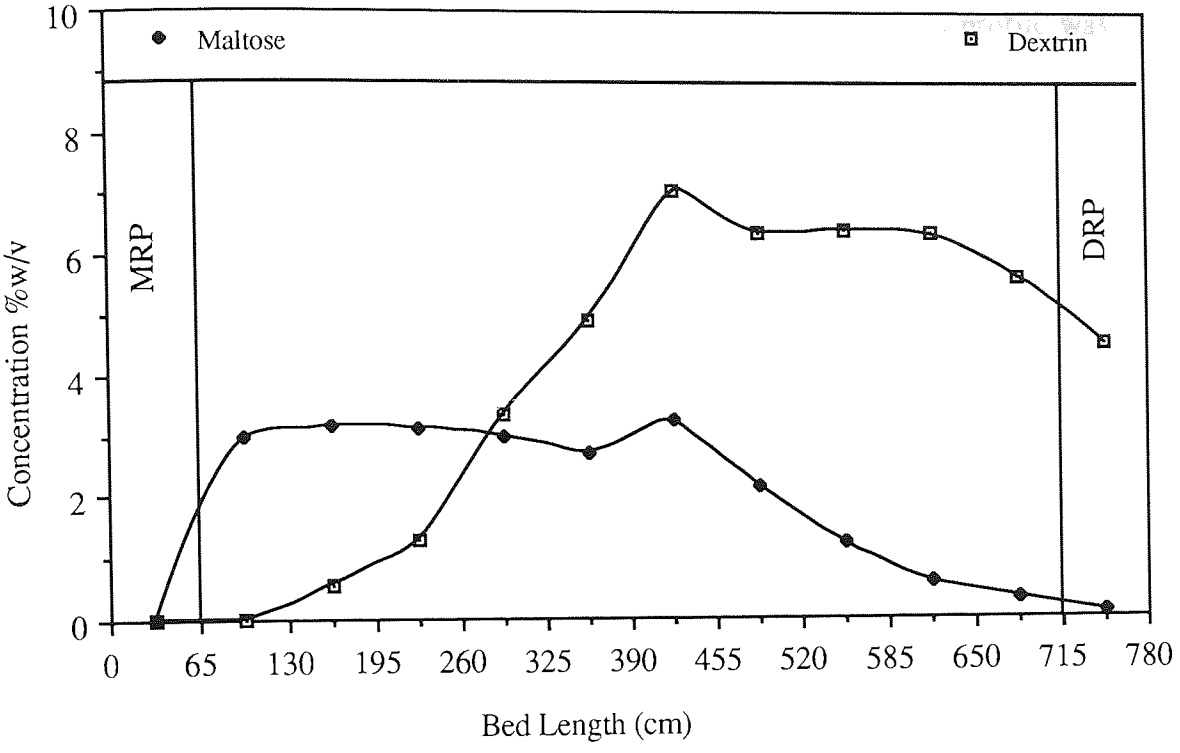


Figure 6.12: On-Column Concentration Profile for Run 9.6-9-25.5-60-19 Cycle 8

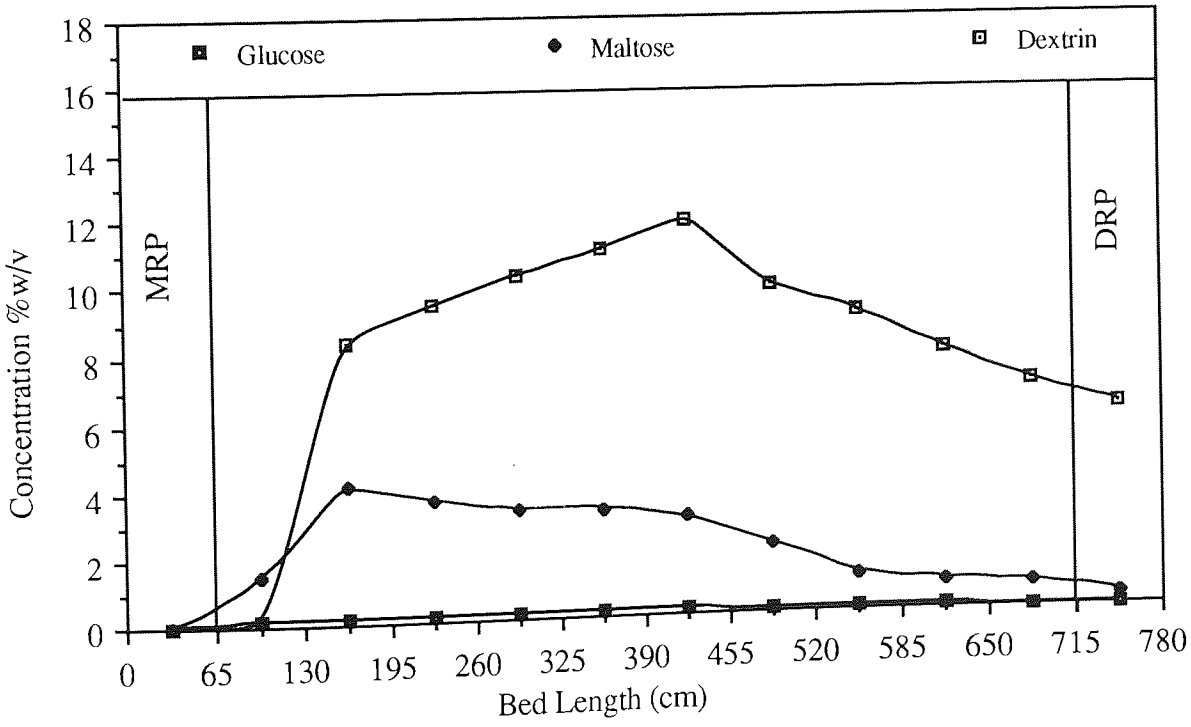


Figure 6.13: On-Column Concentration Profile for Run 20.9-9-25.5-60-19 Cycle 13

Figures 6.12 and 6.13 show that at higher feed concentrations, the dextrin profile was broader and the "cross over" point shifted three columns towards the MRP product, from column 5 to column 2 resulting in the poorer MRP product purities.

6.6.5 Effect of Dextrose Equivalent (DE) of the Starch Feed

To investigate the effect of the DE of the feed starch on the performance of the SCCR-S, two types of feed starch were used. The Crystal Gum UK, a tapioca starch (Starch A) with a DE of 3 and the NADEX 8755, a potato starch (Starch B) with a DE of 7. Both types were supplied by National Starch and Chemical, Manchester UK. Starch A had an average molecular weight of $5x10^5$ daltons while Starch B had an average molecular weight of $2.3x10^5$ daltons.

The experimental conditions and results are shown in Tables 6.14 and 6.15. It was observed that using a feed of higher DE (Starch B), the starch conversion to maltose was lower eventhough Starch B contained less amylopectin than Starch A. The average value of amylopectin content is 82% for tapioca starch and 79% for potato starch[117]. The higher the amylopectin content, the greater the number of α-(1-6) glucosidic linkages and the lower is the equilibrium conversion to maltose. This observation was in agreement with the results reported on maltose production by Takasaki *et al.*[118] and Sarmidi *et al.*[60] using the enzyme maltogenase. Takasaki *et al.*[118] and Saha *et al.*[119] showed that to obtain high starch conversions to maltose, the modified starch should have a DE as low as possible. However, at a low DE, starch is more viscous and easily retrograded.

Figures 6.14 and 6.15 show the on-column concentration profiles for runs 20.3A-9-25.5-60-20 and 20.26B-9-25.5-60-20 respectively. It was observed that at low DE values (Figure 6.14) the dextrin profile was broader. This resulted in a higher MRP concentration and poorer purity because the higher viscosity at low DE causes more dextrin to be retained on the pre-feed section of the system. At higher DE (lower viscosity), the dextrin profile was more prominent in the post-feed section of the system and resulted in a higher DRP purity and concentration being obtained.

Table 6.14: Effect of Dextrose Equivalence (DE) of Feed Starch: Experimental Conditions

Experimental Run	Average Flowrates cm^3min^{-1}			Starch Feed Conc.	Enzyme Activity in Eluent	Switch Time	Lm/P	Cycle No	Feed Throughput
	Feed	Eluent	Purge	%w/v	(U/cm^3)	(min)			($Kg/h/m^3$ resin)
20.3A-9-25.5-60-20	9.0	25.5	76.0	9.6	60	20.0	0.077	15	6.09
20.26B-9-25.5-60-20	9.0	25.5	76.0	20.9	60	20.0	0.077	11	6.09

A - Indicating feed starch of DE=3 (starch A)
B - Indicating feed starch of DE=7 (starch B)

Table 6.15: Effect of Dextrose Equivalence (DE) of Feed Starch: Experimental Results

Experimental Run	MALTOSE RICH PRODUCT				DEXTRIN RICH PRODUCT				Conversion	Relative Enzyme usage	Mass Balance
	Maltose purity %	Impurities % D	G	Total product conc. %w/v	Dextrin purity %	Impurities % M	G	Total product conc. % w/v	%	Actual / Theoretical	Output / Input %
20.3A-9-25.5-60-20	80.0	12.0	8.0	1.33	92.4	7.6	0.0	2.36	50.9	0.583	96.6
20.26B-9-25.5-60-20	87.7	0.0	12.3	0.73	97.0	3.0	0.0	3.60	32.7	0.883	99.5

M = Maltose, D = Dextrin, G = Glucose

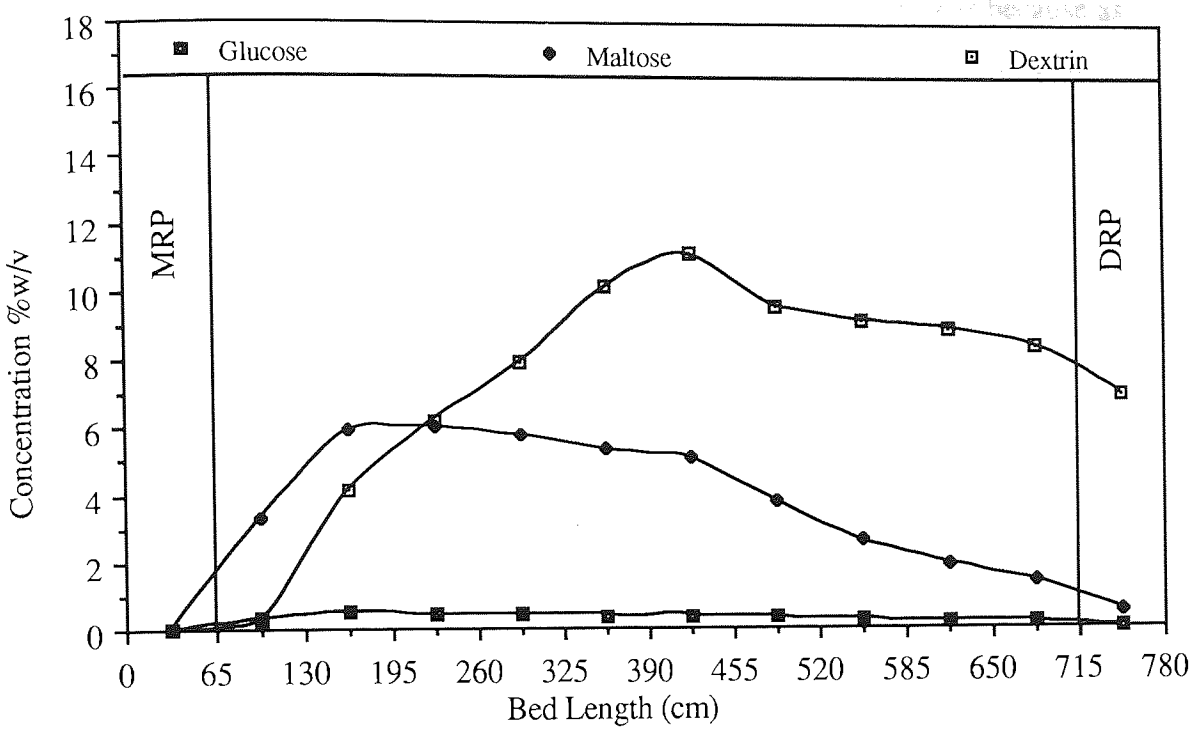


Figure 6.14: On-Column Concentration Profile for Run 20.3A-9-25.5-60-20 Cycle 15

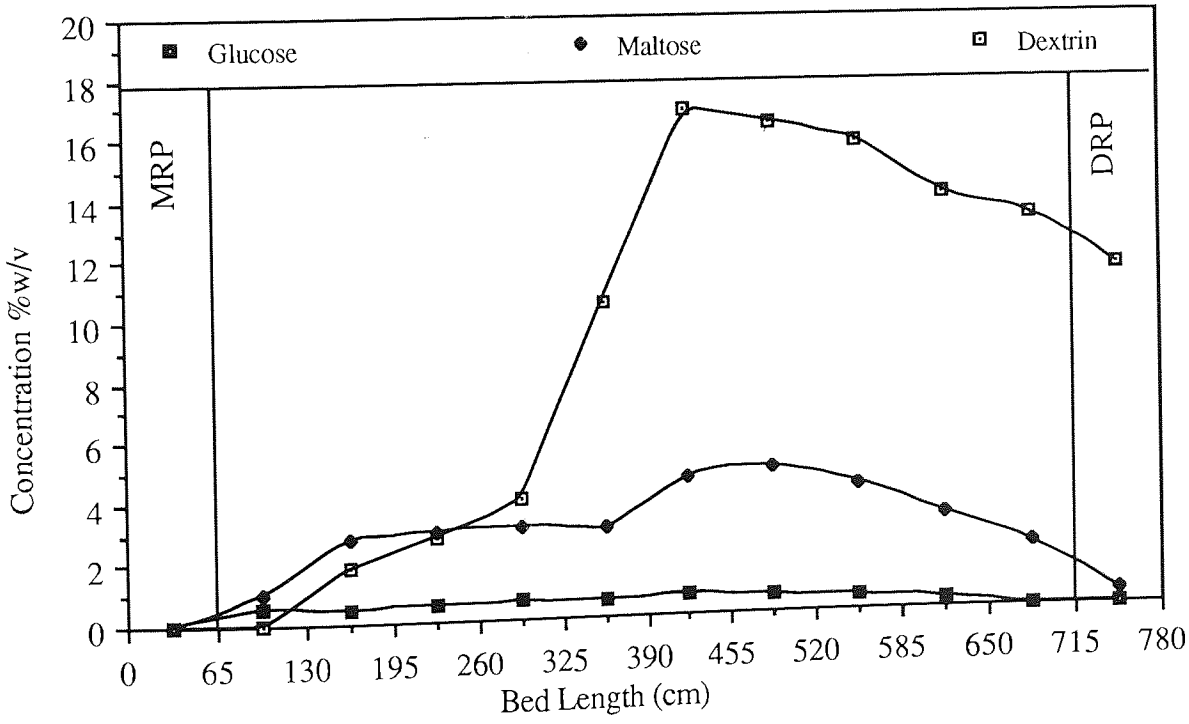


Figure 6.15: On-Column Concentration Profile for Run 20.26B-9-25.5-60-20 Cycle 11

It was found that using a feed of higher DE produced more glucose. This was because as the DE of the substrate increases, the greater the proportion of D-glucose polymers with uneven chains thus causing more glucose to be formed and less maltose. This was also observed by Outtrup *et al.*[68] using the enzyme maltogenase for starch with DE ranging from 7 to 21.

6.6.6 Effect of Enzyme Activity

The effect of enzyme activity on product purities and the performance of the SCCR-S system was investigated by varying the enzyme activity in the eluent whilst all other conditions remained constant.

The experimental conditions and results are shown in Tables 6.16 and 6.17. Increasing the enzyme activity increased the enzyme-to-substrate ratio thus increasing the reaction rate. At high reaction rates, the length of the system required to hydrolyse the α-(1-4) glucosidic linkages of the starch to produce maltose was reduced and therefore a longer length was available for separation thus producing products of better purities. At higher reaction rates, the viscosity of the feed starch was quickly reduced causing a reduction in the amount of dextrin retained in the pre-feed Section of the system. This rapid reduction in viscosity also contributed to the production of maltose with better purities.

At higher enzyme activities, more starch was converted to maltose and more glucose was produced. This resulted in higher MRP concentrations. This was due to the increased reaction rate and the hydrolysis of more α-(1-6) glucosidic linkages in the substrate releasing more glucose.

Figures 6.16 and 6.17 show the on-column concentration profiles for runs 9.6-9-27-60-19.5 and 20.26B-9-25.5-30-20 respectively. Comparing Figure 6.16 to Figure 6.8, it can be seen that the concentration of maltose in the pre-feed Section of the SCCR-S system at a higher enzyme activity was higher. This was due to the increased reaction rate producing more maltose which then complexed with calcium ions on the resin and moved with the

Table 6.16: Effect of Enzyme Activity: Experimental Conditions

Experimental Run	Average Flowrates cm^3min^{-1}			Starch Feed Conc.	Enzyme Activity in Eluent (U/cm^3)	Switch Time	Lm/P	Cycle No	Feed Throughput ($Kg/h/m^3$ resin)
	Feed	Eluent	Purge	%w/v		(min)			
9.5-9-27-30-19.5	9.0	27.0	76.0	9.5	30	19.5	0.092	16	2.85
9.6-9-27-60-19.5	9.0	27.0	76.0	9.6	60	19.5	0.092	8	2.88
20.3-9-25.5-60-20	9.0	25.5	76.0	20.3	60	20.0	0.077	15	6.09
20.3-9-25.5-90-20	9.0	25.5	76.0	20.3	90	20.0	0.077	9	6.09
20.26B-9-25.5-30-20	9.0	25.5	76.0	20.26	30	20.0	0.077	17	6.09
20.26B-9-25.5-60-20	9.0	25.5	76.0	20.26	60	20.0	0.077	11	6.09

B - Indicating starch feed of DE=7 (Starch B)

Table 6.17: Effect of Enzyme Activity: Experimental Results

Experimental Run	MALTOSE RICH PRODUCT				DEXTRIN RICH PRODUCT				Conversion	Relative Enzyme usage Actual / Theoretical	Mass Balance Output / Input
	Maltose purity %	Impurities % D	G	Total product conc. %w/v	Dextrin purity %	Impurities % M	G	Total product conc. % w/v	%		%
9.5-9-27-30-19.5	99.9	0.0	0.0	0.57	84.6	15.4	0.0	1.17	58.3	0.556	99.9
9.6-9-27-60-19.5	99.9	0.0	0.0	0.70	98.6	1.4	0.0	1.01	60.8	0.970	102.7
20.3-9-25.5-60-20	80.0	12.0	8.0	1.33	92.4	7.6	0.0	2.36	50.9	0.583	96.6
20.3-9-25.5-90-20	90.4	0.0	9.6	1.45	99.5	0.5	0.0	2.00	61.5	0.712	98.1
20.26B-9-25.5-30-20	75.3	16.4	8.3	0.73	95.1	4.9	0.0	3.68	28.8	0.498	100.1
20.26B-9-25.5-60-20	87.7	0.0	12.3	0.73	97.0	3.0	0.0	3.60	32.7	0.883	99.5

M = Maltose, D = Dextrin, G = Glucose

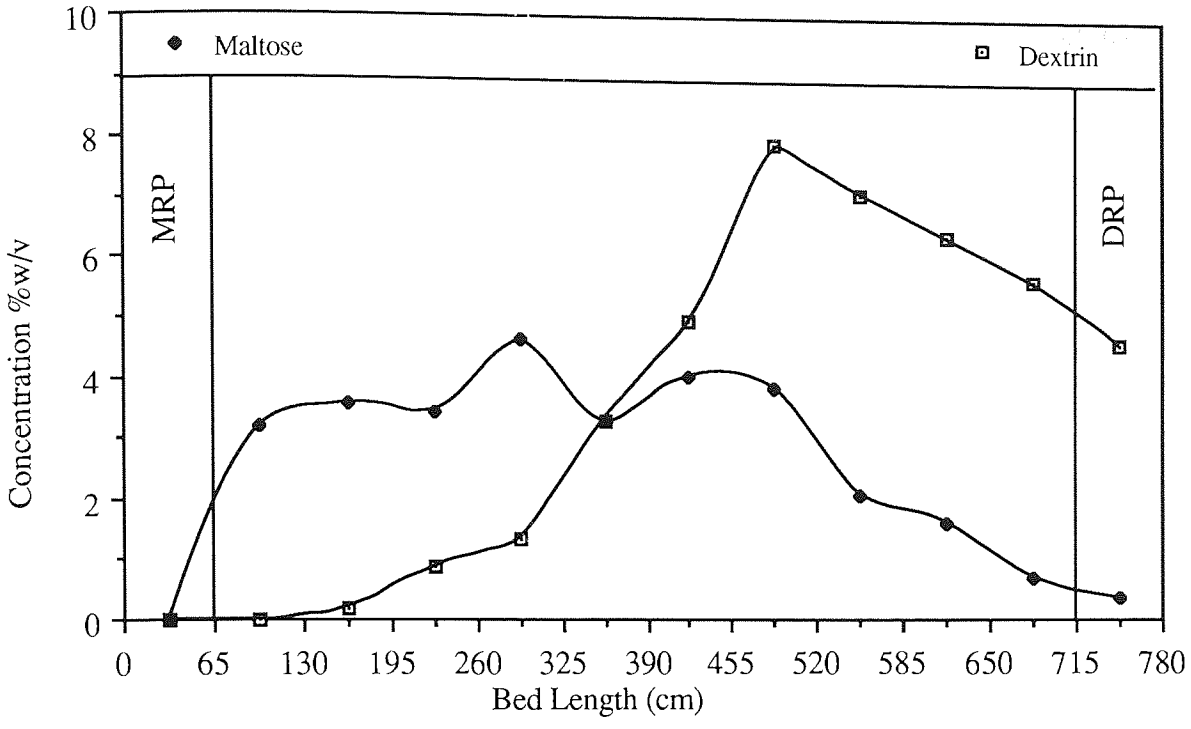


Figure 6.16: On-Column Concentration Profile for Run 9.6-9-27-60-19.5 Cycle 8

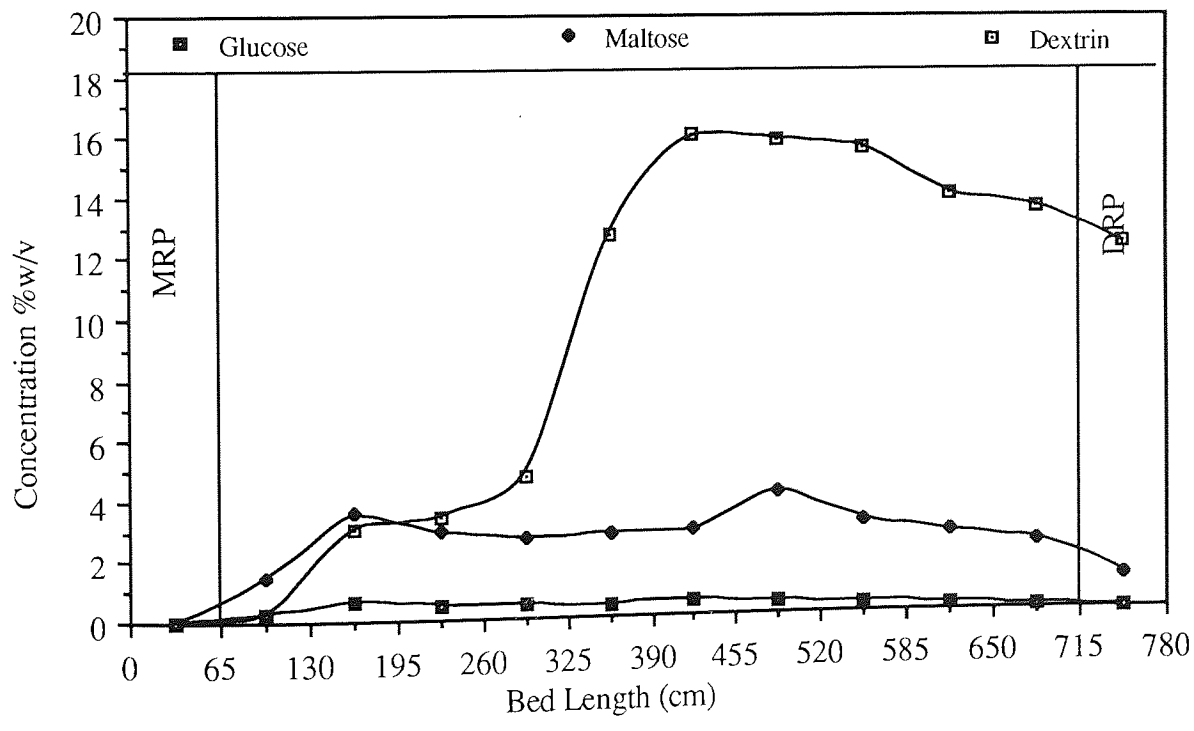


Figure 6.17: On-Column Concentration Profile for Run 20.26B-9-25.5-30-20 Cycle 17

stationary phase. The increased conversion rate at higher enzyme activity also reduced the concentration of dextrin in the system (Figure 6.16 compared to Figure 6.8).

Reducing the enzyme activity (runs 20.26B-9-25.5-60-20 and 20.26B -9-25.5-30-20), reduced the reaction rate and conversion of the feed starch to maltose. This resulted in an increase in viscosity in the system causing more dextrin to be retained in the pre-feeed columns thus shifting the "cross over" point towards the MRP offtake (Figures 6.15 and 6.17). When the enzyme activity was reduced, more of the system length was required to completely hydrolyse the α-(1-4) glucosidic linkages producing maltose, the reaction time was therefore the limiting factor resulting in the MRP being contaminated.

6.6.7 Effect of Adding Pullulanase Enzyme

As mentioned previously, maltogenase is not able to hydrolyse the α-(1-6) glucosidic linkages in amylopectin, limit dextrins or to bypass them. Maltose yield would obviously be improved if a debranching enzyme such as pullulanase is added in the saccharification process.

The effect of the pullulanase on the saccharification and performance of the SCCR-S system was investigated by adding pullulanase to the eluent containing the maltogenase enzyme. Two types of starch feeds (Starch A and Starch B) were used for these experiments. The experimental conditions and results are shown in Tables 6.18 and 6.19.

The results show that the presence of the enzyme pullulanase increased the amount of glucose produced (Figures 6.18 and 6.19) and also improved the conversion of starch to maltose. The increased glucose production caused a reduction in the purity of the MRP. The MRP concentration was higher because the improved starch conversion resulted in more maltose and glucose.

The improvement in starch conversion and the increase in amount of glucose produced was due to the ability of the pullulanase to hydrolyse the α-(1-6) glucosidic linkages in

Table 6.18: Effect of Adding Pullulanase Enzyme : Experimental Conditions

Experimental Run	Average Flowrates cm^3min^{-1}			Starch Feed Conc.	Enzyme Activity in Eluent	Switch Time	Lm/P	Cycle No	Feed Throughput
	Feed	Eluent	Purge	%w/v	(U/cm^3)	(min)			($Kg/h/m^3$ resin)
9.5-9-27-30-19.5	9.0	27.0	76.0	9.5	30	19.5	0.092	16	2.85
9.3-9-27-30-19.5P	9.0	27.0	76.0	9.3	30+1	19.5	0.092	7	2.79
25B-9-25.5-120-21	9.0	25.5	76.0	25.0	120	21.0	0.108	7	7.50
25B-9-25.5-120-21P	9.0	25.5	76.0	25.0	120+3	21.0	0.108	13	7.50

P - Indicating experiments with pullulanase enzyme
B - Indicating experiments with feed starch of DE=7 (Starch B)

Table 6.19: Effect of Adding Pullulanase Ezyme : Experimental Results

Experimental Run	MALTOSE RICH PRODUCT				DEXTRIN RICH PRODUCT				Conversion	Relative Enzyme usage	Mass Balance
	Maltose purity %	Impurities % D	Impurities % G	Total product conc. %w/v	Dextrin purity %	Impurities % M	Impurities % G	Total product conc. % w/v	%	Actual / Theoretical	Output / Input %
9.5-9-27-30-19.5	99.9	0.0	0.0	0.57	84.6	15.4	0.0	1.17	58.3	0.556	99.9
9.3-9-27-30-19.5P	90.6	0.0	9.4	0.65	96.8	3.2	0.0	0.91	60.6	0.549	99.5
25B-9-25.5-120-21	91.8	0.0	8.2	0.85	96.0	4.0	0.0	4.45	32.4	1.480	96.8
25B-9-25.5-120-21P	87.9	0.0	12.1	1.07	95.7	4.3	0.0	4.65	36.5	1.190	107.4

M = Maltose, D = Dextrin, G = Glucose

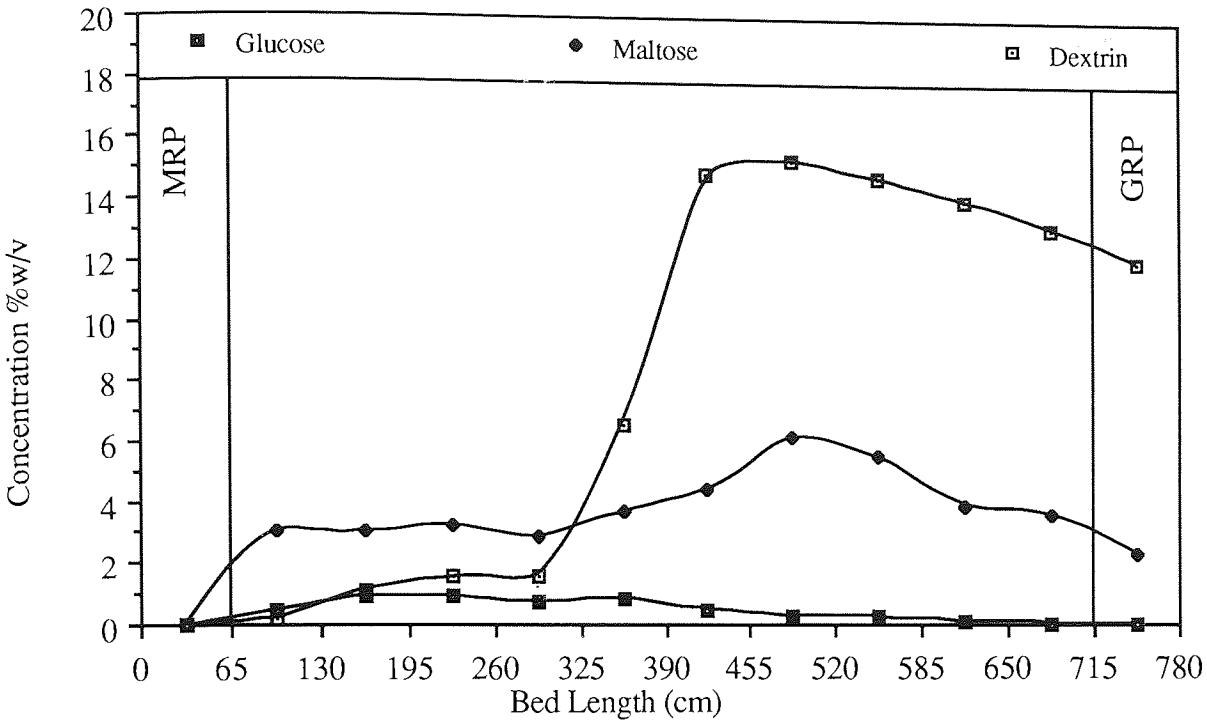


Figure 6.18: On-Column Concentration Profile for Run 25B-9-25.5-120-21 Cycle 7

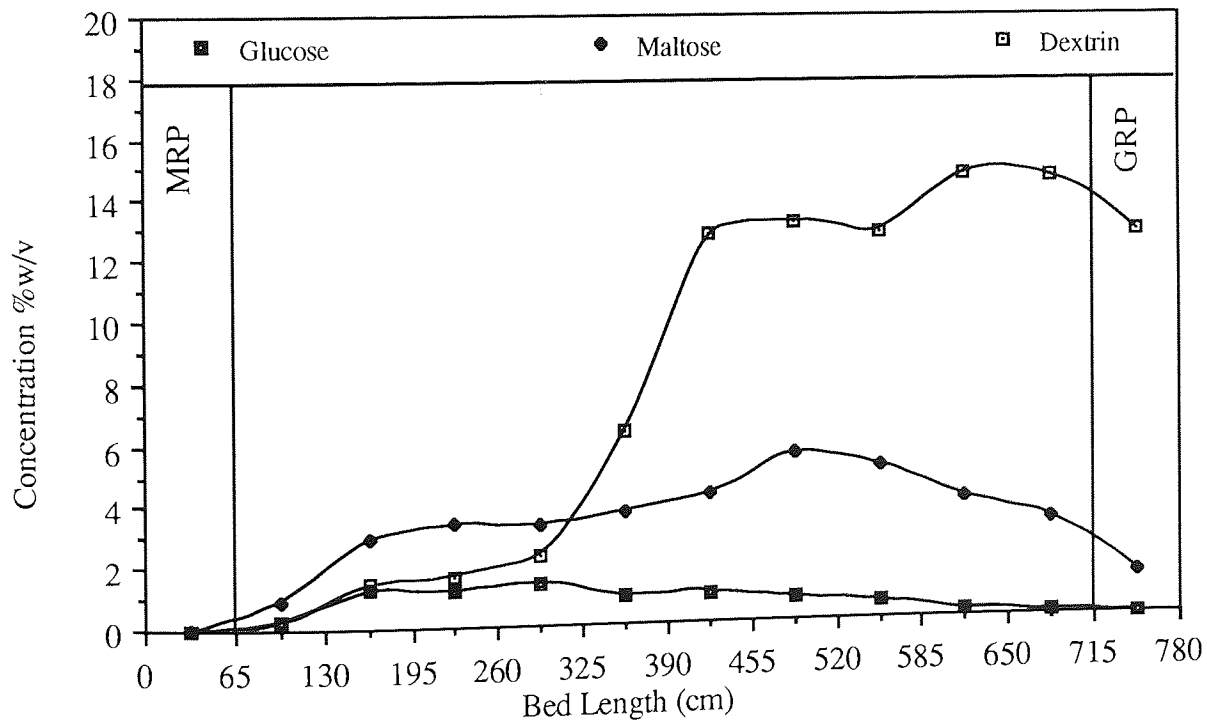


Figure 6.19: On-Column Concentration Profile for Run 25B-9-25.5-120-21P Cycle 13

the feed starch. These observations are in agreement with the results reported by Outtrup *et al.*(68) who showed that the conversion of starch to maltose can be improved by adding pullulanase to the maltogenase enzyme.

6.6.8 Effect of Purge Flowrate

The effect of the purge flowrate on the MRP concentration was investigated. Purge flowrates of 76cm^3/min, 50cm^3/min and 38cm^3/min were used whilst all other parameters remained constant. Starch B was used as the feed and the enzyme pullulanase was added to the eluent containing maltogenase.

The experimental conditions and results are shown in Tables 6.20 and 6.21. It was found that the MRP concentration increased as the purge flowrate was reduced. This was expected as reducing the purge flowrate reduced the amount of de-ionised water diluting the retarded maltose. However, Figures 6.20 and 6.21 and 6.22 show that, at lower purge flowrates the time required to completely purge out the maltose and other retarded components was increased. At a purge flowrate of 76cm^3/min, the components were completely purged out after 13 minutes (Figure 6.20) while at 50 cm^3/min, the full switch time of 20 minutes was required (Figure 6.21). The components were not completely eluted using a purge flowrate of 38cm^3/min and therefore the DRP was more contaminated by the uneluted maltose left in the purge column which becomes the next DRP column.

These results agree with those obtained by previous workers(13,16) using the SCCR-S system. They found that for the separation of glucose and fructose on the SCCR-S system, fructose retarded was completely eluted during the first half of the switch time (0-15mins) for a switch time of 30 minutes using a purge flowrate of 76cm^3/min.

The purge flowrate of 50cm^3/min produced a more concentrated product but did not allow for a reduction in the switch time. From these results it was decided to use the purge flowrate of 76cm^3/min with a recycling technique to concentrate the MRP.

Table 6.20: Effect of Purge Flowrate: Experimental Conditions

Experimental Run	Average Flowrates cm^3min^{-1}			Starch Feed Conc.	Enzyme Activity in Eluent	Switch Time	Lm/P	Cycle No	Feed Throughput
	Feed	Eluent	Purge	%w/v	(U/cm^3)	(min)			($Kg/h/m^3$ resin)
25B-9-25.5-120-21P	9.0	25.5	76.0	25.0	120+3	21.0	0.108	13	7.50
25B-9-25.5-120-21P	9.0	25.5	50.0	25.0	120+3	21.0	0.108	16	7.50
25B-9-25.5-120-21P	9.0	25.5	38.0	25.0	120+3	21.0	0.108	20	7.50

B - Indicating experiments with feed starch of DE=7 (starch B)
P - Indicating experiments with pullulanase enzyme

Table 6.21: Effect of Purge Flowrate : Experimental Results

Experimental Run	MALTOSE RICH PRODUCT				DEXTRIN RICH PRODUCT				Conversion	Relative Enzyme usage Actual / Theoretical	Mass Balance Output / Input
	Maltose purity %	Impurities % D	Impurities % G	Total product conc. %w/v	Dextrin purity %	Impurities % M	Impurities % G	Total product conc. % w/v	%		%
25B-9-25.5-120-21P	87.9	0.0	12.1	1.07	95.7	4.3	0.0	4.65	36.5	1.190	107.4
25B-9-25.5-120-21P	87.0	0.0	13.0	1.58	96.5	3.5	0.0	4.50	36.1	1.240	104.1
25B-9-25.5-120-21P	88.0	0.0	12.0	2.08	91.9	8.1	0.0	4.55	35.6	1.250	104.8

M = Maltose, D = Dextrin, G = Glucose

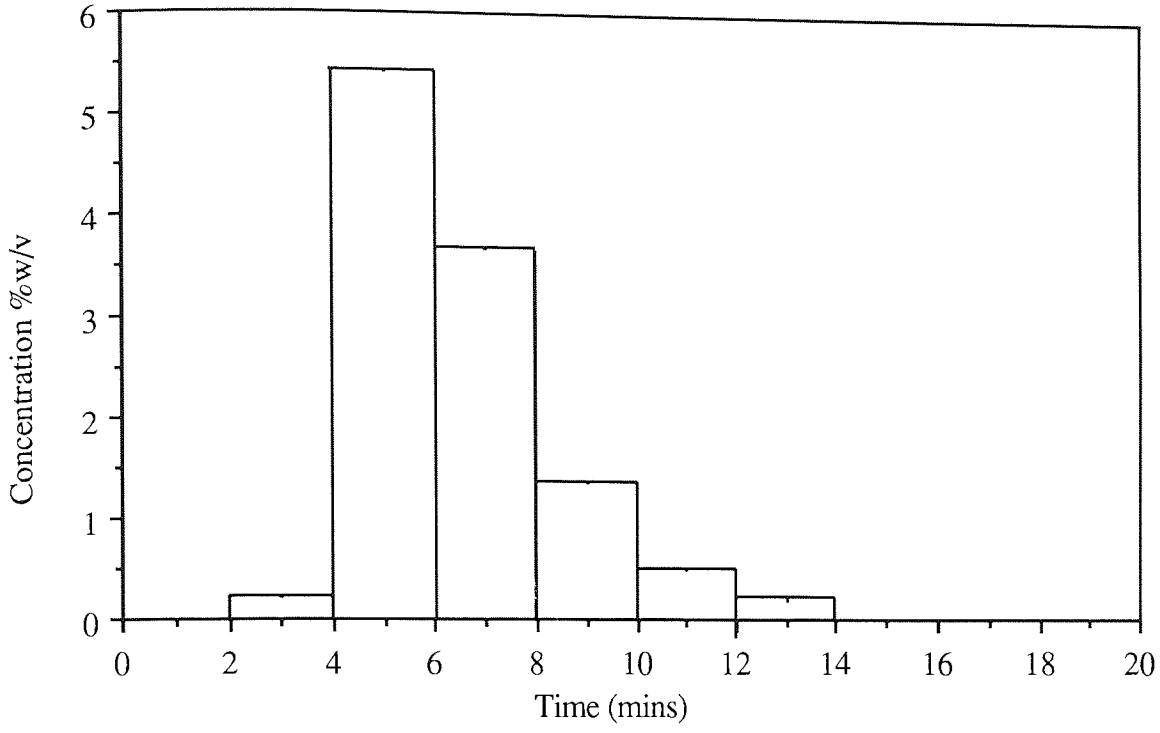


Figure 6.20: MRP Elution Profile at Purge Flowrate of 76cm^3/min.

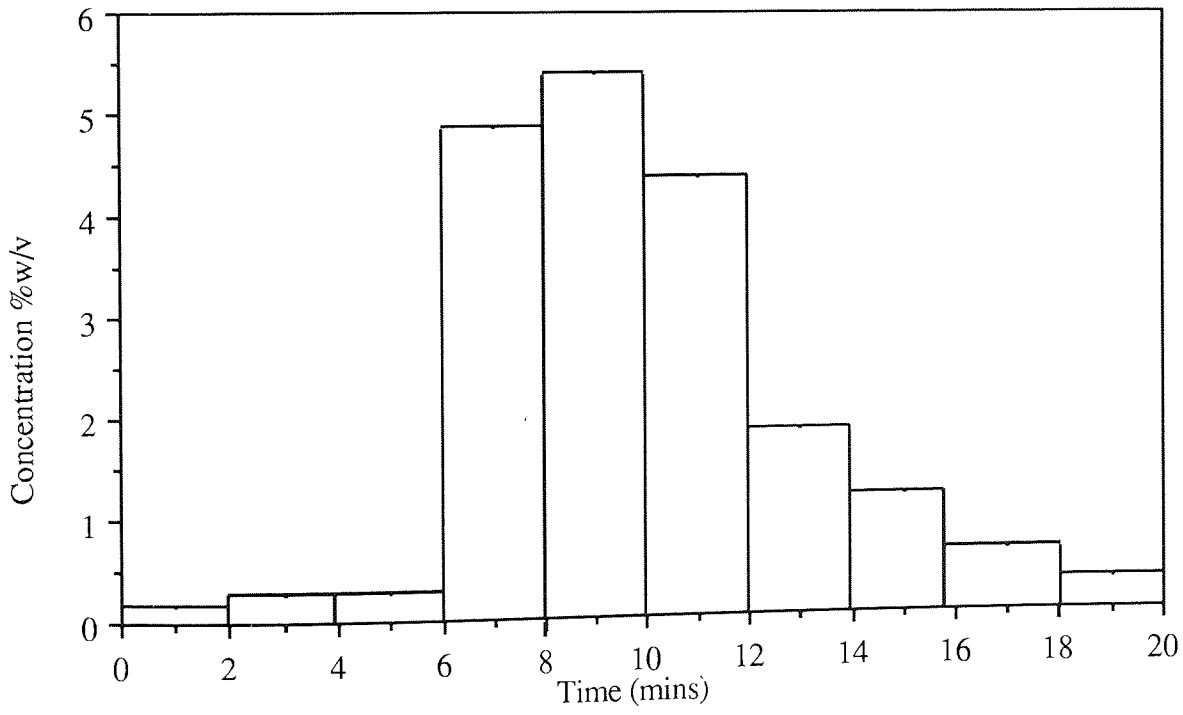


Figure 6.21: MRP Elution Profile at Purge Flowrate of 50cm^3/min.

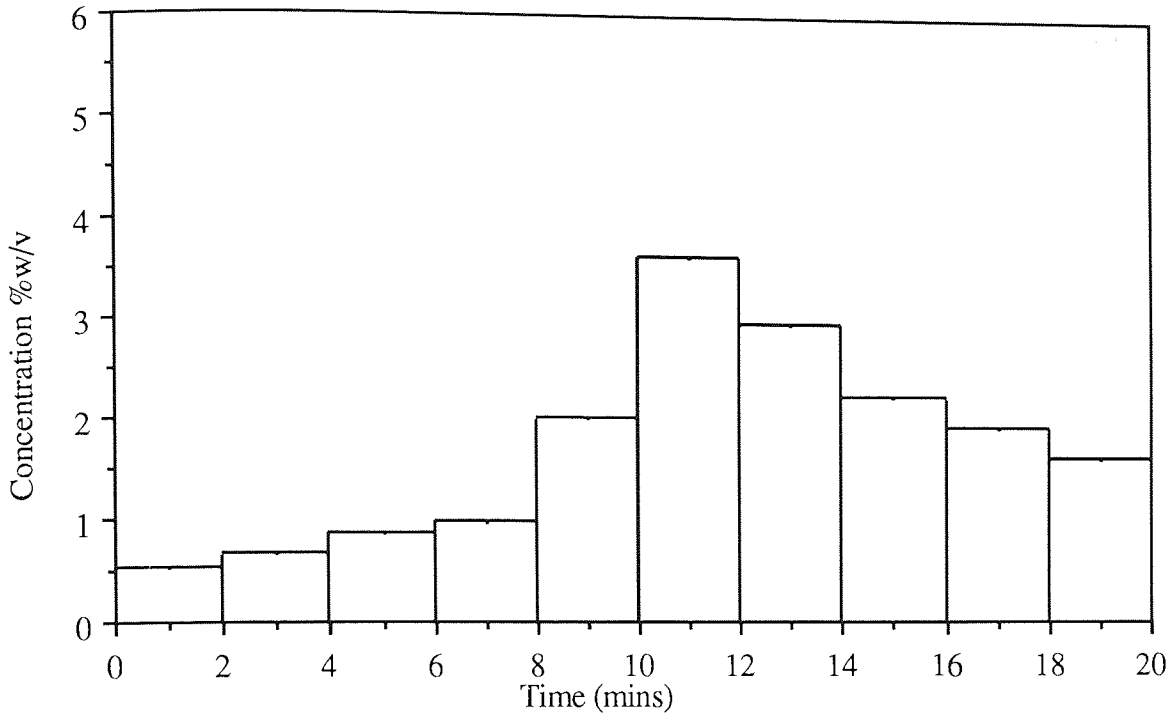


Figure 6.22: MRP Elution Profile at Purge flowrate of 38cm^3/min.

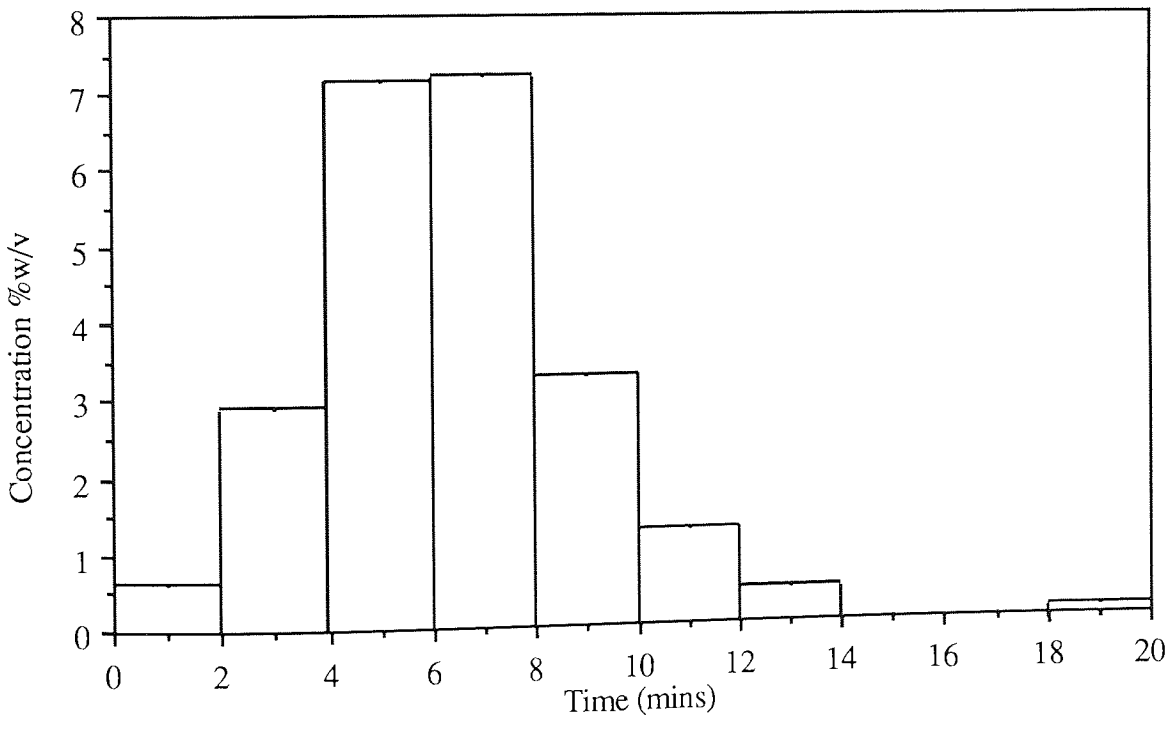


Figure 6.23: DRP Elution Profile

The elution profile of the DRP product is shown in Figure 6.23. It was observed that most of the DRP was eluted during the first 13 minutes of the switch. These results do not agree with the results observed by previous workers[13,16] who showed that most of the glucose rich product during the separation of glucose and fructose on the SCCR-S system was eluted during the last half of the switch (8-25 minutes). The difference observed was due to the relatively lower switch times and mobile phase flowrates used during this study.

6.6.9 Effect of Recycling the MRP Dilute Fraction

The commercial viability of the SCCR-S system as a bioreactor-separator can be improved by improving the product concentration, reducing the amount of enzyme and deionised water used. An attempt to improve the product concentration and reduce the amount of deionised water used was made by employing a combination of product splitting and the recycling of the dilute fraction of the MRP. The splitting technique was achieved using a 3-way solenoid valve and a timer in each product line (Chapter 5). This allowed the product exiting from each line to be split into two fractions (i.e the dilute and concentrated fractions).

Previous workers[13] have shown that recycling the dilute fraction of the DRP contaminated the MRP. It was therefore decided not to recycle the dilute fraction of the DRP since maltose was the main product and will be contaminated.

From the MRP and DRP elution profiles (Figures 6.20 and 6.23), it was apparent that the most of the concentrated MRP and DRP products can be collected in the initial part of the switch. Tables 6.22 and 6.23 show the concentrations of the concentrated and dilute fractions of the MRP and DRP products after product splitting of run 20.3-9-25.5-60-20. It was observed that the fractions collected during the first 10 minutes were more concentrated than the fractions collected during the last 10 minutes of the switch. From these results it was decided to recycle the dilute fraction of the MRP collected during the last 10 minutes of the switch. The DRP dilute fraction was not recycled.

Three runs were repeated employing product splitting and recycling of the MRP dilute fractions. Tables 6.24 and 6.25 show the experimental conditions and results. From Table 6.25 it was observed that, although there were no substantial changes in the MRP purities, the concentrations were increased. These results show that it was possible to improve the MRP product concentrations and reduce the amount of deionised water required for purging by about 50% by employing the MRP product splitting and recycling technique.

Table 6.22: MRP Results Ater Splitting the Dilute Fraction

Experimental Run	Collection Period 0-10mins		Collection Period 10-20mins	
	Maltose Purity	Conc. %w/v	Maltose Purity	Conc. %w/v
20.3-9-25.5-60-20	80.4	2.35	99.9	0.05

Table 6.23: DRP Results After Splitting the Dilute Fraction

Experimental Run	Collection Period 0-10mins		Collection Period 10-20mins	
	Dextrin Purity	Conc. %w/v	Dextrin Purity	Conc. %w/v
20.3-9-25.5-60-20	90.8	3.57	87.8	1.23

Table 6.24: Effect of Recycling MRP dilute fraction: Experimental Conditions

Experimental Run	Average Flowrates cm^3min^{-1}			Starch Feed Conc.	Enzyme Activity in Eluent	Switch Time	Lm/P	Cycle No	Feed Throughput
	Feed	Eluent	Purge	%w/v	(U/cm^3)	(min)			($Kg/h/m^3$ resin)
9.3-9-27-30-19.5	9.0	27.0	76.0	9.3	30	19.5	0.092	7	2.79
9.3-9-27-30-19.5R	9.0	27.0	76.0	9.3	30	19.5	0.092	14	2.79
14.78-9-25.5-60-21	9.0	25.5	76.0	14.78	60	21.0	0.108	7	4.43
14.78-9-25.5-60-21R	9.0	25.5	76.0	14.78	60	21.0	0.108	18	4.43
20.3-9-25.5-60-20	9.0	25.5	76.0	20.3	60	20.0	0.077	15	6.09
20.0-9-25.5-60-20R	9.0	25.5	76.0	20.0	60	20.0	0.077	12	6.00

R - Indicates experiments with dilute fraction of MRP Recycled

Table 6.25: Effect of Recycling MRP dilute fraction: Experimental Results

Experimental Run	MALTOSE RICH PRODUCT				DEXTRIN RICH PRODUCT				Conversion	Relative Enzyme usage Actual / Theoretical	Mass Balance Output / Input
	Maltose purity %	Impurities % D	G	Total product conc. %w/v	Dextrin purity %	Impurities % M	G	Total product conc. % w/v	%		%
9.3-9-27-30-19.5	99.9	0.0	0.0	0.59	91.5	8.5	0.0	1.19	55.3	0.572	104.7
9.3-9-27-30-19.5R	99.9	0.0	0.0	1.20	92.0	8.0	0.0	1.09	57.9	0.554	103.2
14.78-9-25.5-60-21	91.4	0.0	8.6	0.88	94.6	5.4	0.0	1.69	54.5	0.798	93.8
14.78-9-25.5-60-21R	92.0	0.0	8.0	1.88	94.2	5.8	0.0	1.76	57.2	0.692	99.3
20.3-9-25.5-60-20	80.0	12.0	8.0	1.33	92.4	7.6	0.0	2.36	50.9	0.583	96.6
20.0-9-25.5-60-20R	79.5	13.0	7.5	2.62	92.0	8.0	0.0	2.50	50.7	0.556	103.0

M = Maltose, D = Dextrin, G = Glucose

6.6.10 Enzyme Usage

The amount of maltogenase used to carry out the bioreaction-separation for all the experiments was calculated relative to the theoretical amount of enzyme required. The calculations were based on the standard definition of the maltogenase enzyme activity unit (U), which is defined as the amount of the enzyme required to produce 1μmol of maltose in 1 minute at a pH of 5.3 and temperature 60°C. The actual rate of enzyme activity entering the system (U/min) was compared to the theoretical amount of enzyme activity required to produce the same amount of maltose in the system per minute (g/min).

The relative amounts of enzyme used in the various runs are shown in Tables 6.9, 6.11, 6.13, 6.15, 6.17, 6.21 and 6.25. From these results it was found that the SCCR-S system required less enzyme than a conventional batch bioreactor to produce the same amount of maltose over the same period. An example of the calculations of the relative amount of enzyme is given in Appendix B. These results show the efficiency of the SCCR-S system.

6.7 COMPARISON OF BIOREACTION AND SEPARATION PERFORMANCE IN THE SCCR-S SYSTEM AND A CONTINUOUS ROTATING ANNULAR CHROMATOGRAPH (CRAC)

Sarmidi[60] studied the performance of a continuous rotating annular chromatograph (CRAC) as a bioreactor-separator for the production of maltose from modified starch using the enzyme maltogenase. The comparison of reaction and separation performance on the SCCR-S system and the CRAC (Section 2.7.2.2) was based on the feed throughput, enzyme usage and starch conversion. The comparison was for the same type of feed starch, the Crystal Gum UK (starch A).

The two systems were comparable in terms of throughput and the SCCR-S was better than the CRAC in terms of enzyme usage. From Table 6.26, it can be seen that the SCCR-S required only 34.6-47.3% of the amount of enzyme required by the CRAC system. The lower enzyme requirement and better conversion obtained using the SCCR-S system were due to the longer time the substrate was in contact with the enzyme. There was separation

between the enzyme and the degraded starch in the CRAC thus reducing the contact time between the enzyme and the substrate.

The CRAC has the ability to handle multicomponent mixtures and the products can be collected into any number of fractions. It was therefore possible for the CRAC to produce maltose of higher purities by separating the glucose produced from the maltose which was not possible using the SCCR-S system.

In terms of equipment constructions, the SCCR-S system was more complex than the CRAC system. Although the capital costs for both equipments were similar, the bed length of the CRAC was 1.35 m whereas for the SCCR-S the bed length was 7.8 m. Sarmidi[50] compared the SCCR-S to the CRAC in terms of product concentrations for the hydrolysis of sucrose using the enzyme invertase and showed that the SCCR-S system produced products of higher concentrations.

Tables 6.26 : Comparison between the SCCR-S and CRAC Systems

SCCR-S SYSTEM			CRAC SYSTEM			Relative Enzyme Usage SCCR-S / CRAC
Throughput (Kg/h/m^3 resin)	Conversion %	Enzyme Usage (U/g)	Throughput (Kg/h/m3 resin)	Conversion %	Enzyme Usage (U/g)	
2.91	63.7	1786.9	2.12	63.3	5161.3	0.346
4.43	54.5	1150.2	4.28	53.1	2580.7	0.446
6.27	60.1	813.4	6.41	51.4	1720.4	0.473

CHAPTER 7

CONTINUOUS AND BATCH OPERATION OF THE SCCR-S SYSTEM AS BIOREACTOR-SEPARATOR FOR THE HYDROLYSIS OF LACTOSE

7.1 INTRODUCTION

The performance of the SCCR-S system as a bioreactor-separator was further investigated by studying the hydrolysis of lactose using the enzyme lactase from *Aspergillus oryzae* and simultaneously separating the products galactose and glucose. The SCCR-S system was first operated in the continuous mode, then modified and operated in the batch mode for comparison between batch and continuous modes of operation of the SCCR-S system. The results obtained during this study are presented in this chapter.

The hydrolysis of lactose by lactase from *A. oryzae* produces galactose and glucose. The galactose is known to strongly inhibit the lactase[70,71] and therefore requires large amounts of enzyme to achieve complete conversion in a conventional batch reactor. By continuously separating the galactose from the reaction medium, it was anticipated that the amount of enzyme required could be substantially reduced.

Galactose is known to form a complex with calcium ions and therefore by using a chromatographic system packed with resin in the Ca^{2+} form, the galactose can be separated from the reaction medium as it is formed.

7.2 PREPARATION OF THE LACTOSE FEED AND THE ENZYME LACTASE

The lactose used for this study was D(+) lactose monohydrate supplied by Fisons Scientific, Loughborough, UK. A feed solution of the required concentration was prepared by dissolving the calculated weight of the lactose in de-ionised water. The solution was heated with continuous stirring to 100°C. This was to completely dissolve the lactose and deaerate the feed solution. The pH of the solution was then adjusted to 5.0.

The lactase from *A. oryzae* was supplied by Quest (Biocon), Co. Cork, Ireland. The enzyme was supplied in a powder form with activity of 15,000-20,000 U/g. Enzyme solution of a required strength was prepared by dissolving the calculated weight of the enzyme in deionised water and the pH was adjusted to 5.0. The enzyme solution was filtered (sintered glass filter, Whatman) and transferred to the enzyme container. The enzyme was stable with less than 2% loss of declared activity over a period of six months when stored at 4°C.

7.3 SIMULTANEOUS BIOREACTION AND SEPARATION IN A BATCH (1.96cm i.d) CHROMATOGRAPHIC COLUMN

Before carrying out the hydrolysis of lactose on the SCCR-S system, it was desirable to first test the possibility of the bioreaction and separation taking place in a chromatographic column packed with a polystyrene resin in the Ca^{2+} form (DOWEX 50W-X4). The column was 1.96cm i.d and 2m long and made of a jacketed glass column. The arrangement of the equipment was similar to that of Zafar *et al.*[9,10] (Figure 2.8).

The separation of equimolar mixtures of galactose and glucose was first performed on the batch chromatographic column before attempting the bioreaction and separation . The galactose and glucose mixtures were prepared by dissolving the calculated weight of the sugars in de-ionised water. All the experiments were carried out at a temperature of 55°C and pH 5 which are the optimum conditions required for the enzyme reaction. The feed was injected as a pulse and the outlet stream was monitored using a differential refractometer linked to a chart recorder. The samples collected during the elution of the components were analysed using the HPLC.

7.3.1 Experimental Results and Discussion

The experimental runs are represented in the coded form, 10-1.5-3.5 where the code represents feed concentration (%w/v), pulse size (as % of total column volume (TCV)) and the eluent flowrate (cm^3/min) respectively for the separation experiments. Two experiments were carried out on the separation of galactose and glucose. The results of

runs 10-1.5-3.5 and 20-3-3.5 are shown in Figures 7.1 and 7.2. These results showed that it was possible to separate galactose and glucose in a chromatographic column packed with DOWEX 50W-X4 resin in the Ca^{2+} form.

The experimental runs for the simultaneous bioreaction and separation are represented in the coded form, 10-3-3.5-60 where the code represents lactose feed concentration (%w/v), pulse size (%TCV), eluent flowrate (cm^3/min) and the enzyme activity in the eluent (U/cm^3) respectively. Two simultaneous bioreaction and separation experiments, runs 10-3-3.5-60 and 20-3-3.5-60 were carried out on the batch chromatographic column. The results obtained are shown in Figures 7.3 and 7.4. The results indicated that it was possible to carry out simultaneous hydrolysis of lactose using lactase and separating the products galactose and glucose in a chromatographic bioreactor-separator. The results also showed that it was possible to achieve complete conversion of the lactose using an enzyme activity of $60U/cm^3$ in the eluent.

Comparing the elution profiles from runs 20-3-3.5 and 20-3-3.5-60, it was observed that the separation was better for the physical separation of galactose and glucose than for the simultaneous bioreaction and separation experiments. This was because in run 20-3-3.5 all the column length was used for the separation while for run 20-3-3.5-60, some of the length was used for reaction thus reducing the length available for separation of the products. More tailing was also observed in the elution profiles obtained during the simultaneous bioreaction and separation experiments. This was possibly due to the reaction rate and mechanism of the enzyme, lactase.

From the bioreaction and separation experiments it was concluded that a longer column length than 2m was required to achieve a reasonable separation between the products, glucose and galactose.

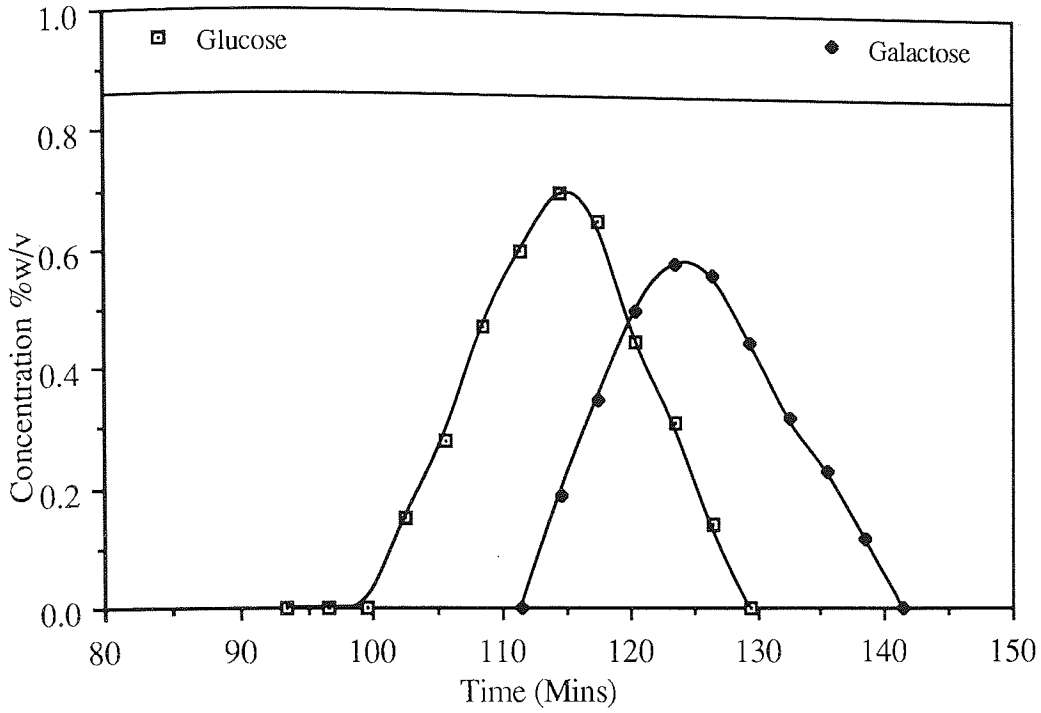


Figure 7.1: Elution Profile for the Separation of Galactose and Glucose Mixture in a Batch Chromatographic Column Run 10-1.5-3.5

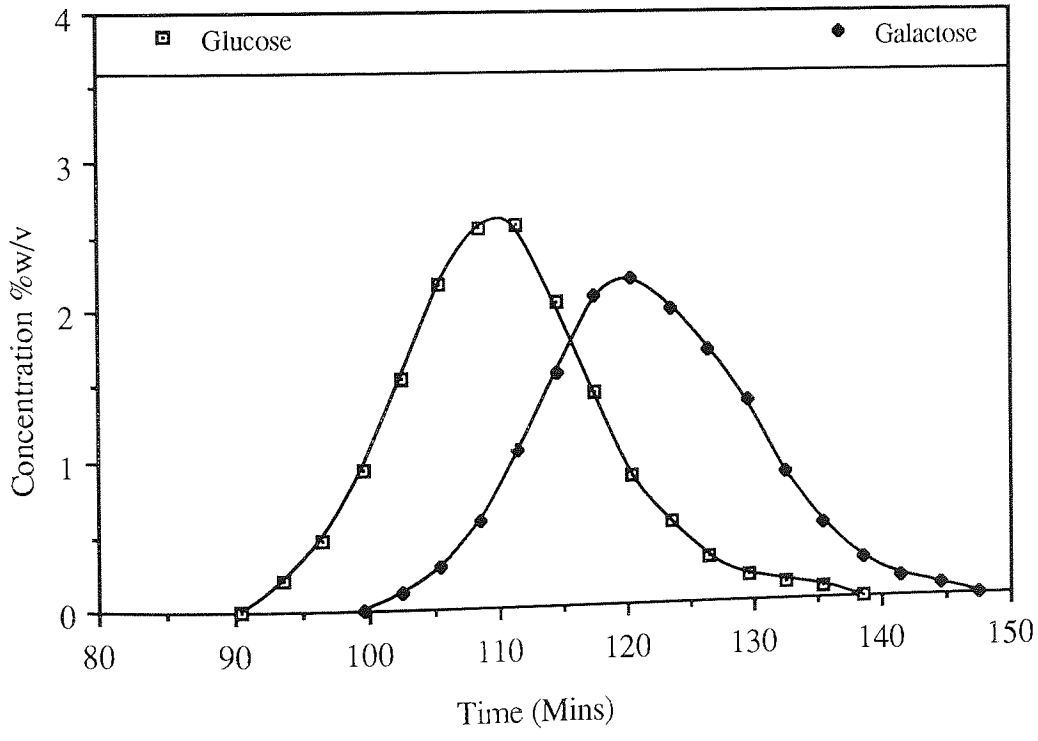


Figure 7.2: Elution Profile for the Separation of Galactose and Glucose Mixture in a Batch Chromatographic Column Run 20-3-3.5

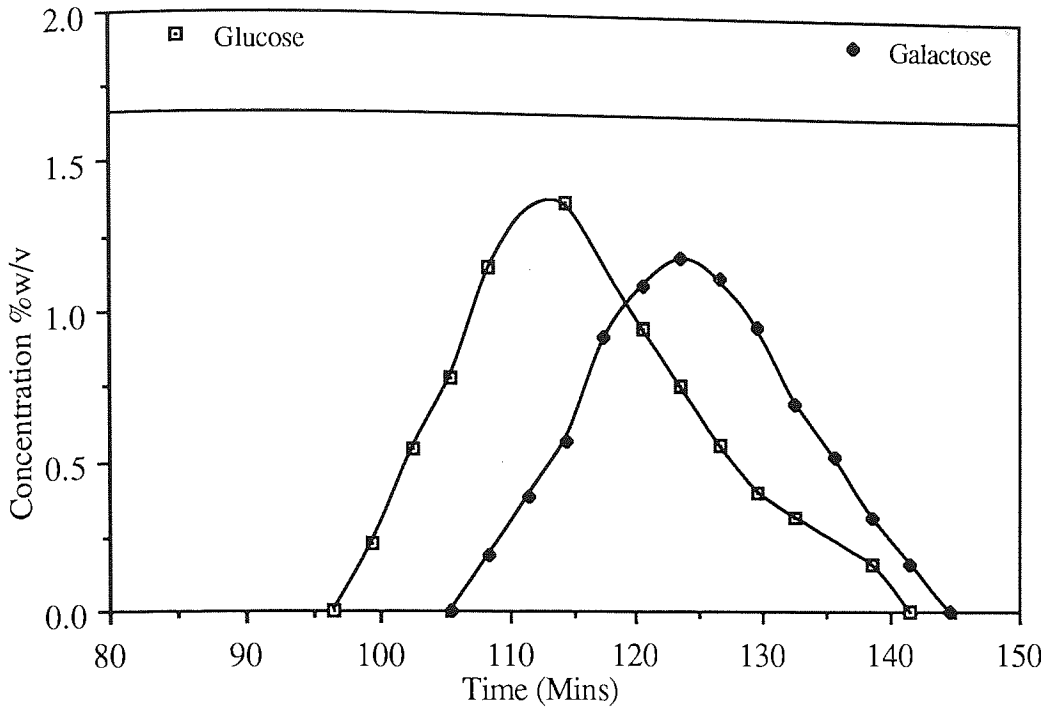


Figure 7.3: Elution Profile for Run 10-3-3.5-60 on a 1.96cm i.d Batch Column Bioreactor-Separator

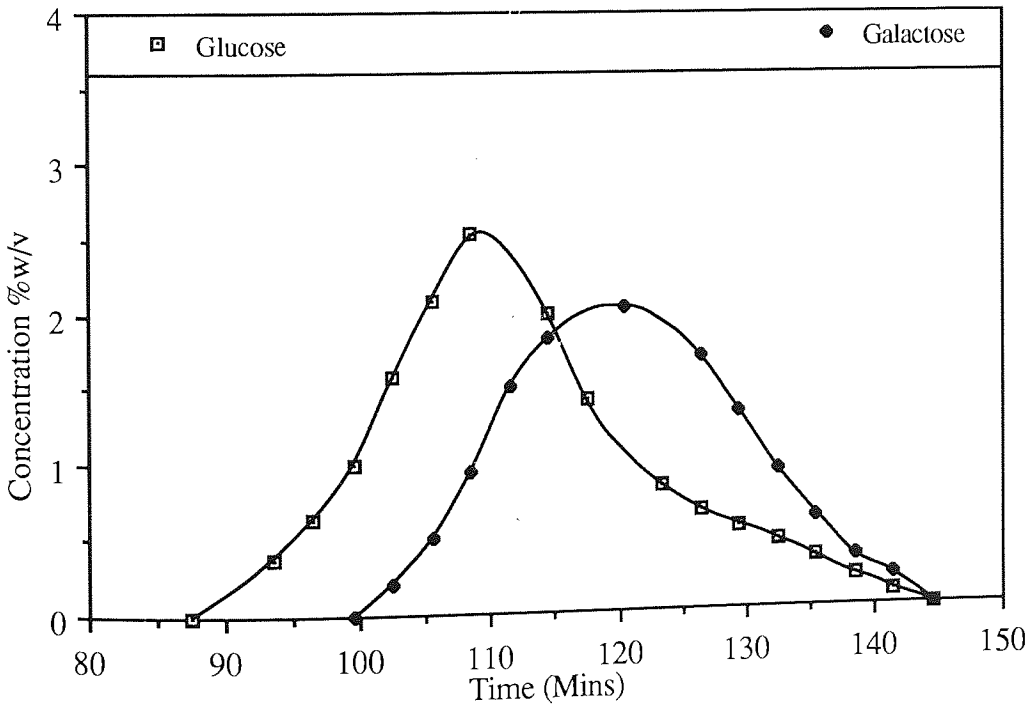


Figure 7.4: Elution Profile for Run 20-3-3.5-60 on a 1.96cm i.d Batch Column Bioreactor-Separator

7.4 CONTINUOUS OPERATION OF THE SCCR-S SYSTEM AS A BIOREACTOR-SEPARATOR FOR THE HYDROLYSIS OF LACTOSE

After carrying out the simultaneous bioreaction and separation in a batch chromatographic bioreactor-separator, the SCCR-S system was repacked with the same resin used in the batch column (DOWEX 50W-X4) and characterised as outlined in Chapter 5.

The parameters investigated during this study were the eluent flowrate, switch time, feed concentration and enzyme activity. The performance of the SCCR-S system was analysed in terms of enzyme usage, throughputs, product purities and product concentrations. The enzyme usage was related to the theoretical amount of enzyme required to produce the same amount of glucose in a batch reactor over the same time period.

7.4.1 Commissioning Runs with the Enzyme in the Eluent

The operating parameters such as the eluent flowrate and switch time were estimated from the Lm/P ratio (Equation 6.3). The temperature and pH were the same as those used in the batch experiments. The eluent used for these runs was a dilute enzyme solution similar to that used for the saccharification experiments.

The experimental conditions and results from these commissioning runs are shown in Tables 7.1 and 7.2. The experimental runs are represented in a coded form. For example 5.65-9-30-60-30 represents the feed concentration (%w/v), feed flowrate (cm^3/min), eluent flowrate (cm^3/min), enzyme activity in the eluent (U/cm^3) and the switch time (mins) respectively.

From the results it was observed that 1.96 times the theoretical amount of enzyme required by a conventional batch process was needed to achieve complete conversion. Although this very high amount of enzyme was used (run 5.5-9-30-90-30), the galactose rich product (GalRP) and the glucose rich product (GRP) purities were only 87% and 65.8% respectively.

Table 7.1: Commisioning Runs With Enzyme in the Eluent: Experimental Conditions

Experimental Run	Average Flowrates cm^3min^{-1}				Lactose Feed Conc %w/v	Enzyme Activity in Eluent U/cm^3	Switch Time (min)	Lm/P	Cycle No	Feed Throughput ($Kg/h/m^3$ resin)
	Feed	Enzyme	Eluent	Purge						
5.65-9-30-60-30	9.0	-	30.0	76.0	5.65	60	30.0	0.50	7	1.70
5.5-9-30-90-30	9.0	-	30.0	76.0	5.50	90	30.0	0.50	13	1.65

Table 7.2: Commisioning Runs With Enzyme in the Eluent: Experimental Results

Experimental Run	GALACTOSE RICH PRODUCT.				GLUCOSE RICH PRODUCT				Conversion %	Relative Enzyme usage Actual / Theoretical	Mass Balance Output / Input %
	Galact. purity %	Impurities %		Total product conc. %w/v	Glucose purity %	Impurities %		Total product conc. % w/v			
		G	L			GAL	L				
5.65-9-30-60-30	84.6	15.4	0.0	0.13	56.0	36.7	7.3	1.01	93.7	1.36	103.0
5.5-9-30-90-30	87.0	13.0	0.0	0.23	65.8	34.2	0.0	0.79	100	1.96	95.0

G = Glucose, GAL = Galactose, L = Lactose

Figures 7.5 and 7.6 show the on-column concentration profiles for runs 5.65-9-30-60-30 and 5.5-9-30-90-30. Figure 7.5 shows that the conversion of the lactose to galactose and glucose was incomplete resulting in the poorer GRP purity obtained. Figure 7.6 shows that by increasing the enzyme from 60U/cm^3 to 90U/cm^3 a complete conversion was obtained although a substantial length of the system was used for the reaction thus reducing the length available for separation.

These experiments showed that the amount of enzyme required by the SCCR-S system was higher than the theoretical amount of enzyme required by a conventional batch process to achieve complete conversion over the same time period when the enzyme was added to the eluent. It was therefore decided to carry out experiments with the enzyme and the feed introduced into the system together.

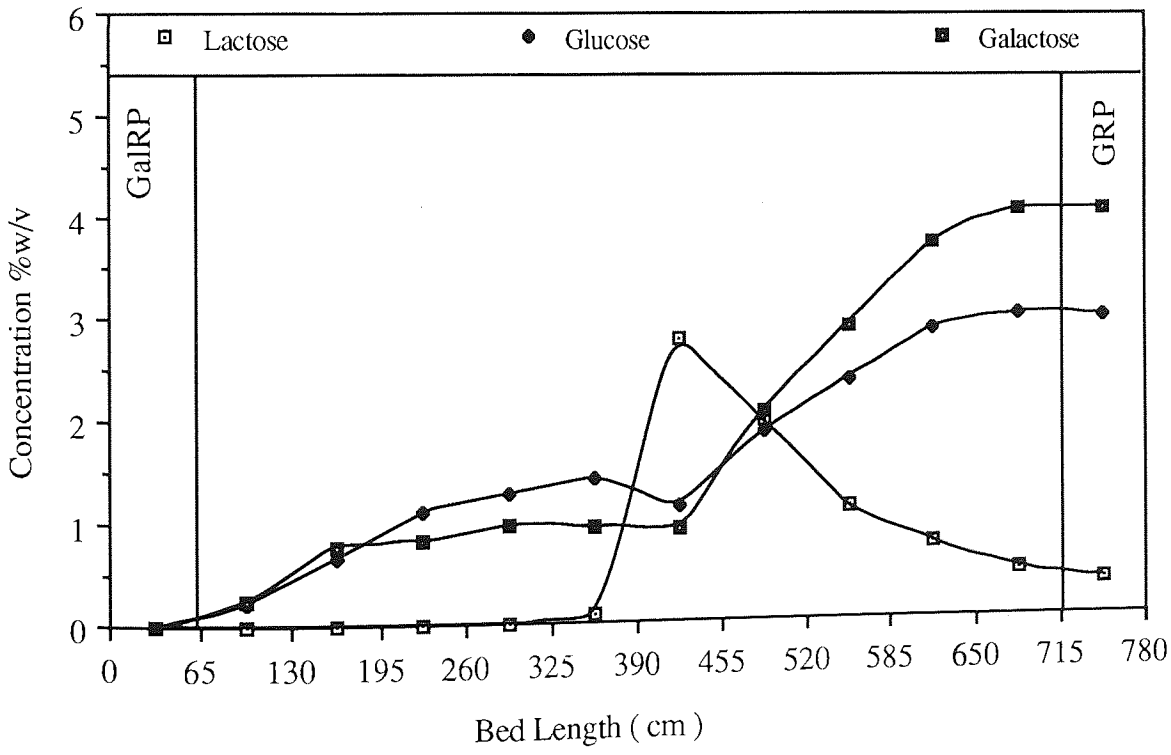


Figure 7.5: On-Column Concentration Profile for Run 5.65-9-30-60-30 Cycle 13

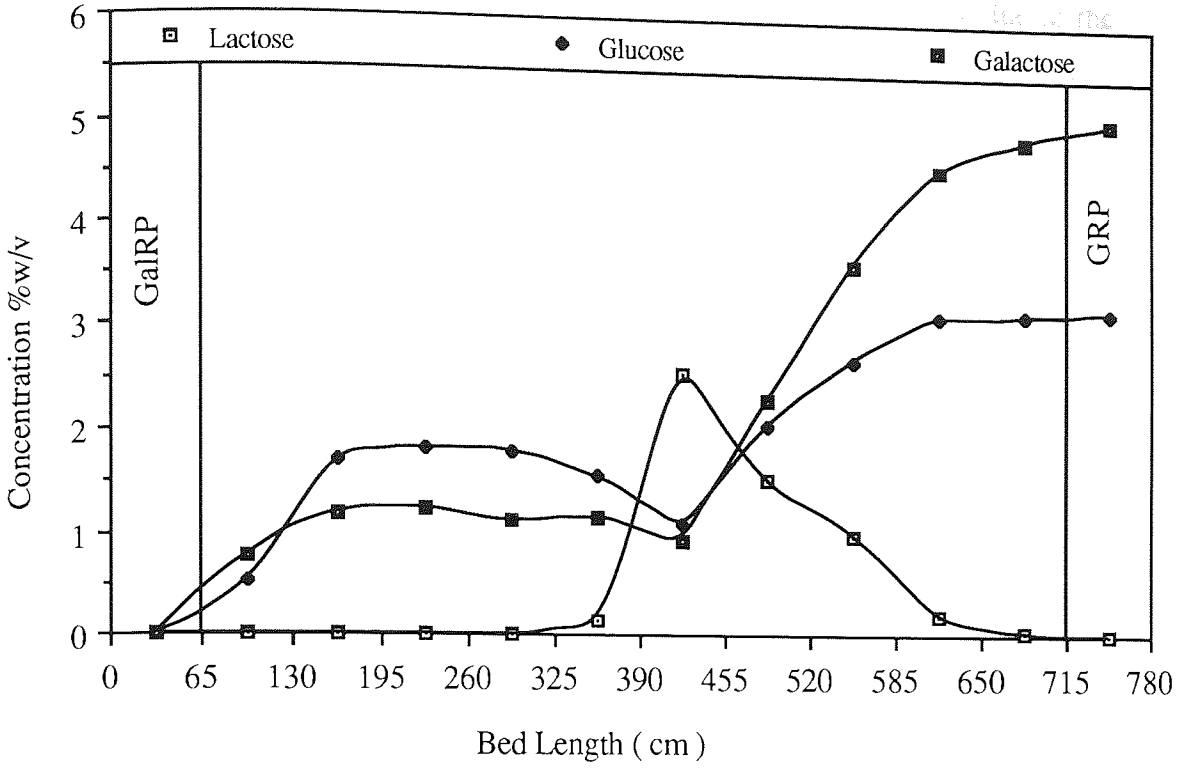


Figure 7.6: On-Column Concentration Profile for Run 5.5-9-30-90-30 Cycle 13

7.4.2 Commissioning Runs with Enzyme and Feed Entering the System Together

During these runs, the enzyme and the feed were fed into the SCCR-S system through the same port. The enzyme and the lactose feed were pumped separately from their respective containers and were mixed at the inlet to the system. The eluent was de-ionised water.

The experimental conditions and results are shown in Tables 7.3 and 7.4. The experimental runs are represented in a coded form, 5.1-4.5-4.5-30-100-26 where the code represents, the lactose feed concentration (%w/v), feed flowrate (cm^3/min), enzyme flowrate (cm^3/min), eluent flowrate (cm^3/min), enzyme activity (U/cm^3) and the switch time (mins) respectively.

From Table 7.4 it was found that by introducing the feed and the enzyme together into the SCCR-S system, the amount of enzyme required to achieve complete conversion was lower than was required when the enzyme was added in the eluent (Table 7.2) at similar

throughputs and all other operating conditions the same. This was possibly due to the stability of the enzyme, lactase, in the SCCR-S system. When the enzyme was added to the eluent, it was introduced into the system at column 2 while the feed was introduced at column 7. The enzyme had to pass through five columns (Figures 7.5 and 7.6) to be in contact with the substrate, lactose. It was therefore possible for some of the enzyme activity to be lost due to instability and adsorption of the enzyme in the system. When the enzyme was introduced with the feed, the enzyme was immediately in contact with the feed at the inlet to the system thus reducing the time the enzyme spent in the system without being in contact with the feed. This caused the reduction in the amount of enzyme required by the SCCR-S system when the enzyme and feed were introduced into the system together.

Figures 7.7 and 7.8 show the on-column concentration profile for runs 10-4.5-4.5-30-200-30 and 10-4.5-4.5-30-300-30. Comparing Figure 7.7 to 7.8, it was observed that there was a better separation between the galactose and glucose when the enzyme activity was increased. This was reflected in the better GRP purity obtained at higher enzyme activity. This was because by increasing the enzyme activity, the length of the system required for the reaction was reduced thus a longer length was available for separation resulting in better products.

Figures 7.9 and 7.10 show the on-column concentration profiles for two consecutive cycles (cycles 7 and 8) for run 5.1-4.5-4.5-30-100-26 which are very similar. This confirmed that the SCCR-S system had reached a pseudo-steady state after 7 cycles of continuous operation.

From the commissioning runs it was found that it was better to introduce the enzyme and the feed into the SCCR-S system at the same port while using an eluent of de-ionised water. Although the amount of enzyme required by the SCCR-S system was still higher than the amount required by a conventional batch reactor, there was scope for further optimization by studying the effects of the operating parameters with the aim of reducing the amount of enzyme required by the SCCR-S system. This method of introducing the

Table 7.3: Commisioning Runs With Enzyme and Feed Entering the System at the same port: Experimental Conditions

Experimental Run	Average Flowrates cm^3min^{-1}				Lactose Feed Conc %w/v	Enzyme Activity U/cm^3	Switch Time (min)	Lm/P	Cycle No	Feed Throughput ($Kg/h/m^3$ resin)
	Feed	Enzyme	Eluent	Purge						
5.1-4.5-4.5-30-100-26	4.5	4.5	30.0	76.0	5.1	100	26.0	0.39	7	0.77
10-4.5-4.5-30-200-30	4.5	4.5	30.0	76.0	10.0	200	30.0	0.50	11	1.50
10-4.5-4.5-30-300-30	4.5	4.5	30.0	76.0	10.0	300	30.0	0.50	10	1.50

Table 7.4: Commisioning Runs With Enzyme and Feed Entering the System at the same port: Experimental Results

Experimental Run	GALACTOSE RICH PRODUCT.				GLUCOSE RICH PRODUCT				Conversion %	Relative Enzyme usage Actual / Theoretical	Mass Balance Output / Input %
	Galact. purity %	Impurities %		Total product conc. %w/v	Glucose purity %	Impurities %		Total product conc. % w/v			
		G	L			GAL	L				
5.1-4.5-4.5-30-100-26	57.6	42.4	0.0	0.25	99.9	0.0	0.0	0.10	100	0.71	99.7
10-4.5-4.5-30-200-30	90.9	9.1	0.0	0.13	58.5	33.7	7.8	0.83	93.9	0.72	99.4
10-4.5-4.5-30-300-30	90.6	9.4	0.0	0.22	75.0	25.0	0.0	0.72	100	1.08	99.6

G = Glucose, GAL = Galactose, L = Lactose

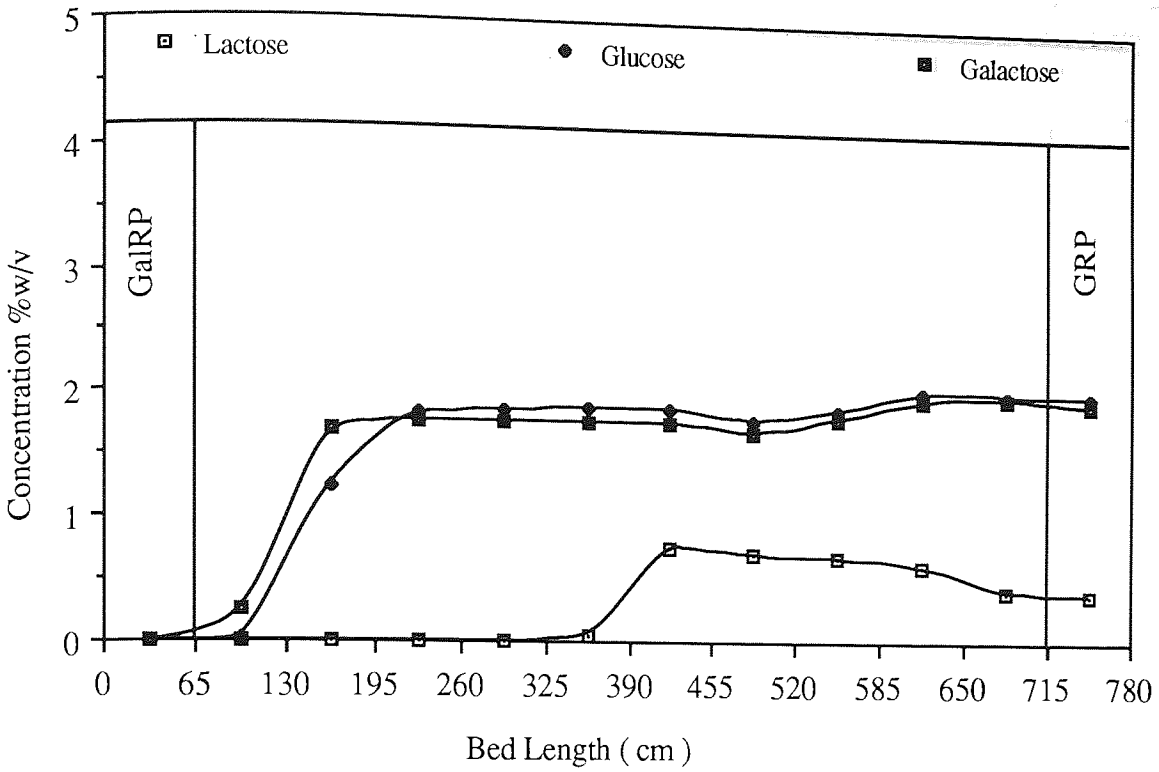


Figure 7.7: On-Column Concentration Profile for Run 10-4.5-4.5-30-200-30 Cycle 11

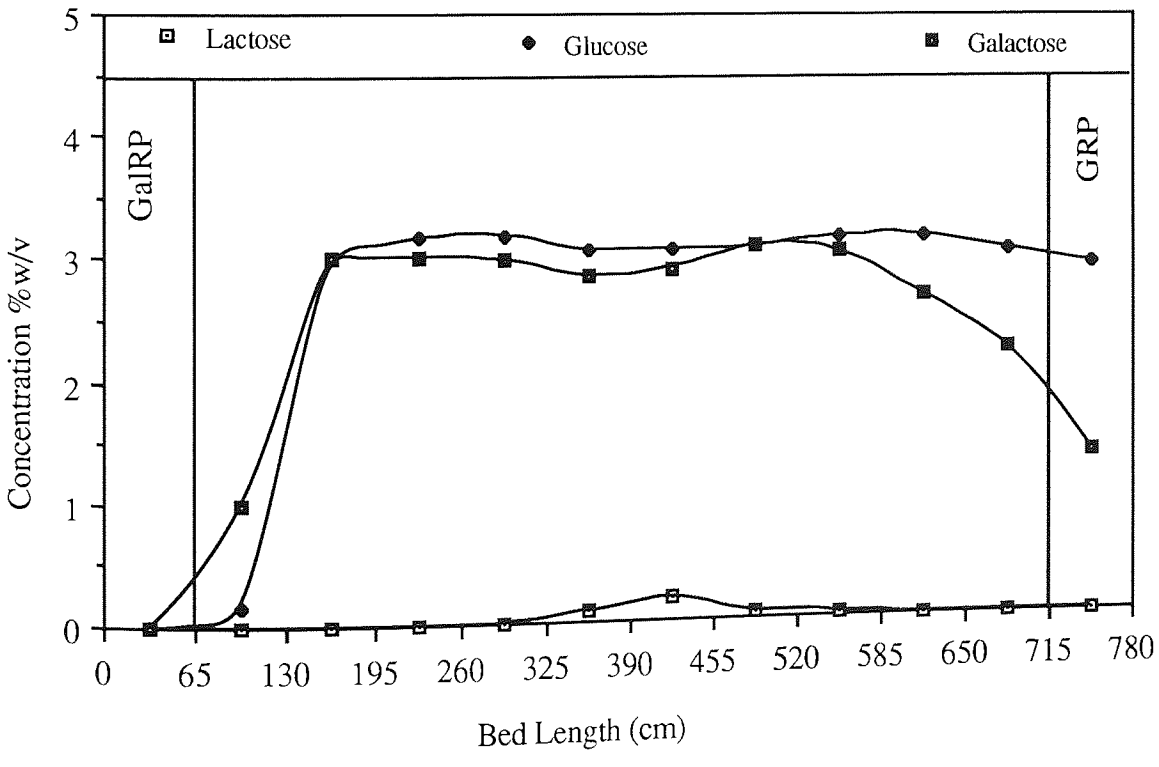


Figure 7.8: On-Column Concentration Profile for Run 10-4.5-4.5-30-300-30 Cycle 10

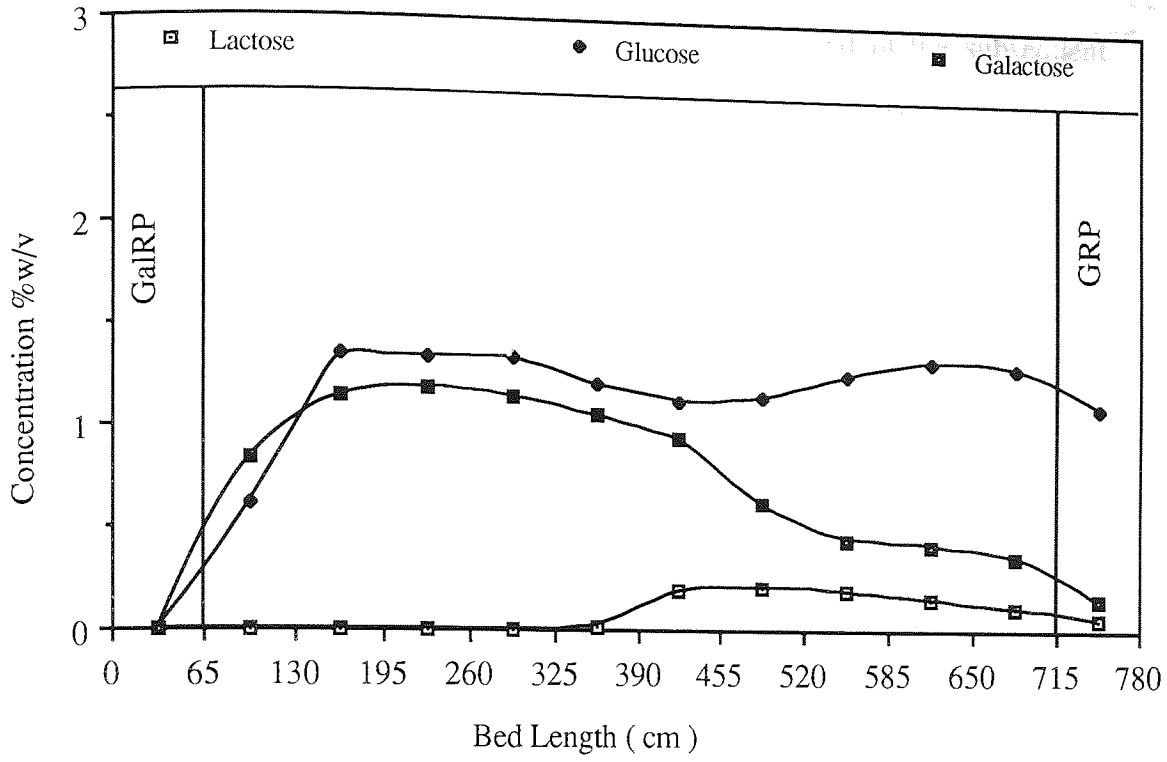


Figure 7.9: On-Column Concentration Profile for Run 5.1-4.5-4.5-30-100-26 Cycle 7

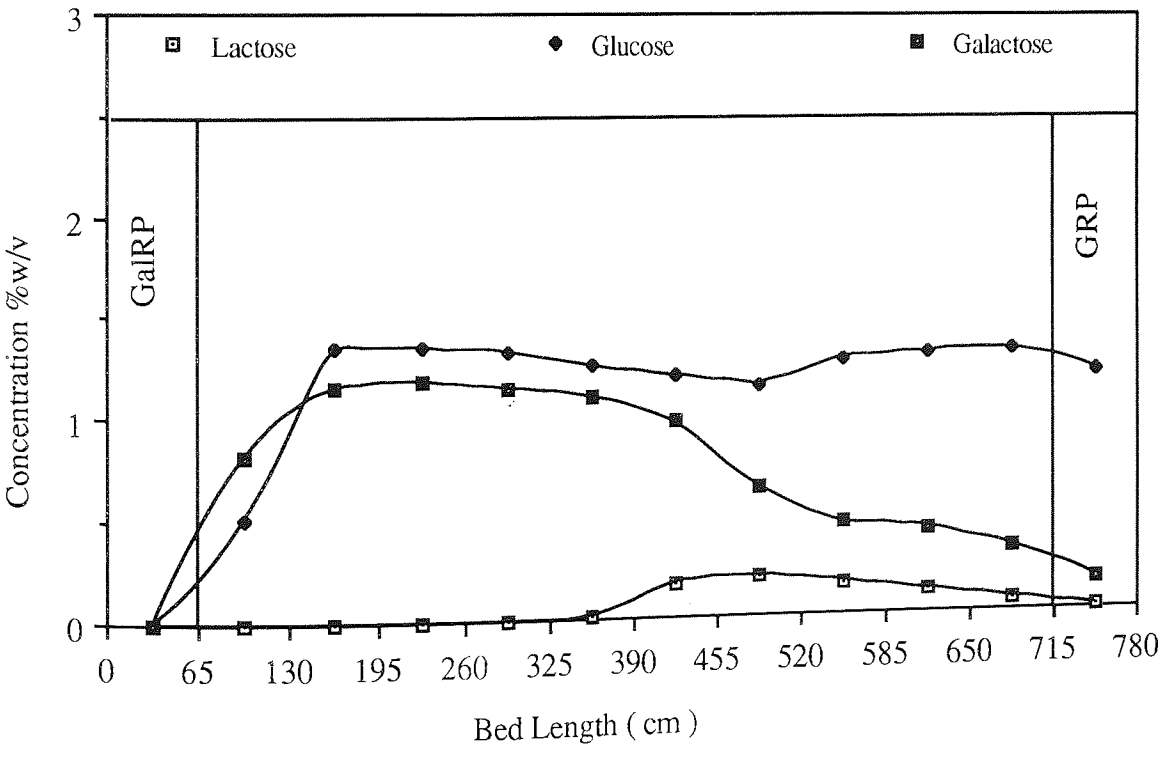


Figure 7.10: On-Column Concentration Profile for Run 5.1-4.5-4.5-30-100-26 Cycle 8

enzyme and the feed into the system at the same port was used in the subsequent experiments. Samples were collected from the system from the seventh cycle during which a pseudo-steady state had been reached. When a parameter was changed, the operation was continued for at least four cycles for the system to regain its steady state before samples were collected.

7.4.3 Effect of Eluent Flowrate

The eluent flowrate was found to be one of the controlling parameters that affected the performance of the SCCR-S system used for the saccharification of modified starch. The effect of the eluent flowrate on the performance of the SCCR-S system in terms of enzyme usage, conversion, product purities and concentrations was further studied when the SCCR-S system was used for the hydrolysis of lactose.

Tables 7.5 and 7.6 show the experimental conditions and results. At high eluent flowrates, the movement of the mobile phase relative to the stationary phase was increased and more galactose was stripped off the resin and moved forward with the mobile phase. This caused most of the glucose and some galactose to be removed in the glucose rich product (GRP) thus reducing the purities of the GRP and increasing its concentration and as a result led to high galactose rich product (GalRP) purities at lower concentrations.

The on-column concentration profiles in Figures 7.11, 7.12, 7.13 and 7.14 showed that although increasing the eluent flowrate did not affect the overall conversion in the SCCR-S system, the length of the system used for the reaction was increased thus reducing the length available for separation. This effect combined with the increased stripping of galactose that had complexed on the resin and moved forward resulted in the increased broadening of the galactose profile in the SCCR-S system thus reducing the GRP purities and increasing the concentration.

Comparing Figures 7.13 and 7.14 it can be observed that, the "cross over" point between the galactose and glucose profiles was shifted towards the GRP end of the system at a higher eluent flowrates.

Table 7.5: Effect of Eluent Flowrate: Experimental Conditions

Experimental Run	Average Flowrates cm^3min^{-1} Feed	Enzyme	Eluent	Purge	Lactose Feed Conc %w/v	Enzyme Activity U/cm^3	Switch Time (min)	Lm/P	Cycle No	Feed Throughput ($Kg/h/m^3$ resin)
5.1-4.5-4.5-27-100-26	4.5	4.5	27.0	76.0	5.1	100	26.0	0.310	13	0.77
5.1-4.5-4.5-30-100-26	4.5	4.5	30.0	76.0	5.1	100	26.0	0.390	7	0.77
10-4.5-4.5-28-300-28	4.5	4.5	28.0	76.0	10.0	300	28.0	0.404	14	1.50
10-4.5-4.5-30-300-28	4.5	4.5	30.0	76.0	10.0	300	28.0	0.460	8	1.50
15-4.5-4.5-28-300-29	4.5	4.5	28.0	76.0	15.0	300	29.0	0.437	14	2.25
15-4.5-4.5-32-300-29	4.5	4.5	32.0	76.0	15.0	300	29.0	0.550	8	2.25

Table 7.6: Effect of Eluent Flowrate: Experimental Results

Experimental Run	GALACTOSE RICH PRODUCT. Galact. purity %	Impurities % G	L	Total product conc. %w/v	GLUCOSE RICH PRODUCT Glucose purity %	Impurities % GAL	L	Total product conc. % w/v	Conversion %	Relative Enzyme usage Actual / Theoretical	Mass Balance Output / Input %
5.1-4.5-4.5-27-100-26	55.6	44.4	0.0	0.27	99.9	0.0	0.0	0.07	100	0.71	100.4
5.1-4.5-4.5-30-100-26	57.6	42.4	0.0	0.25	99.9	0.0	0.0	0.10	100	0.71	100.1
10-4.5-4.5-28-300-28	63.6	36.4	0.0	0.44	99.9	0.0	0.0	0.30	100	1.08	99.0
10-4.5-4.5-30-300-28	87.5	12.5	0.0	0.36	90.3	9.7	0.0	0.46	100	1.08	100.7
15-4.5-4.5-28-300-29	62.8	37.2	0.0	0.70	97.4	2.6	0.0	0.39	100	0.72	100.2
15-4.5-4.5-32-300-29	99.9	0.0	0.0	0.17	62.9	38.0	0.0	1.29	100	0.72	97.5

G = Glucose, GAL = Galactose, L = Lactose

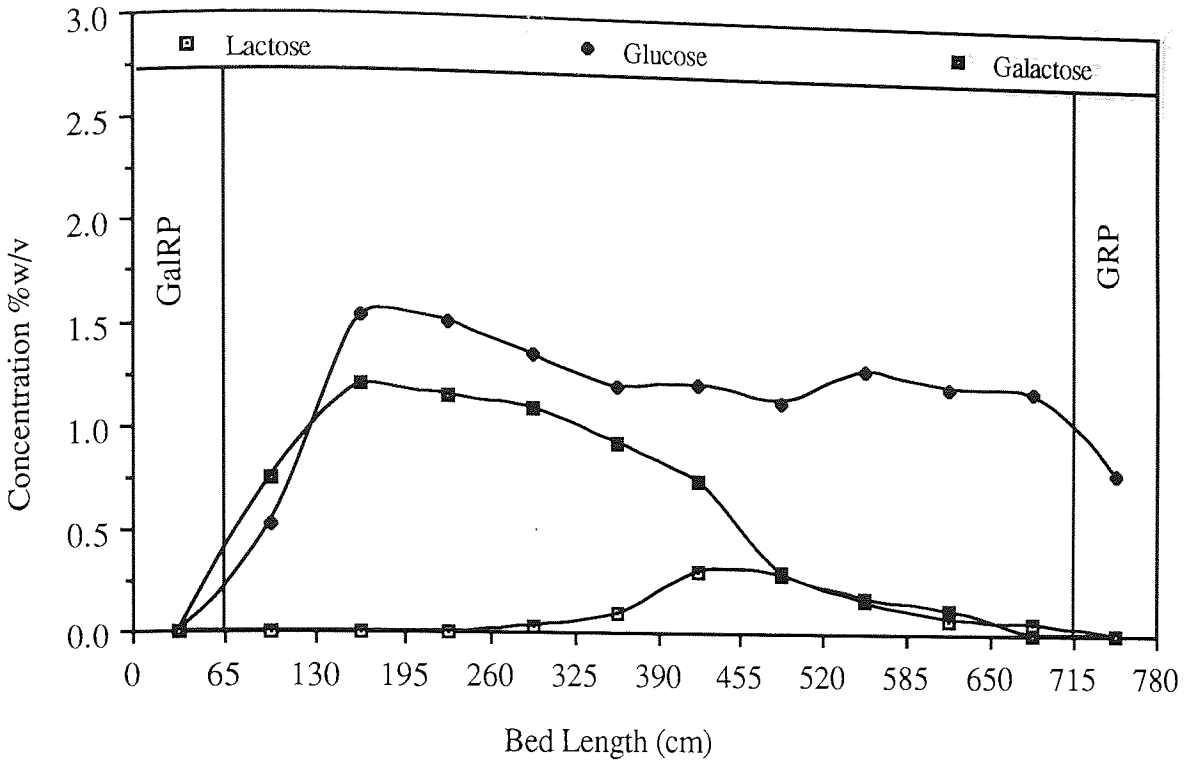


Figure 7.11: On-Column Concentration Profile for Run 5.1-4.5-4.5-27-100-26 Cycle 13

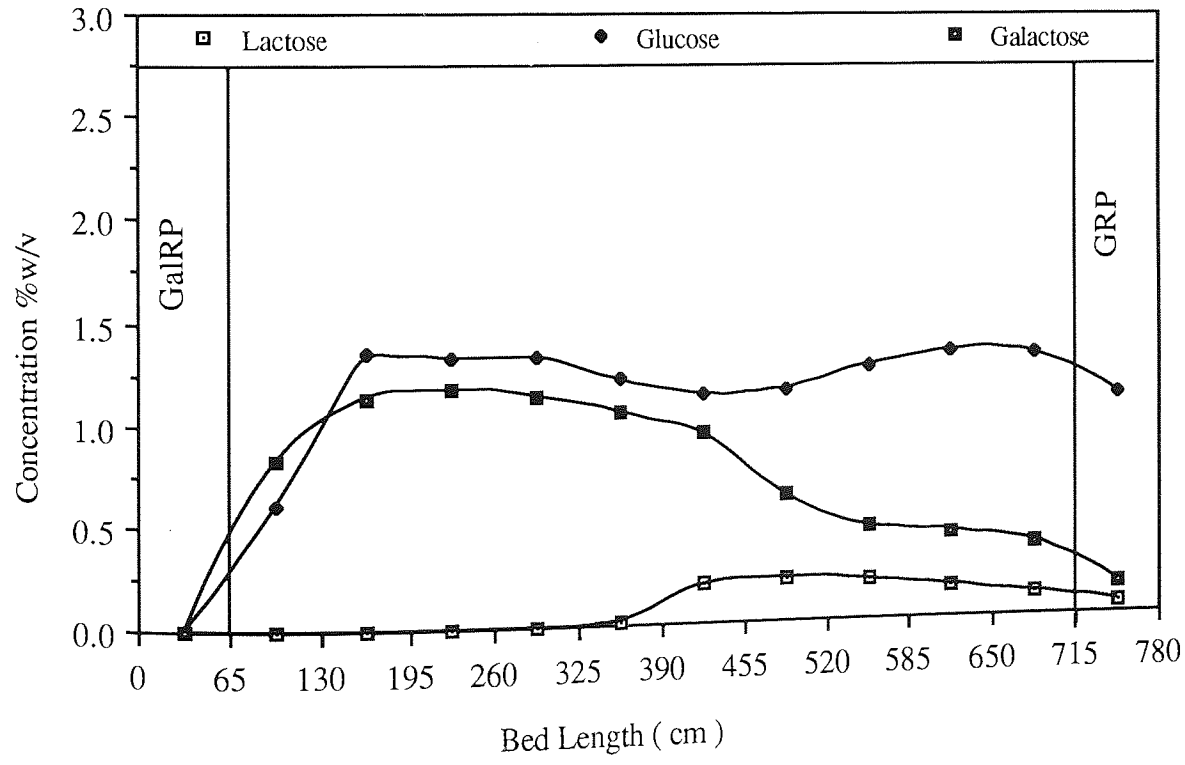


Figure 7.12: On-Column Concentration Profile for Run 5.1-4.5-4.5-30-100-26 Cycle 7

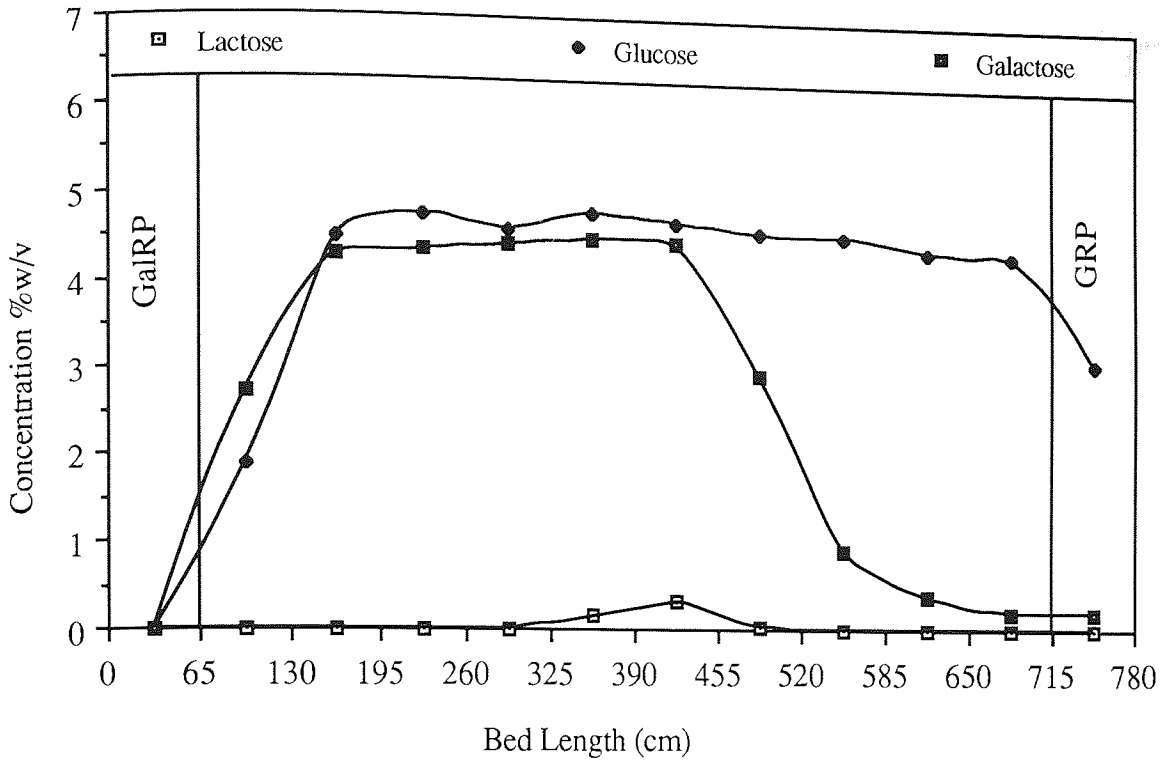


Figure 7.13: On-Column Concentration Profile for Run 15-4.5-4.5-28-300-29 Cycle 14

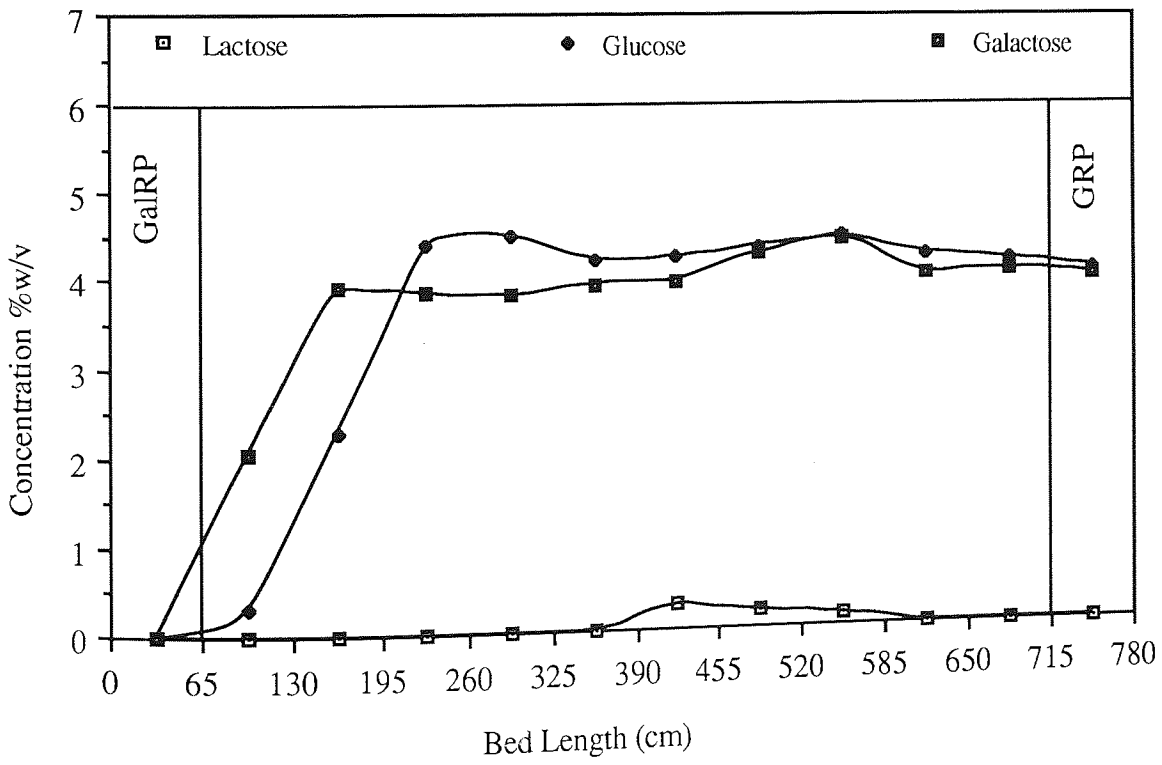


Figure 7.14: On-Column Concentration Profile for Run 15-4.5-4.5-32-300-29 Cycle 8

7.4.4 Effect of Switch Time

The switch time has been shown to affect the performance of the SCCR-S system since it controls the residence time in the system. The effect of the switch time on the performance of the SCCR-S used for the hydrolysis of lactose was investigated. The experimental conditions and results are shown in Tables 7.7 and 7.8.

By increasing the switch time, the time spent by the eluent in a column is increased causing less complexing of the galactose with the Ca^{2+} on the resin and stripping of the galactose that had complexed on the resin along the system. This led to more galactose being eluted with the mobile phase resulting in a reduction in the purities of the GRP and increasing its concentrations while the purities of the GalRP were improved with reduced concentrations (Table 7.8).

From runs 10-4.5-4.5-30-200-26 and 10-4.5-4.5-30-200-30, it was found that increasing the switch time caused a reduction in the conversion of the lactose. This was also observed when studying the saccharification of modified starch in the SCCR-S system. This was due to the lactose which has very low affinity for the stationary phase being eluted out of the system faster resulting in lower conversions. The reduction in the conversion of lactose in run 10-4.5-4.5-200-30 also contributed to the reduction in the GRP purity.

Figures 7.15, 7.16, 7.17 and 7.18 showed that by increasing the switch time, the broadness of the galactose concentration profile in the system was increased. The more the broadness the poorer the GRP purity the greater the GRP concentration (run 10-4.5-4.5-30-200-30). This effect was also observed when studying the saccharification of modified starch to maltose (Chapter 6). The "cross over" point shifted towards the GRP end from column 3 to column 4 when the switch time was changed from 26 to 30 minutes (Figures 7.17 and 7.18). Akintoye[16] studying the inversion of sucrose to glucose and fructose in the SCCR-S system found that the "cross over" point between the fructose and glucose profiles shifted from column 3 to 5 towards the GRP end when the switch time was increased from 31 to 32 minutes.

Table 7.7: Effect of Switch Time: Experimental Conditions

Experimental Run	Average Flowrates cm^3min^{-1}				Lactose Feed Conc %w/v	Enzyme Activity U/cm^3	Switch Time (min)	Lm/P	Cycle No	Feed Throughput ($Kg/h/m^3$ resin)
	Feed	Enzyme	Eluent	Purge						
5.4-4.5-4.5-30-150-26	4.5	4.5	30.0	76.0	5.4	150	26.0	0.390	7	0.80
5.4-4.5-4.5-30-150-27.5	4.5	4.5	30.0	76.0	5.4	150	27.5	0.443	14	0.80
10-4.5-4.5-30-200-26	4.5	4.5	30.0	76.0	10.0	200	26.0	0.390	13	1.50
10-4.5-4.5-30-200-30	4.5	4.5	30.0	76.0	10.0	200	30.0	0.500	7	1.50

Table 7.8: Effect of Switch Time: Experimental Results

Experimental Run	GALACTOSE RICH PRODUCT.				GLUCOSE RICH PRODUCT				Conversion %	Relative Enzyme usage Actual / Theoretical	Mass Balance Output / Input %
	Galact. purity %	Impurities %		Total product conc. %w/v	Glucose purity %	Impurities %		Total product conc. % w/v			
		G	L			GAL	L				
5.4-4.5-4.5-30-150-26	61.5	38.5	0.0	0.26	99.9	0.0	0.0	0.12	100	1.00	100.6
5.4-4.5-4.5-30-150-27.5	71.4	28.6	0.0	0.22	99.9	0.0	0.0	0.20	100	1.00	99.3
10-4.5-4.5-30-200-26	67.8	32.2	0.0	0.31	70.3	19.9	9.8	0.51	95.7	0.72	96.6
10-4.5-4.5-30-200-30	90.9	9.1	0.0	0.13	58.5	33.7	7.8	0.89	93.9	0.72	99.4

G = Glucose, GAL = Galactose, L = Lactose

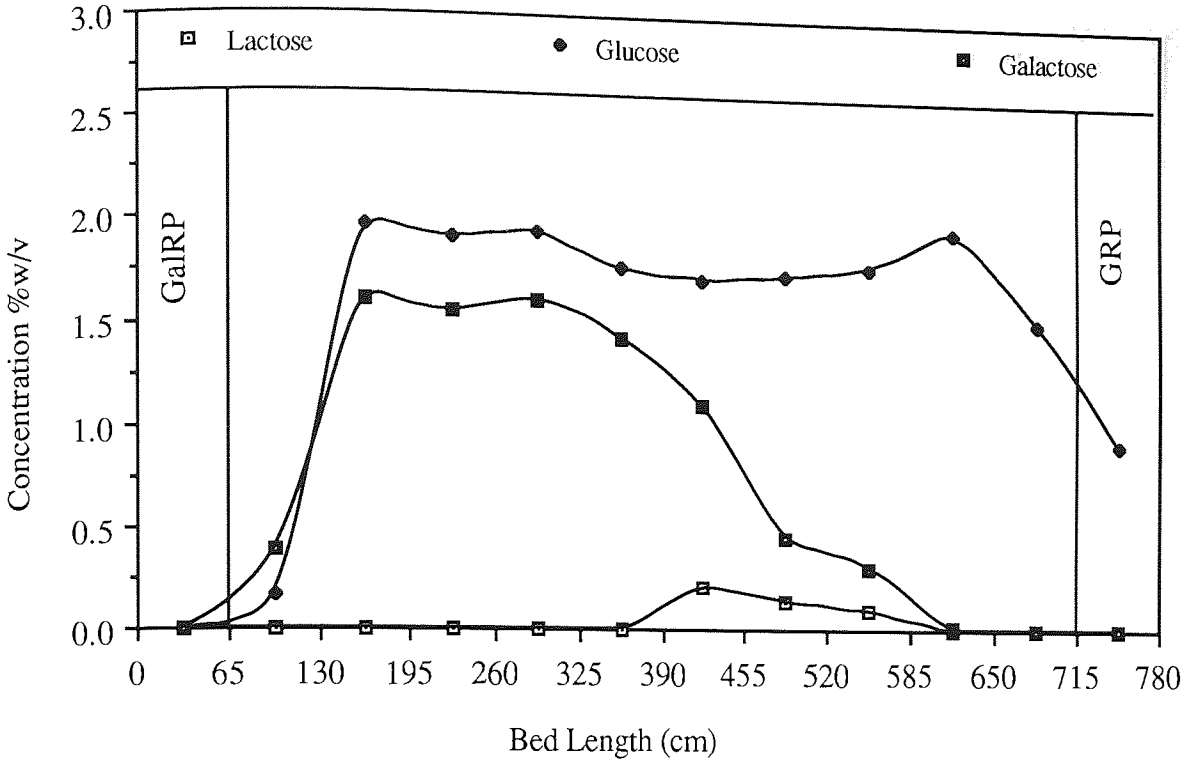


Figure 7.15: On-Column Concentration Profile for Run 5.4-4.5-4.5-30-150-26 Cycle 7

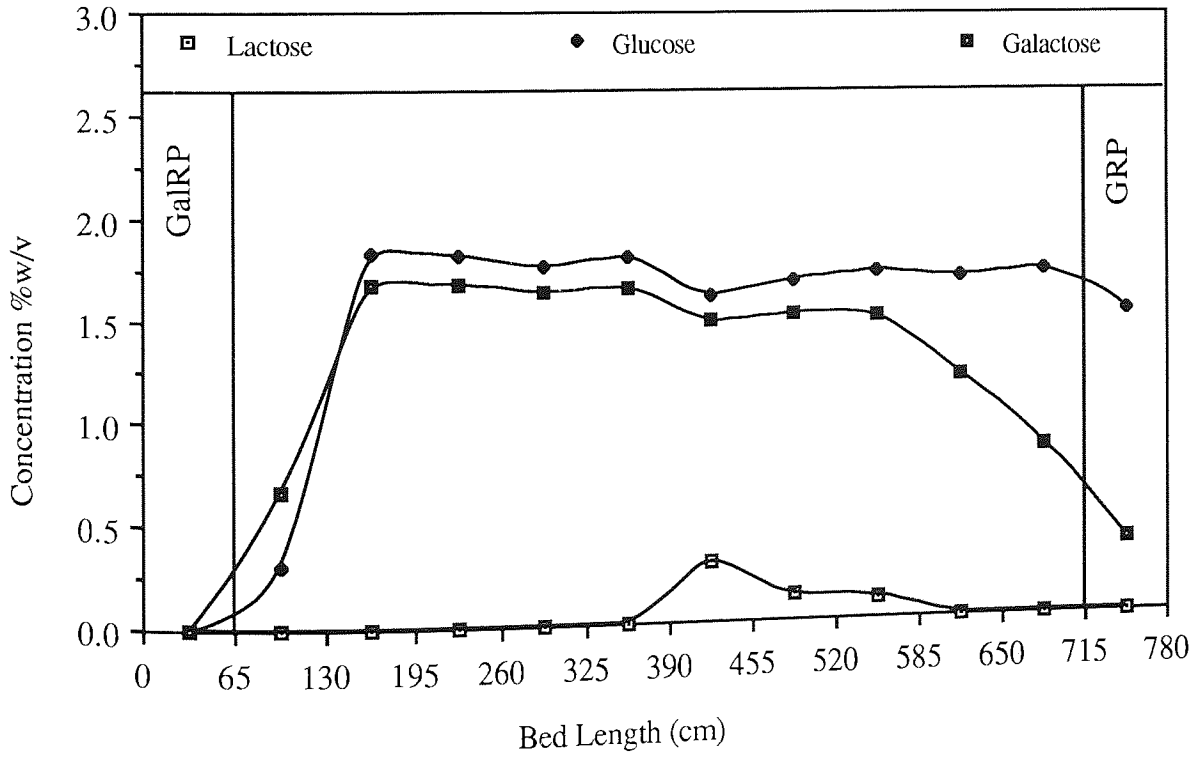


Figure 7.16: On-Column Concentration Profile for Run 5.4-4.5-4.5-30-150-27.5 Cycle 14

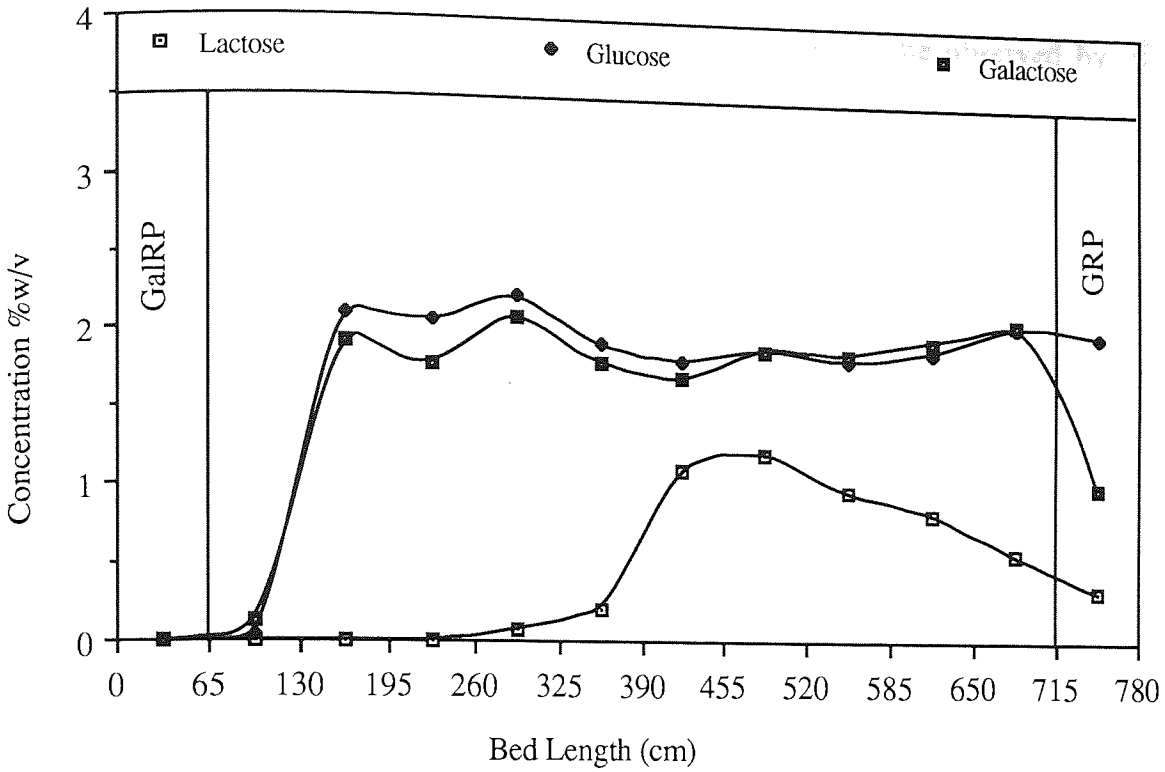


Figure 7.17: On-Column Concentration Profile for Run 10-4.5-4.5-30-200-26 Cycle 13

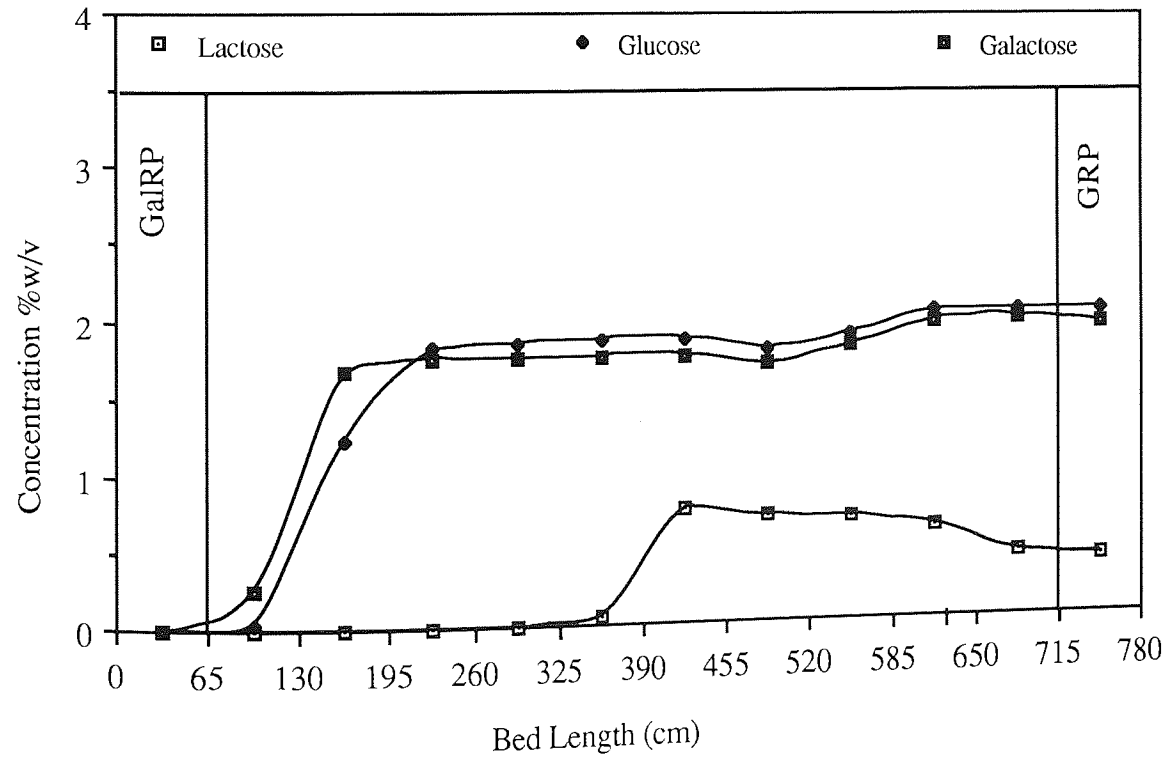


Figure 7.18: On-Column Concentration Profile for Run 10-4.5-4.5-30-200-30 Cycle 7

The higher sensitivity of the SCCR-S to variation in the switch time observed by Akintoye[16] was due to the relatively high separation factor between fructose and glucose of 2.25 compared to 1.37 for galactose and glucose.

7.4.5 Effect of Feed Concentration

The feed concentration was varied to further study the effect of the substrate (lactose) concentration on the performance of the SCCR-S system. Tables 7.9 and 7.10 show the experimental conditions and results respectively. As the feed concentration was increased, the enzyme to feed concentration ratio was reduced. This resulted in low reaction rates and longer bed length being required for the reaction and led to poorer separation of the products, i.e poorer product purities as the feed concentration was increased. At higher feed concentrations, the galactose concentration in the mobile phase also increased leading to product inhibition of the enzyme and lower reaction rates.

It was also observed that as the feed concentration was increased, very small amounts (0.1 to 0.3%) of oligosaccharides (galactobiose and galactotriose) were detected in columns 7, 8 and 9 during analysis. These oligosaccharides were subsequently hydrolysed to galactose causing galactose concentration profile to be more prominent in columns 8 and 9 compared to the glucose profile (Figures 7.19, 7.20 and 7.21). The formation of oligosaccharides during the initial stage of the reaction was also observed when studying the kinetics of the enzyme and also by other workers[70,120].

Table 7.9: Effect of Feed Concentration: Experimental Conditions

Experimental Run	Average Flowrates cm^3min^{-1} Feed	Enzyme	Eluent	Purge	Lactose Feed Conc %w/v	Enzyme Activity U/cm^3	Switch Time (min)	Lm/P	Cycle No	Feed Throughput ($Kg/h/m^3$ resin)
10-4.5-4.5-28-300-28	4.5	4.5	28.0	76.0	10.0	300	28.0	0.404	14	2.25
15-4.5-4.5-28-300-28	4.5	4.5	28.0	76.0	15.0	300	28.0	0.404	19	3.00
10-4.5-4.5-30-300-28	4.5	4.5	30.0	76.0	10.0	300	28.0	0.460	7	1.50
20-4.5-4.5-30-300-28	4.5	4.5	30.0	76.0	20.0	300	28.0	0.460	8	3.00
32-4.5-4.5-30-300-28	4.5	4.5	30.0	76.0	32.0	300	28.0	0.460	9	4.80

Table 7.10: Effect of Feed Concentration: Experimental Results

Experimental Run	GALACTOSE RICH PRODUCT. Galact. purity %	Impurities % G	L	Total product conc. %w/v	GLUCOSE RICH PRODUCT Glucose purity %	Impurities % GAL	L	Total product conc. % w/v	Conversion %	Relative Enzyme usage Actual / Theoretical	Mass Balance Output / Input %
10-4.5-4.5-28-300-28	63.6	36.4	0.0	0.44	99.9	0.0	0.0	0.30	100	1.08	99.0
15-4.5-4.5-28-300-28	60.0	40.0	0.0	0.66	99.9	0.0	0.0	0.48	100	0.72	100
10-4.5-4.5-30-300-28	87.5	12.5	0.0	0.36	90.3	9.7	0.0	0.46	100	1.08	100.7
20-4.5-4.5-30-300-28	74.3	25.7	0.0	0.74	85.7	14.3	0.0	0.91	100	0.54	102.0
32-4.5-4.5-30-300-28	67.6	32.4	0.0	1.14	79.9	20.1	0.0	1.16	100	0.34	91.6

G = Glucose, GAL = Galactose, L = Lactose

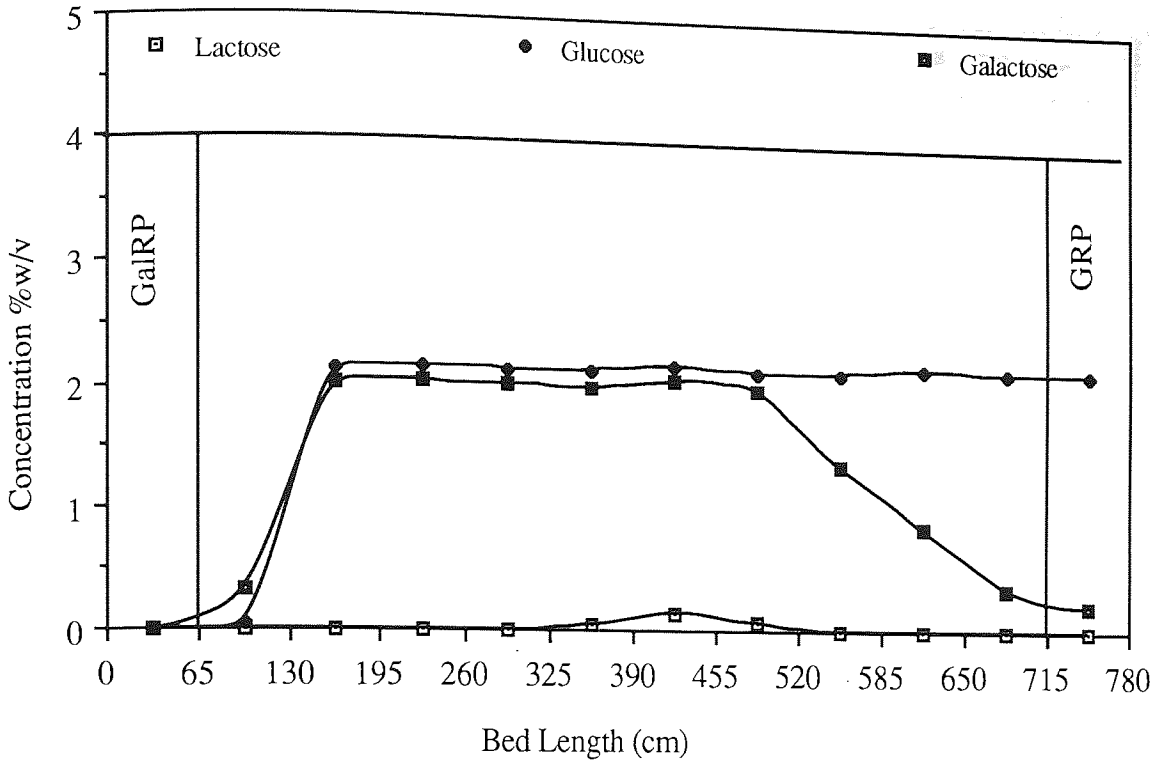


Figure 7.19: On-Column Concentration Profile for Run 10-4.5-4.5-30-300-28 Cycle 7

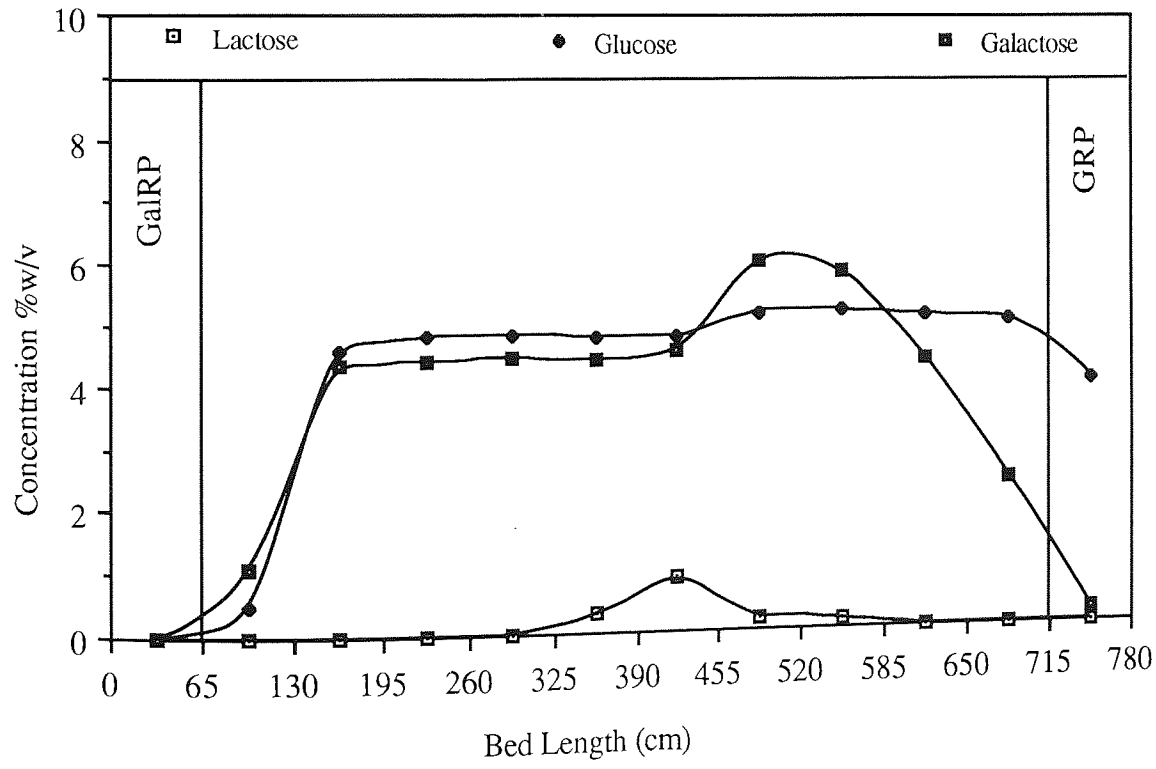


Figure 7.20: On-Column Concentration Profile for Run 20-4.5-4.5-30-300-28 Cycle 8

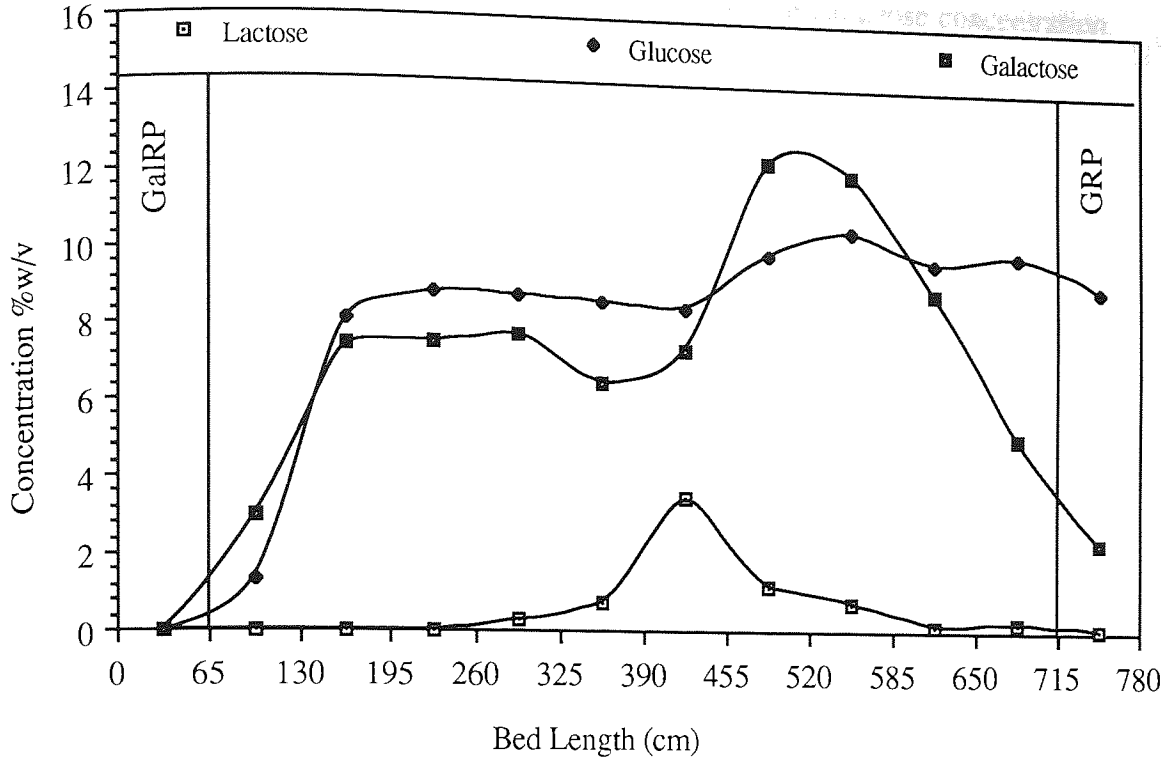


Figure 7.21: On-Column Concentration Profile for Run 32-4.5-4.5-30-300-28 Cycle 9

7.4.6 Effect of Enzyme Activity

The enzyme activity was found to affect the performance of the SCCR-S system used for the saccharification of modified starch to maltose when the enzyme was added to the eluent. The effect of the enzyme activity on the performance of the SCCR-S was further studied when the system was used for the hydrolysis of lactose. The enzyme in this case was introduced into the system with the feed. The pH in the system was kept around 5.0 and steady state was established well before samples were collected and analysed.

The experimental conditions and results are shown in Tables 7.11 and 7.12 respectively. As the enzyme activity was increased, the enzyme to substrate ratio was increased. This resulted in higher reaction rates resulting in rapid lactose conversion leaving longer bed length for separation of the products. The availability of longer bed length for separation resulted in better GalRP and GRP. Reduced amounts of the oligosaccharides (<0.1%) observed in columns 7, 8 and 9 at higher enzyme activities also contributed to products of

better purities being obtained and caused less broadening of the galactose concentration profile.

The on-column concentration profiles shown in Figures 7.22, 7.23,7.24 and 7.25 show that by increasing the enzyme activity, the "cross over" point shifted towards the GRP end. Comparing Figure 7.22 (run 5.4-4.5-4.5-30-150-27.5) and Figure 7.23 (run 5.7-4.5-4.5-30-200-27.5), it can be observed that the "cross over" point shifted from column 3 to column 5 when the enzyme activity was increased from 150U/cm^3 to 200U/cm^3 resulting in an improvement in the GalRP purity. Figure 7.23 shows that during run 5.7-4.5-4.5-30-200-27.5, the hydrolysis reaction was so fast that the limiting factor was the separating power of the equipment.

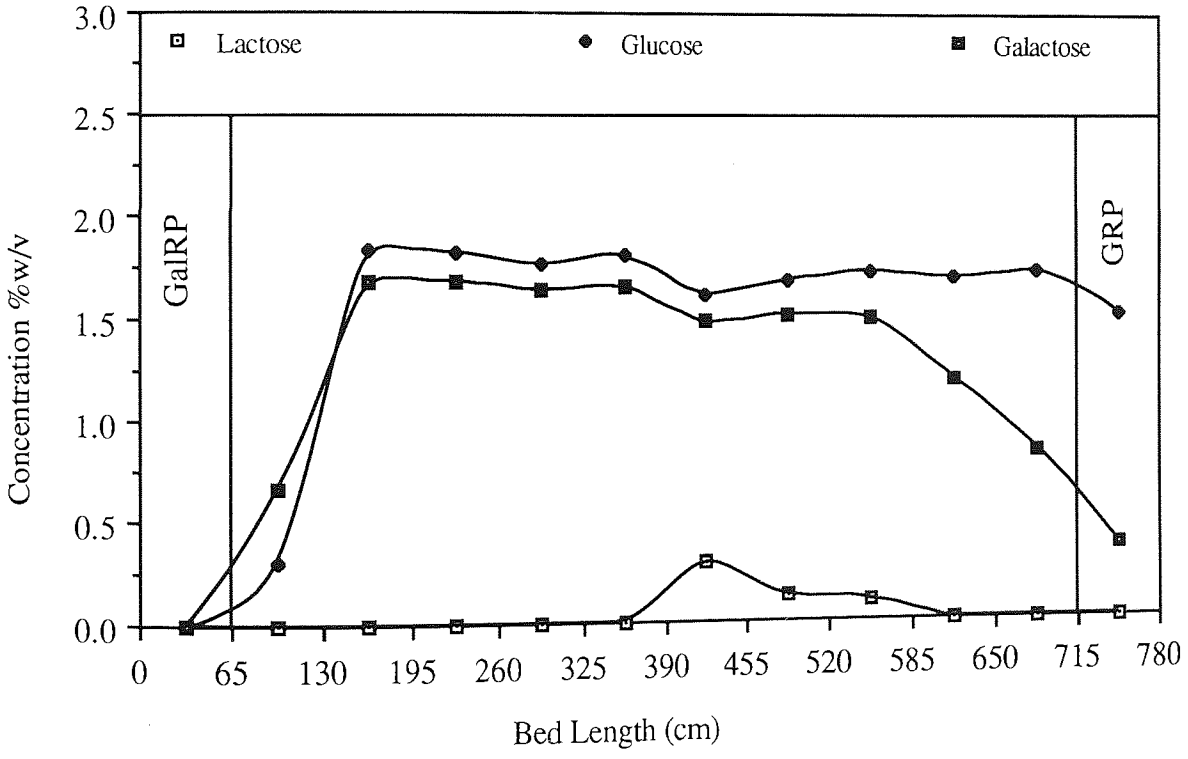


Figure 7.22: On-Column Concentration Profile for Run 5.4-4.5-4.5-30-150-27.5 Cycle 14

Table 7.11: Effect of Enzyme Activity: Experimental Conditions

Experimental Run	Average Flowrates cm^3min^{-1} Feed	Enzyme	Eluent	Purge	Lactose Feed Conc %w/v	Enzyme Activity U/cm^3	Switch Time (min)	Lm/P	Cycle No	Feed Throughput ($Kg/h/m^3$ resin)
5.4-4.5-4.5-30-150-27.5	4.5	4.5	30.0	76.0	5.4	150	27.5	0.443	14	0.81
5.7-4.5-4.5-30-200-27.5	4.5	4.5	30.0	76.0	5.7	200	27.5	0.443	8	0.81
20-4.5-4.5-30-200-28	4.5	4.5	30.0	76.0	20.0	200	28.0	0.460	7	3.00
20-4.5-4.5-30-300-28	4.5	4.5	30.0	76.0	20.0	300	28.0	0.460	8	3.00
32-4.5-4.5-30-300-28	4.5	4.5	30.0	76.0	32.0	300	28.0	0.460	9	4.80
30-4.5-4.5-30-400-28	4.5	4.5	30.0	76.0	30.0	400	28.0	0.460	9	4.50

Table 7.12: Effect of Enzyme Activity: Experimental Results

Experimental Run	GALACTOSE RICH PRODUCT. Galact. purity %	Impurities % G	L	Total product conc. %w/v	GLUCOSE RICH PRODUCT Glucose purity %	Impurities % GAL	L	Total product conc. % w/v	Conversion %	Relative Enzyme usage Actual / Theoretical	Mass Balance Output / Input %
5.4-4.5-4.5-30-150-27.5	71.4	28.6	0.0	0.22	99.9	0.0	0.0	0.20	100	1.00	99.3
5.7-4.5-4.5-30-200-27.5	94.4	5.60	0.0	0.18	97.0	3.0	0.0	0.33	100	1.26	103.5
20-4.5-4.5-30-200-28	66.1	33.9	0.0	0.62	64.2	18.4	17.4	1.09	91.8	0.36	99.6
20-4.5-4.5-30-300-28	74.3	25.7	0.0	0.74	85.7	14.3	0.0	0.91	100	0.54	102.0
32-4.5-4.5-30-300-28	67.6	32.4	0.0	1.14	79.9	20.1	0.0	1.16	100	0.34	91.6
30-4.5-4.5-30-400-28	77.1	22.9	0.0	1.14	99.9	0.0	0.0	1.22	100	0.48	99.4

G = Glucose, GAL = Galactose, L = Lactose

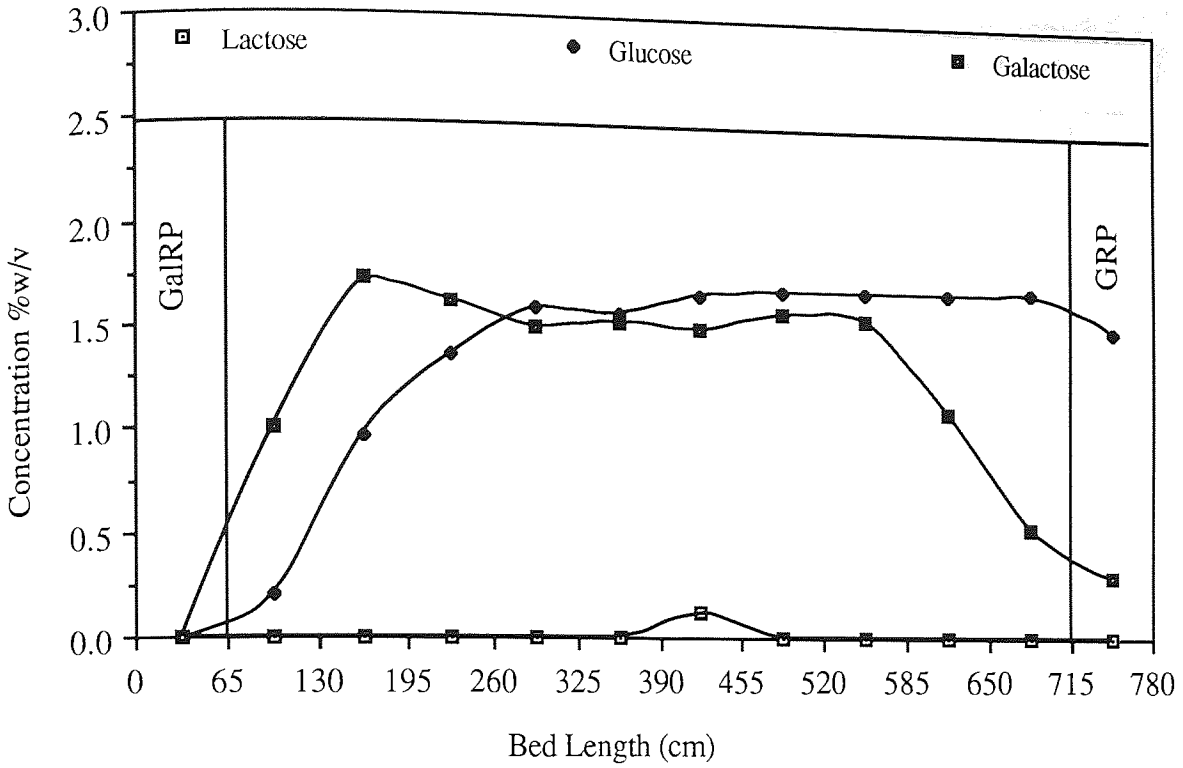


Figure 7.23 On-Column Concentration Profile for Run 5.7-4.5-4.5-30-200-27.5 Cycle 8

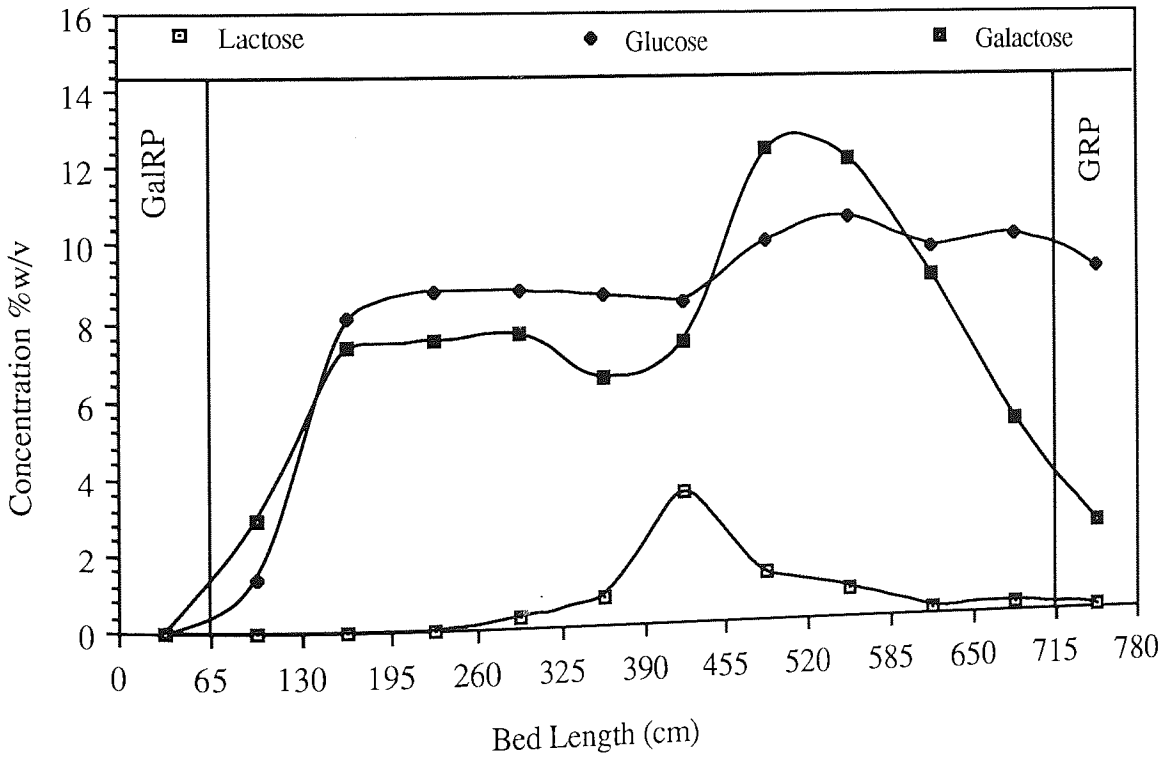


Figure 7.24: On-Column Concentration Profile for Run 32-4.5-4.5-30-300-28 Cycle 9

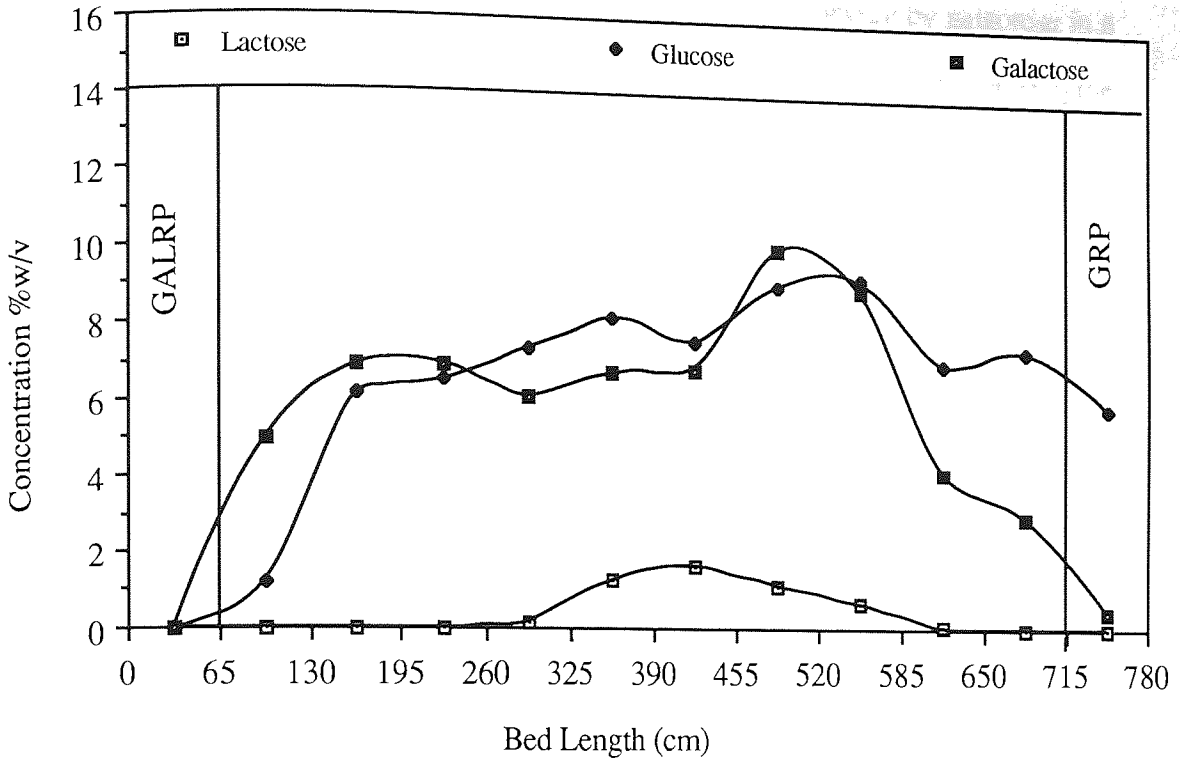


Figure 7.25: On-Column Concentration Profile for Run 30-4.5-4.5-30-400-28 Cycle 9

7.4.7 Enzyme Usage

The amount of enzyme used was found to affect the performance of the SCCR-S system. Tables 7.6, 7.8, 7.10 and 7.12 show the amount of enzyme required by the SCCR-S relative to the theoretical amount of enzyme required to produce 1μmole of glucose in 1 minute at a pH of 5.0 and a temperature of 55°C. The rate of enzyme entering the system (U/min.) was compared to the theoretical enzyme activity required to hydrolyse the lactose fed into the system per minute.

As reported in Tables 7.6, 7.8, 7.10 and 7.12, the SCCR-S system required only 34% of the theoretical amount of enzyme required by a conventional batch process to achieve complete hydrolysis of lactose in the SCCR-S system. This was due to the ability of the SCCR-S to minimize the inhibition effect of the galactose on the enzyme by reducing the amount of galactose in the reaction medium. However, to achieve better product purities, enzyme amounts higher than the 34% were required because of the low separation factor between galactose and glucose.

Friend *et al.*[71] studied the inhibition of lactase enzyme from *A. oryzae* by galactose in a batch process and found that the presence of only 5mM galactose decreased the enzyme activity by 50%.

The enzyme activity was determined under initial reaction velocity conditions during which the amount of galactose was very small predicting higher enzyme activities than in the later stage of the reaction. This was confirmed when the reaction time during the enzyme activity determination was increased from 10 to 20 minutes and the enzyme activity obtained was reduced by about 50%. Taking these factors into consideration gives support to the statement that the SCCR-S required much less enzyme than a conventional batch process eventhough more separating length was required to achieve good separation and therefore products of better purities.

7.5 BATCH OPERATION OF THE SCCR-S SYSTEM FOR THE HYDROLYSIS OF LACTOSE

After operating the SCCR-S in the continuous mode of operation for the hydrolysis of lactose using the enzyme, lactase, the SCCR-S system was modified and operated in the batch mode for the hydrolysis of lactose. The data obtained during the batch operation of the SCCR-S was used to compare the performance of the SCCR-S operating in the batch and continuous modes. The comparison was based on throughputs, enzyme consumption, product concentration and the amount of pure (99.9%) glucose recovered for a single charge of the feed into the SCCR-S system when converted to batch mode operation.

During this study, the effect of eluent flowrate, feed concentration, enzyme activity and pulse size on the performance of the SCCR-S operating in the batch mode was also investigated.

7.5.1 Modification of the SCCR-S System to Operate in the Batch Mode

The arrangement of the valves on the SCCR-S when it was operated in the continuous mode has been described in Chapter 5. Figure 7.27 shows the arrangement of valves when the eluent enters into column 1 of the SCCR-S when it was operated in the continuous mode. To convert the SCCR-S system to operate in the batch mode a few adjustments were made, namely:

1) A direct connection was made between adjacent columns except between columns 12 and 1. The transfer valves were by-passed and the transfer line tubes were reduced to minimise the liquid hold-up in the system. To separate columns 12 and 1, the transfer valve between the two was kept closed.

2) The feed and the eluent were introduced directly into column 1 *via* valves which allowed an input of either the lactose feed or the eluent to the column.

3) The valves (see Figure 7.27)
   (i) the feed entry to column 6
   (ii) the product exit from column 11
   (iii) the purge inlet to column 12

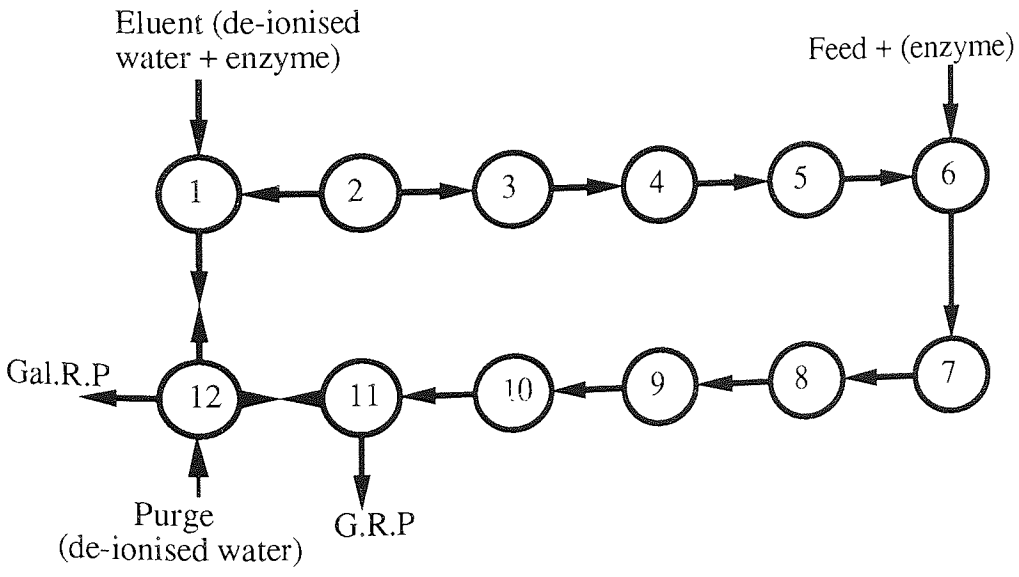
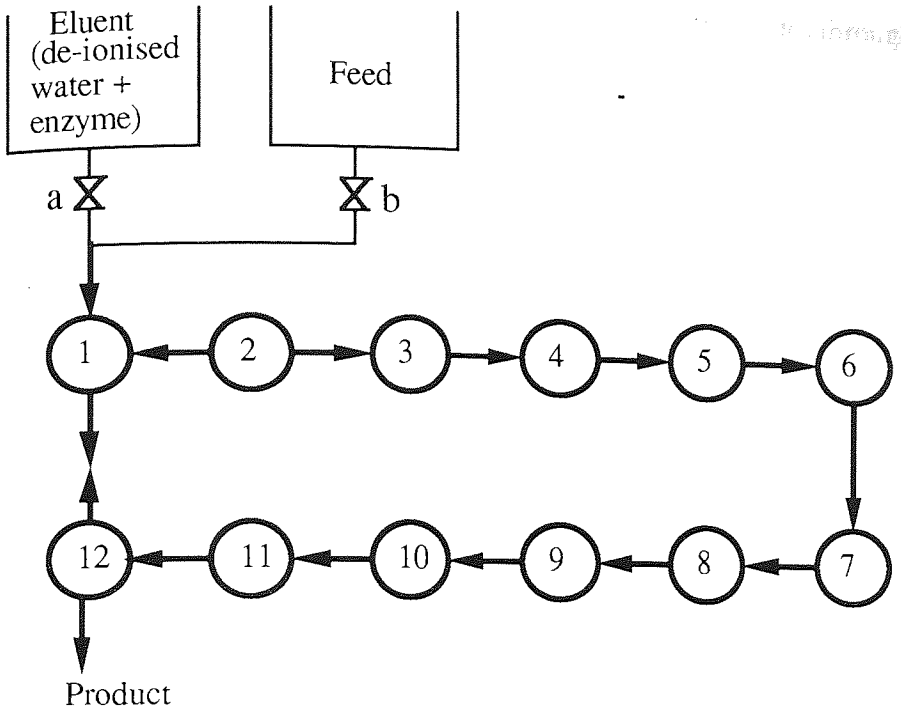


Figure 7.26: Arrangement of the SCCR-S system in the continuous mode



Valve "a" opened and "b" closed simultaneously for eluent entry

Valve "b" opened and "a" closed simultaneously for feed entry

Figure 7.27: Arrangement of the SCCR-S system in the batch mode

were closed allowing only one product outlet valve from column 12 to remain open. The arrangement of the SCCR-S system in the batch mode is shown in Figure 7.28. A refractometer and chart recorder were used in the product line for identification of the solutes in the eluent stream.

7.5.2 Experimental Techniques

The lactose feed and the enzyme were prepared as in Section 7.2. The temperature and pH were the same as those used during the continuous operation of the SCCR-S. The eluent (dilute enzyme solution) was pumped into the system and the feed was injected as a pulse.

Before each run, the SCCR-S was first flushed with the eluent for at least 5 hours to ensure that the system had the same enzyme activity as in the incoming eluent and to ensure reproducibility of the results.

The feed and product samples collected at an interval of 10 minutes throughout the duration of the chromatogram were analysed using the HPLC.

7.5.3 Experimental Results and Discussion

The results obtained during the operation of the SCCR-S in the batch mode are presented in this Section. The experimental runs are represented in the coded form 20-2-30-45 where

20 = feed concentration (%w/v)

2 = pulse size (% of total column volume)

30 = eluent flowrate (cm^3/min)

45 = enzyme activity in the eluent (U/cm^3)

The concentrations of samples collected during each run were plotted against time to give the elution profiles. From these elution profiles the galactose rich products (GalRP) and the glucose rich products (GRP) purities and concentrations were calculated as shown in Figure 7.28. The effects of eluent flowrate, feed concentration, enzyme activity and pulse size were investigated.

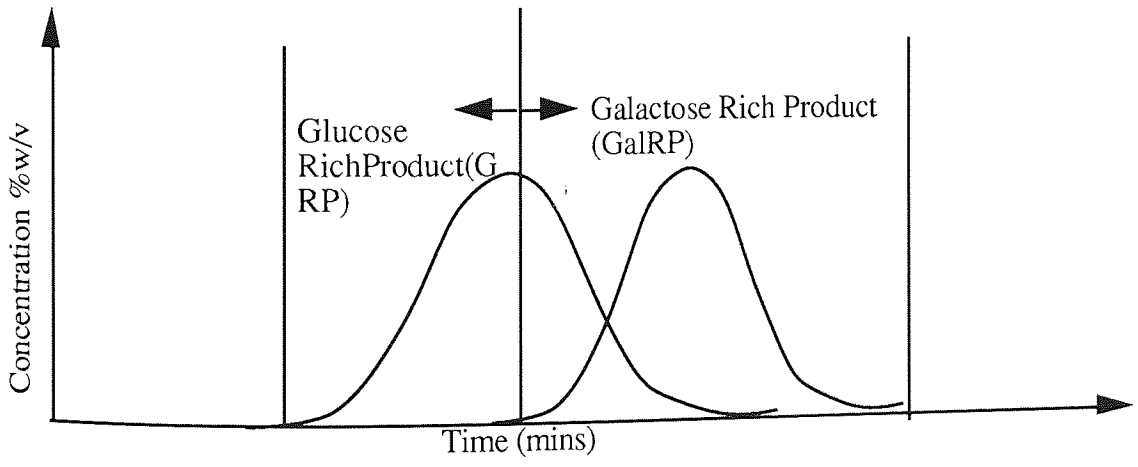


Figure 7.28: Calculation of product purities

7.5.3.1 Effect of Eluent Flowrate

Tables 7.13 and 7.14 show the experimental conditions and results respectively. By reducing the eluent flowrate, the residence time in the system was increased, resulting in better separation (Figures 7.29 and 7.30) and better GalRP purities were observed. The product concentrations also increased due to the reduction in the amount of eluent diluting the products.

Reducing the flowrate increased the elution time (T1-T0) of the products (Figures 7.29 and 7.30). This resulted in a reduction in the throughput observed at lower eluent flowrates. The same observation was also made by Zafar[9] who studied the biosynthesis of dextran in a batch chromatographic system.

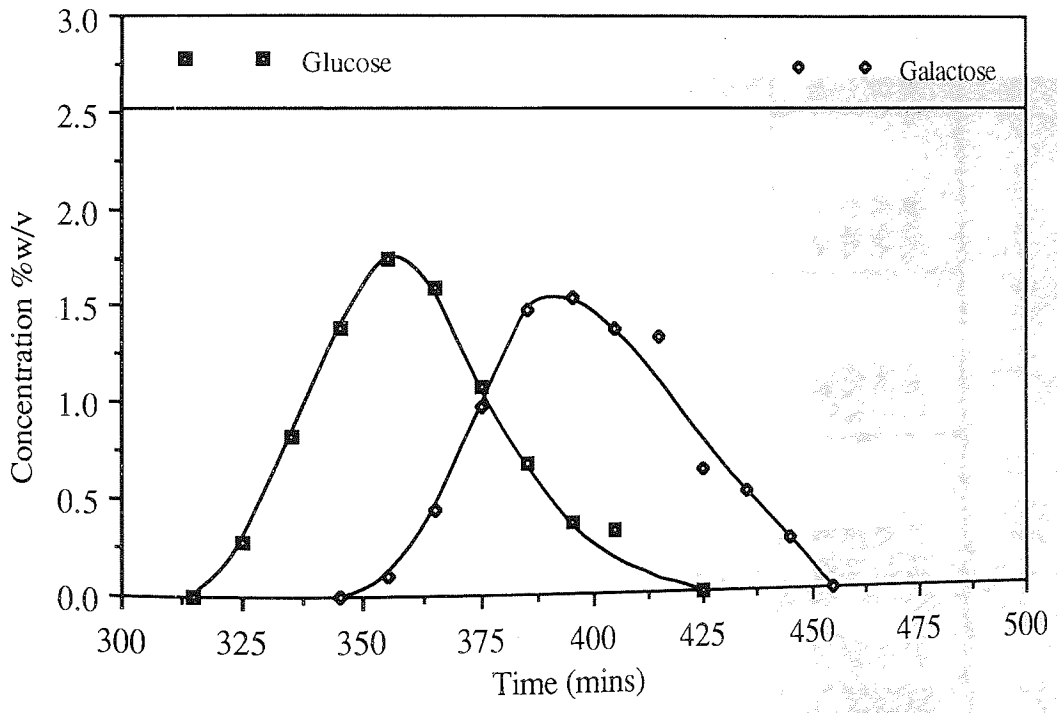


Figure 7.29: Elution Profile for Run 30-1-30-45

Table 7.13: Effect of Eluent Flowrate: Experimental Condition

Experimental Run	Eluent flowrate cm^3/min	Feed Conc. %w/v	Enzyme Activity in Eluent U/cm^3	Feed Volume Injected (cm^3)	Pulse Size % of Total system Volume
20-2-30-45	30.0	20.0	45.0	360.0	2.0
20-2-25-45	25.0	20.0	45.0	360.0	2.0
30-1-30-45	30.0	30.0	45.0	180.0	1.0
30-1-25-45	25.0	30.0	45.0	180.0	1.0

Table 7.14: Effect of Eluent Flowrate: Experimental Results

Experimental Run	GALACTOSE RICH PRODUCT		GLUCOSE RICH PRODUCT		Conver -sion	Elution Time T1-T0	Through-put	Relative Enzyme Usage
	Galactose. Purity %	Product Conc. %w/v	Glucose Purity %	Product Conc. %w/v	(%)	(mins)	$Kg/h/m^3$ Resin	Actual / Theoretical
20-2-30-45	56.8	1.66	99.9	0.76	100	180	1.33	1.21
20-2-25-45	59.3	1.93	99.9	1.10	100	210	1.14	1.18
30-1-30-45	57.9	1.36	99.9	0.63	100	150	1.20	1.35
30-1-25-45	64.5	1.44	99.9	0.80	100	200	0.90	1.50

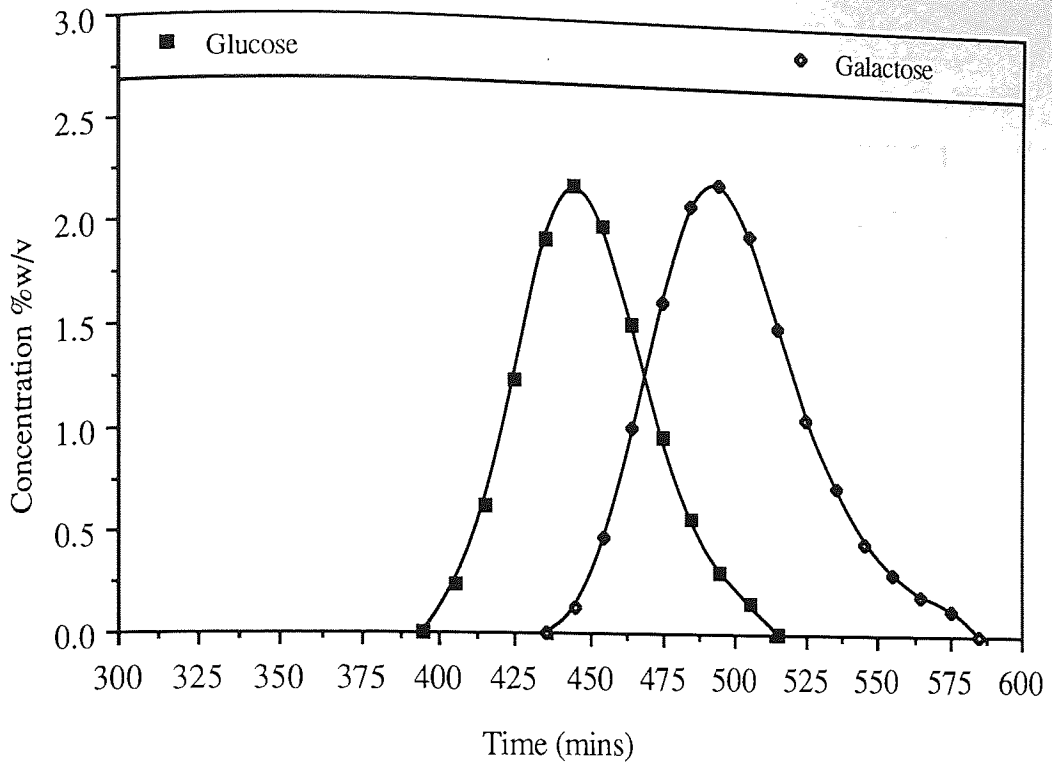


Figure 7.30: Elution Profile for Run 30-1-25-45

7.5.3.2 Effect of Feed Concentration

Zafar[9] in his work found that by increasing the feed concentration, product purities were reduced due to a reduction in the conversion of the substrate, sucrose, in the batch bioreactor-separator. The experimental conditions and results obtained during this study are shown in Tables 7.15 and 7.16 respectively. The results recorded in Table 7.16 agree with Zafar's observations that increasing the feed concentration resulted in poor GalRP purities although complete substrate conversions were achieved during this study.

By increasing the feed concentration, the enzyme-to-substrate ratio was reduced. This caused the reaction to take place over a longer bed length in the SCCR-S system reducing the length available for separating the reaction products and resulting in poorer separation (Figures 7.31, 7.32, 7.33 and 7.34). This led to poorer GalRP purities at higher feed concentrations. The product concentrations increased and the relative enzyme usage reduced as expected when the feed concentration was increased.

Table 7.15: Effect of Feed Concentration: Experimental Conditions

Experimental Run	Eluent flowrate cm^3/min	Feed Conc. %w/v	Enzyme Activity in Eluent U/cm^3	Feed Volume Injected (cm^3)	Pulse Size % of Total system Volume
5-2-30-45	30.0	5.0	45.0	360.0	2.0
15-2-30-45	30.0	15.0	45.0	360.0	2.0
10-2-25-45	25.0	10.0	45.0	360.0	2.0
20-2-25-45	25.0	20.0	45.0	360.0	2.0

Table 7.16: Effect of Feed Concentration: Experimental Results

Experimental Run	GALACTOSE RICH PRODUCT		GLUCOSE RICH PRODUCT		Conver -sion	Elution Time T1-T0	Through-put	Relative Enzyme Usage
	Galactose. Purity %	Product Conc. %w/v	Glucose Purity %	Product Conc. %w/v	(%)	(mins)	$Kg/h/m^3$ Resin	Actual / Theoret ical
5-2-30-45	61.6	0.50	99.9	0.38	100	170	0.35	4.60
15-2-30-45	57.1	1.73	99.9	0.80	100	170	1.06	1.53
10-2-25-45	64.9	1.03	99.9	0.61	100	190	0.63	2.14
20-2-25-45	59.3	1.93	99.9	1.10	100	210	1.14	1.18

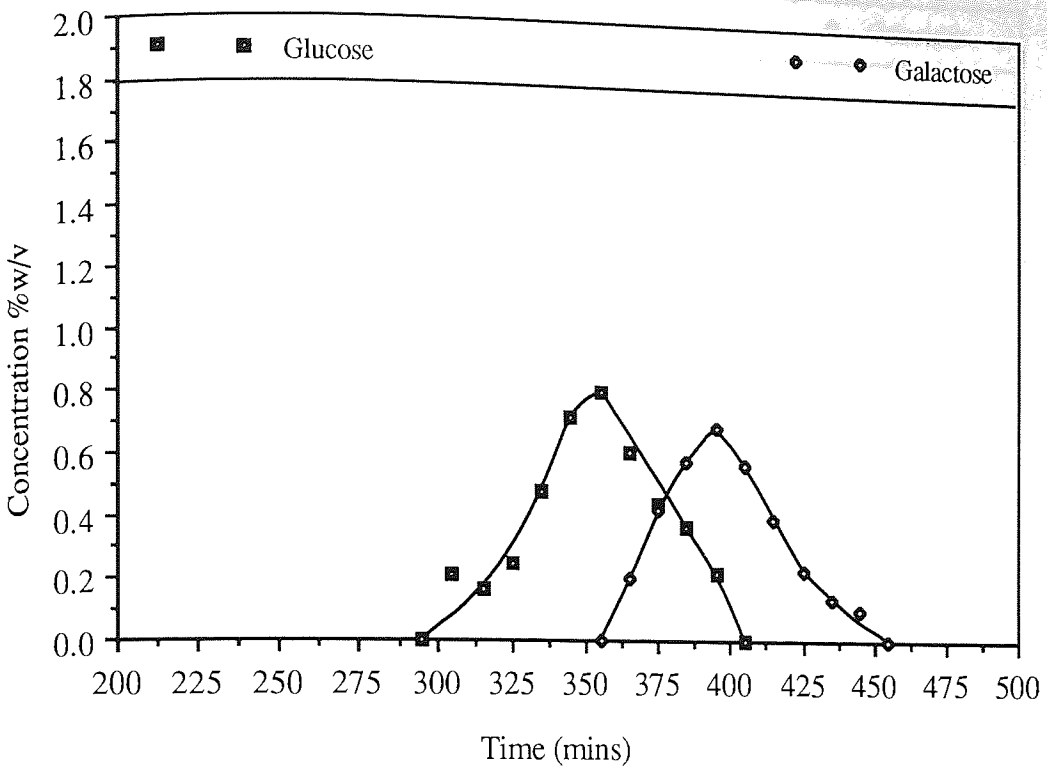


Figure 7.31: Elution Profile for Run 5-2-30 -45

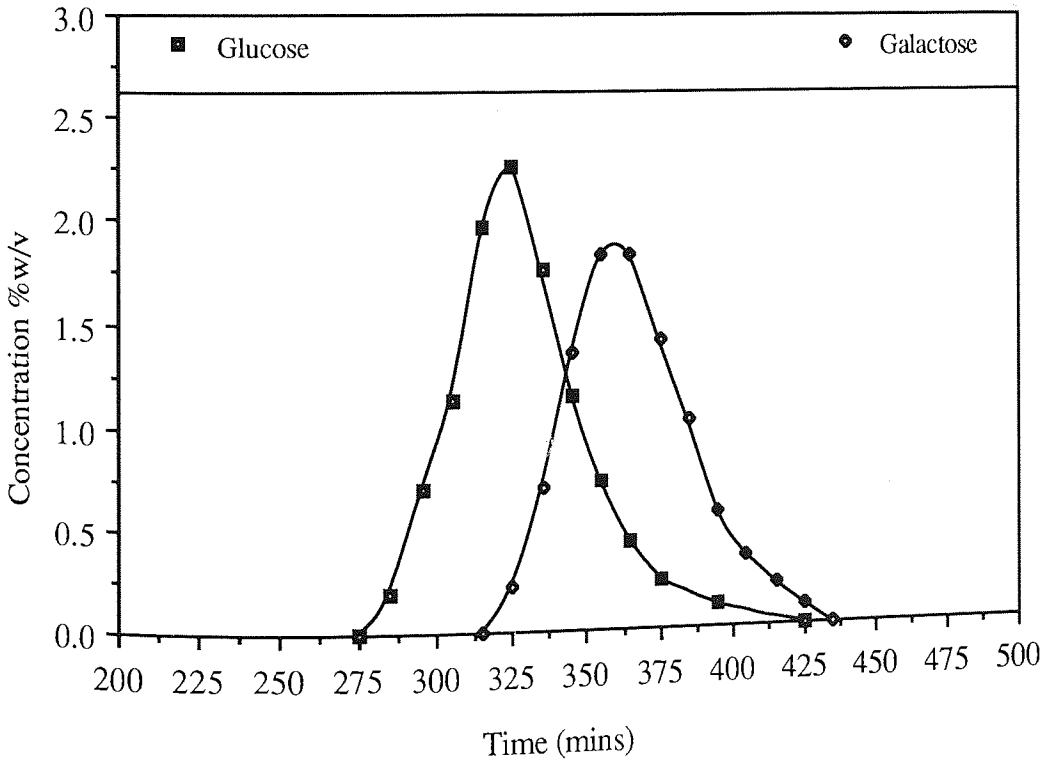


Figure 7.32: Elution Profile for Run 15-2-30-45

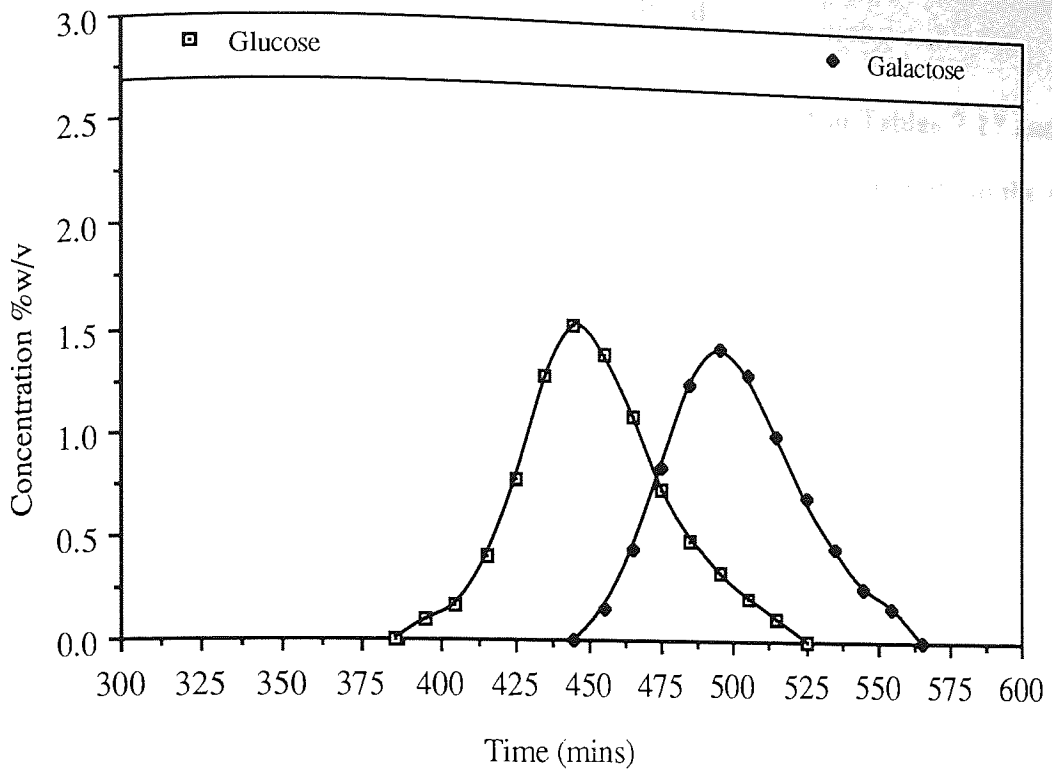


Figure 7.33: Elution Profile for Run 10-2-25-45

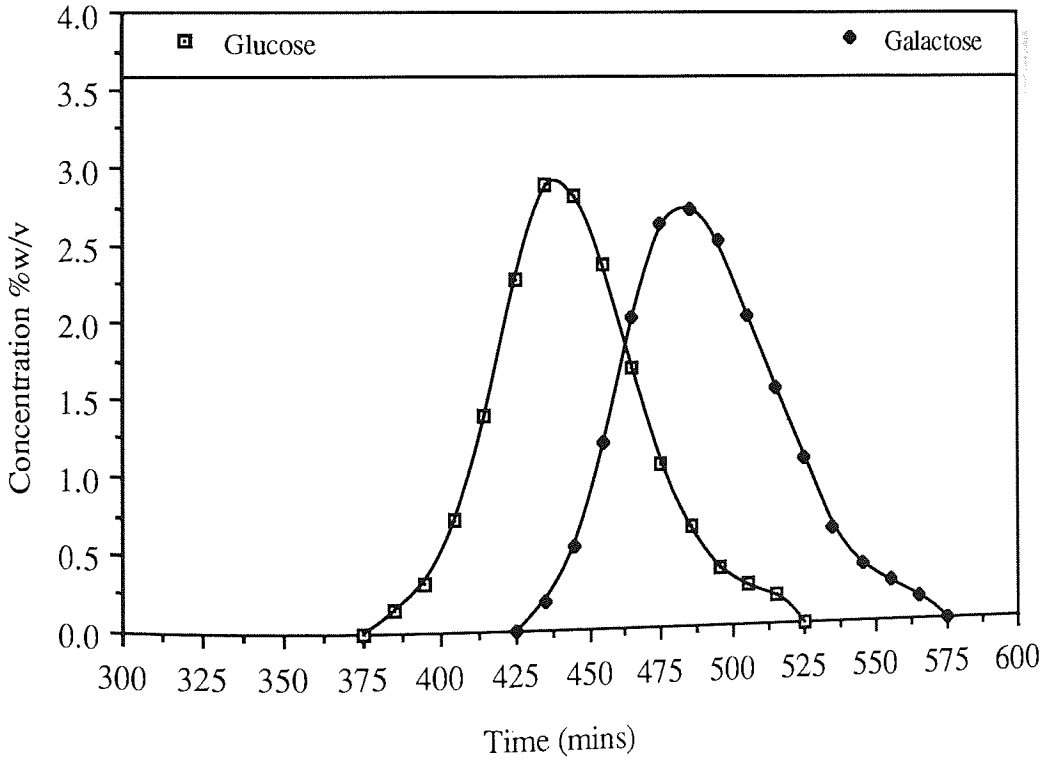


Figure 7.34: Elution Profile for Run 20-2-25-45

7.5.3.3 Effect of Enzyme Activity in the Eluent

The experimental conditions and results obtained are presented in Tables 7.17 and 7.18 respectively. From the results it can be seen that when the enzyme activity in the eluent was increased from 45U/cm^3 to 60U/cm^3, the GalRP purity was increased from 56.8% to 60.5%. This was due to an increase in the reaction rate causing the substrate, lactose, to be converted to galactose and glucose in a shorter time and therefore less of the system length was used for the reaction leaving the remaining length available for separating the products. This resulted in better separation (Figures 7.35 and 7.36) and an increase in the GalRP purity at a higher enzyme activity.

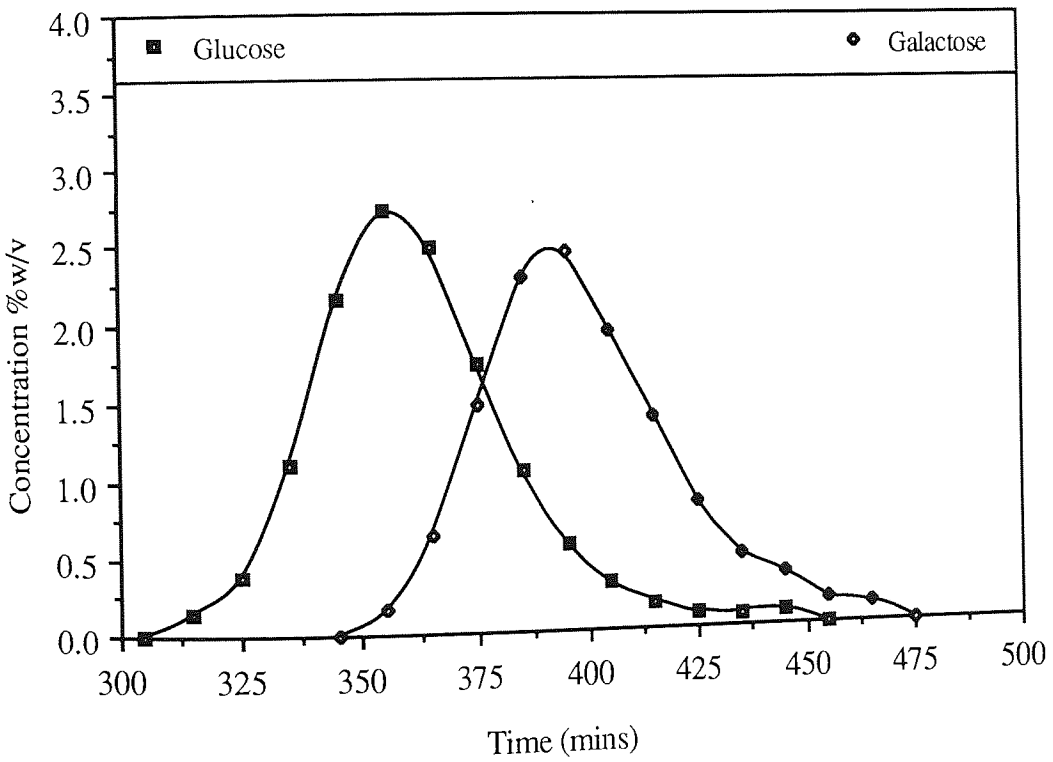


Figure 7.35: Elution Profile for Run 20-2-30-45

Table 7.17: Effect of Enzyme Activity: Experimental Conditions

Experimental Run	Eluent flowrate cm^3/min	Feed Conc. %w/v	Enzyme Activity in Eluent U/cm^3	Feed Volume Injected (cm^3)	Pulse Size % of Total system Volume
20-2-30-45	30.0	20.0	45.0	360.0	2.0
20-2-30-60	30.0	20.0	60.0	360.0	2.0

Table 7.18: Effect of Enzyme Activity: Experimental Results

Experimental Run	GALACTOSE RICH PRODUCT		GLUCOSE RICH PRODUCT		Conver -sion	Elution Time T1-T0	Through-put	Relative Enzyme Usage
	Galactose. Purity %	Product Conc. %w/v	Glucose Purity %	Product Conc. %w/v	(%)	(mins)	$Kg/h/m^3$ Resin	Actual / Theoretical
20-2-30-45	56.8	1.66	99.9	0.76	100	180	1.33	1.21
20-2-30-60	60.5	1.88	99.9	0.98	100	180	1.33	1.61

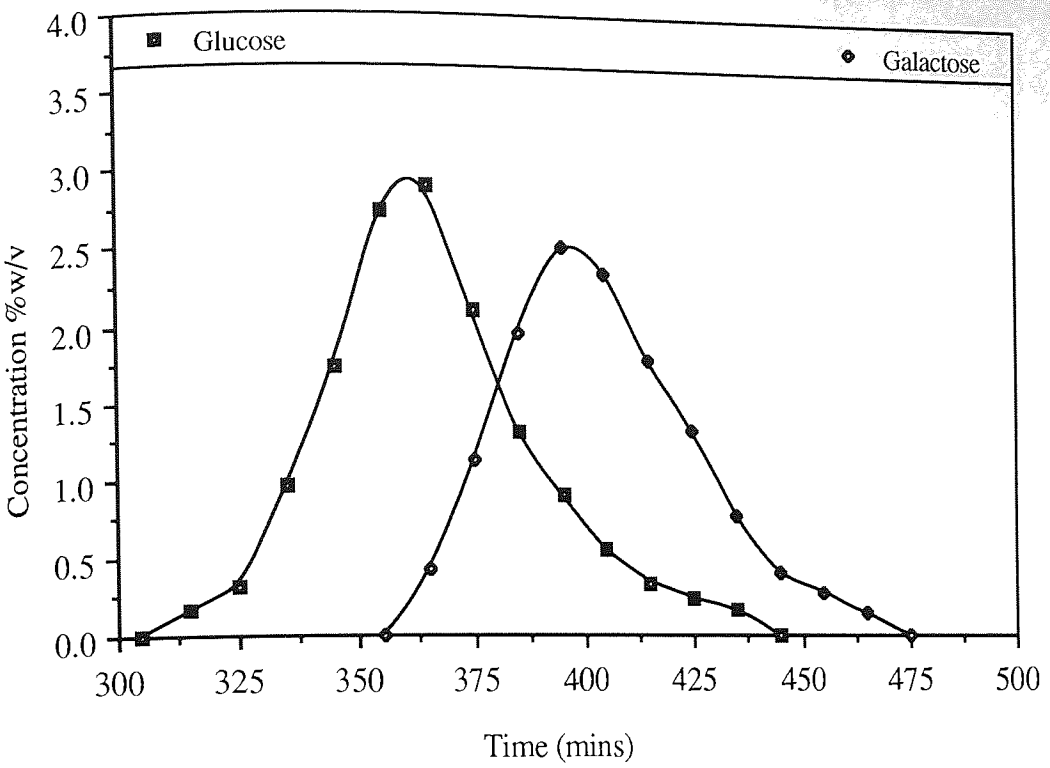


Figure 7.36: Elution Profile for Run 20-2-30-60

7.5.3.4 Effect of Pulse Size

Previous researchers[9,115] working on batch chromatographic systems have shown that pulse size affects separation of the components injected into a chromatographic column. It is normally recommended that sample loading should not exceed 2% of the total column volume for a good resolution of components. However, Thawait[115] in her work on the separation of glucose and fructose, obtained good resolution for pulse sizes up to 20% column volume and 20% solids concentration. She showed that by increasing the pulse size, the separation between the components was poorer. This was confirmed by Zafar[9] who studied the biosynthesis of dextran in batch chromatographic bioreactor-separators. During this study, a pulse size of 2 and 5% column volume were used. The experimental conditions and results are presented in Tables 7.19 and 7.20 respectively. The elution profiles are shown in Figures 7.37 and 7.38. From these results it was found that increasing the pulse size resulted in poorer separation (Figure 7.37 and 7.38) and a

Table 7.19: Effect of Pulse Size: Experimental Conditions

Experimental Run	Eluent flowrate cm^3/min	Feed Conc. %w/v	Enzyme Activity in Eluent U/cm^3	Feed Volume Injected (cm^3)	Pulse Size % of Total system Volume
10-5-30-45	30.0	10.0	45.0	900.0	5.0
10-2-30-45	30.0	10.0	45.0	360.0	2.0

Table 7.20: Effect of Pulse Size: Experimental Results

Experimental Run	GALACTOSE RICH PRODUCT		GLUCOSE RICH PRODUCT		Conver-sion	Elution Time T1-T0	Through-put	Relative Enzyme Usage
	Galactose. Purity %	Product Conc. %w/v	Glucose Purity %	Product Conc. %w/v	(%)	(mins)	$Kg/h/m^3$ Resin	Actual / Theoretical
10-5-30-45	55.0	2.37	99.9	0.59	100	180	1.67	0.97
10-2-30-45	61.8	1.06	99.9	0.74	100	160	0.75	2.16

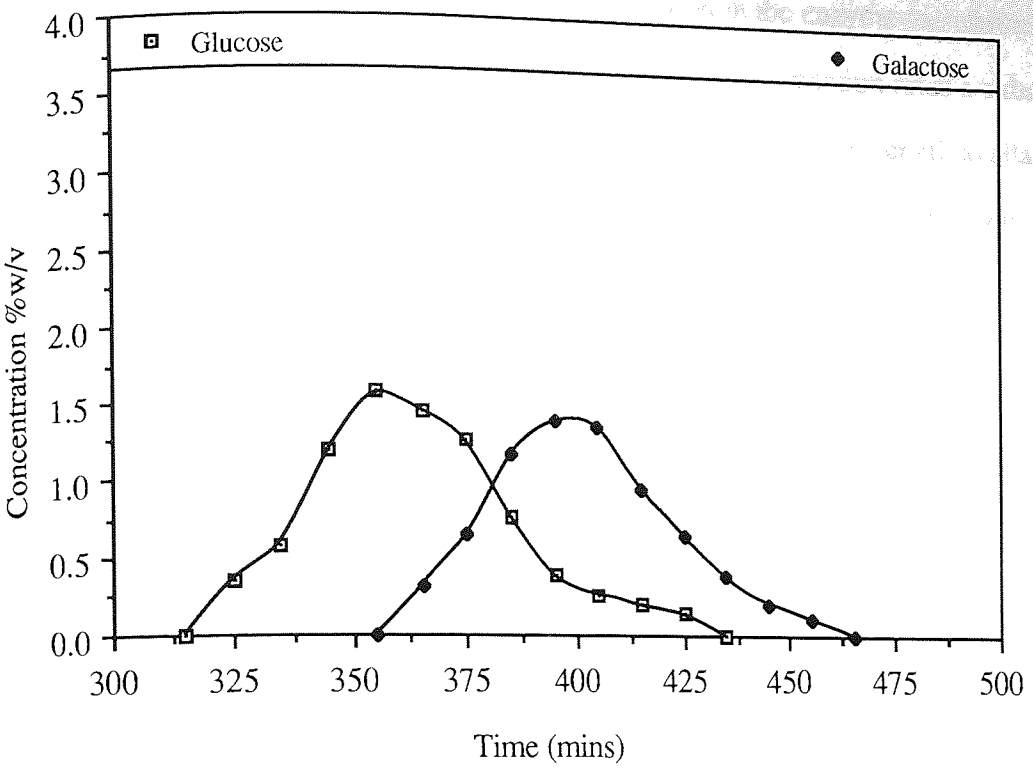


Figure 7.37: Elution Profile for Run 10-2-30-45

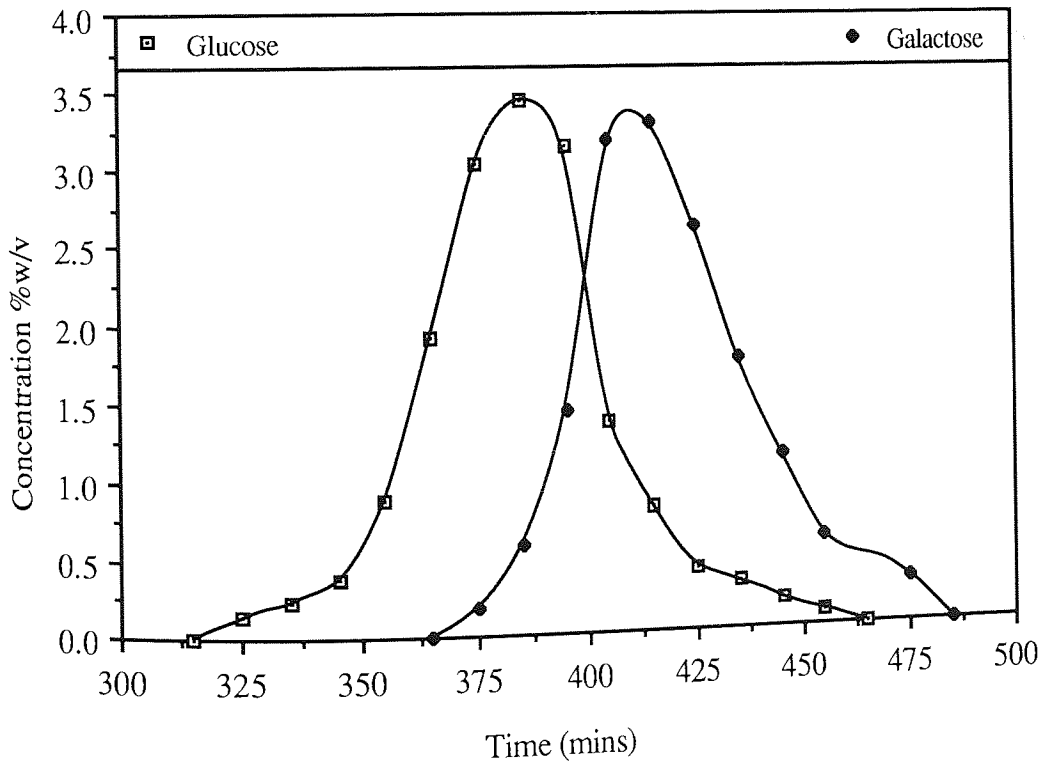


Figure 7.38: Elution Profile for Run 10-5-30-45

reduction in GalRP purity. This was due to the reduction in the enzyme-to-substrate ratio in the system which resulted in lower reaction rate. At lower reaction rates a substantial fraction of the system length is used for the reaction thus reducing the length available for separating the products. These results are in agreement with the results observed by Thawait[115] and Zafar[9].

7.6 COMPARISON OF BATCH AND CONTINUOUS OPERATION OF THE SCCR-S SYSTEM FOR THE HYDROLYSIS OF LACTOSE

The comparison of batch and continuous operation of the SCCR-S system has been based on three main criteria, namely the throughput, enzyme usage and concentration of products.

7.6.1 Results and Discussion

Table 7.21 shows the results from experiments which compare the batch and continuous operation of the SCCR-S system based on throughput. At feed concentrations of 10-20%w/v, the throughputs in the continuous mode were 2.3-2.6 times those of the batch mode. With 30%w/v feed concentration, the throughput ratio of continuous to batch was 5.0. More glucose was recovered in the GRP during the continuous operation of the SCCR-S compared to the batch mode of operation. This shows that the continuous operation of the SCCR-S is superior to the batch mode of operation for the hydrolysis of lactose.

The SCCR-S system required less enzyme operating in the continuous mode than in the batch mode (Table 7.22). The continuous mode required only between 32-51% of the amount of enzyme required for batch mode of operation. The enzyme usage ratio of continuous to batch decreased as the feed concentration was increased indicating that the performance of the SCCR-S system operating in the continuous mode is also better than the batch mode of operation at higher feed concentrations. This was due to the more effective use of the chromatographic bed when the system was operated in the continuous mode.

The lower enzyme usage during the continuous operation of the SCCR-S will result in lower operating costs.

In terms of product concentrations, the batch operation looks promising (Table 7.23). The GalRP and the GRP obtained during the continuous mode of operation were more dilute compared to those obtained during batch operation of the system. The GalRP concentration can be increased by splitting and recycling the dilute fraction.

The development of the SCCR-S system appears to be very promising when used as a bioreactor-separator but further improvements to the product concentration are desirable. A change of the DOWEX50W-X4 resin to a better stationary phase with better selectivity between galactose and glucose might be one possibility.

The continuous operation of the SCCR-S system has more advantages than the batch mode of operation including:

(i) no recycle is necessary; two products continuously exit from the two product lines, while in batch the recycle of the overlapping Section between peaks is essential to produce two pure products.

(ii) repeatable product quality from cycle to cycle is obtainable in the continuous mode while the batch product differs slightly in quality from batch to batch.

(iii) the system is more flexible during continuous operation. The product quality could be altered by changing the switch time and the flowrates.

Table 7.21: Comparison of Batch and Continuous Operation of the SCCR-S

(Throughput of Lactose)

Feed Conc. %w/v	CONTINUOUS OPERATION				BATCH OPERATION				Relative Through-put CONT / BATCH
	Experimental Run	PRODUCT PURITIES %		% Glucose Recovered in GRP	Experimental Run	PRODUCT PURITIES %		% Glucose Recovered in GRP	
		GRP	GALRP			GRP	GALRP		
10	10-4.5-4.5-28-300-28	99.9	63.6	49.3	10-2-25-45	99.9	64.9	49.4	2.38
15	15-4.5-4.5-28-300-28	99.9	60.0	52.6	15-2-25-45	99.9	66.2	46.6	2.38
20	20-4.5-4.5-28-300-29	91.9	68.8	57.4	20-2-25-45	99.9	59.3	45.2	2.63
30	30-4.5-4.5-30-400-28	99.9	77.1	70.4	30-1-25-45	99.9	64.5	34.3	5.00

Table 7.22: Comparison of Batch and Continuous Operation of the SCCR-S (Enzyme Usage)

Feed Conc. %w/v	ENZYME CONSUMPTION (U/g of Lactose)		Relative Enzyme Consumption Continuous / Batch
	CONTINUOUS	BATCH	
10	3000.0	5937.5	0.51
15	2000.0	4250.0	0.47
20	1500.0	3281.3	0.46
30	1333.0	4166.7	0.32

Table 7.23: Comparison of Batch and Continuous Operation of the SCCR-S (Product Concentrations)

Feed Conc. %w/v	CONTINUOUS Product Concentration (%w/v)		BATCH Product Concentration (%w/v)	
	GALRP	GRP	GALRP	GRP
10	0.44	0.30	1.03	0.61
15	0.66	0.48	1.55	1.00
20	0.83	0.76	1.93	1.10
30	1.14	1.22	1.44	0.80

CHAPTER 8

THE BIOSYNTHESIS OF DEXTRAN USING THE SCCR-S SYSTEM

8.0 INTRODUCTION

The biosynthesis of dextran from sucrose using the enzyme dextransucrase on batch chromatographic bioreactor-separators was studied by Zafar[9,10]. During the biosynthesis, the byproduct, fructose, was retarded immediately it was produced by complexing with the calcium ions on the resin while the dextran formed was size excluded and migrated with the mobile phase. By employing the simultaneous bioreaction and separation principle, Zafar[9,10] found that the dextran yield was substantially improved. The reaction is summarised by the equation:

$$\underset{\text{SUCROSE}}{n(C_{12}H_{12}O_{11})} \xrightarrow{\text{dextransucrase}} \underset{\text{DEXTRAN}}{(C_6H_{10}O_5)_n} + \underset{\text{FRUCTOSE}}{n(C_6H_{12}O_6)} \qquad 8.1$$

The biosynthesis of dextran on the SCCR-S system was studied and the results will be presented in this chapter. The dextransucrase required for this study was not commercially available in the quantities required. It was therefore necessary to investigate the production and purification of the enzyme in the quantities required for the bioreaction-separation experiments.

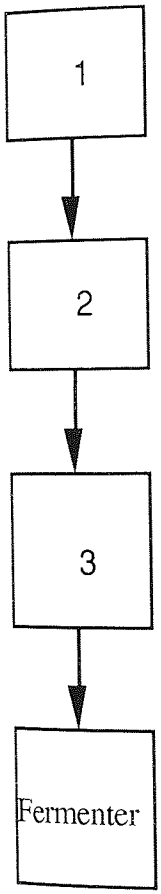
8.1 PRODUCTION OF THE ENZYME DEXTRANSUCRASE

The crude dextransucrase was produced by unaerated fed-batch fermentations of *Leuconostoc mesenteroides* (LM) strain NRRL B512F in a 1000 dm^3 fermenter (working volume 600 dm^3) at Fisons Pharmaceutical plc, Holmes Chapel Cheshire. These fermentations were performed with Dr J Ajongwen and other colleagues in the laboratory.

The culture was maintained on MRS (Deman-Regosa-Sharpe) agar slopes. The inoculation of the fermenter with the culture followed the stages shown in Figure 8.1. Transfer from

one stage to another was aseptically carried out (in laminar air flow cabinets). For each stage for the preparation of the inoculum, the bottles with medium before inoculation were sterilised at 118-121°C for 20-30 minutes in an autoclave. The empty fermenter was sterilised using steam at 120 °C for 30 minutes in the jacket of the fermenter.

During the fermentation, the pH was controlled at 6.7-7.0 by the continuous addition of sucrose-alkali solution. The temperature set point was 23 °C and a constant agitation of about 120 rpm was used. Tables 8.1, 8.2, 8.3 and 8.4 show the compositions of the inocula, fermenter media and the sucrose-alkali solution used during the fermentation.



8 x 9ml MRS broth bottles inoculated with slopes of L.M. 12hrs incubation at 23-25°C.

8 x 500ml MRS broth bottles inoculated with inucolum from stage 1. 16hrs, (23-25°C) incubation.

6 x 10L of MRS broth bottles inoculated with broth from stage 2. 15 hrs incubation at 23-25°C.

Fermenter inoculated with all 6 bottles from stage 3. 18 hrs fermentation. pH adjusted to about 7.1.

Figure 8.1 Inoculum preparation stages for the 1000dm^3 fermenter

Table 8.1: Inoculum bottles medium composition

Material	Amount used (for each bottle)
MRS broth	520g
Water	to 10 dm^3
NaOH	to pH 7.5

Table 8.2: Composition of fermenter medium

Material	Composition	Amount Used
Sucrose	10 g/dm^3	6 Kg
Di-potassium phosphate	20 g/dm^3	12 Kg
Yeast extract (GISTEX)	40 g/dm^3	24 Kg
R* Salts	0.5% v/v	3 dm^3
Water		To 600 dm^3
HCL		To pH about 7-7.1

Table 8.3: Composition of sucrose-alkali solution

Material	Amount used
Sucrose	180 Kg
Water	240 dm^3
NaOH (17M)	57 dm^3

Table 8.4: R* Salts composition

R* Salts	Amount Used
$MgSO_4.7H2O$	4.0 g
NaCl	0.2 g
$FeSO_4.H_2O$	0.2 g
$MnSO_4.H_2O$	0.2 g
Water	To 100 cm^3

The fermentation was monitored by taking samples and assaying for cell growth (OD_{590}) and activity (by the Hostettler method)[107]. The fermentation was stopped when the cell growth peaked as shown by Figure 8.2.

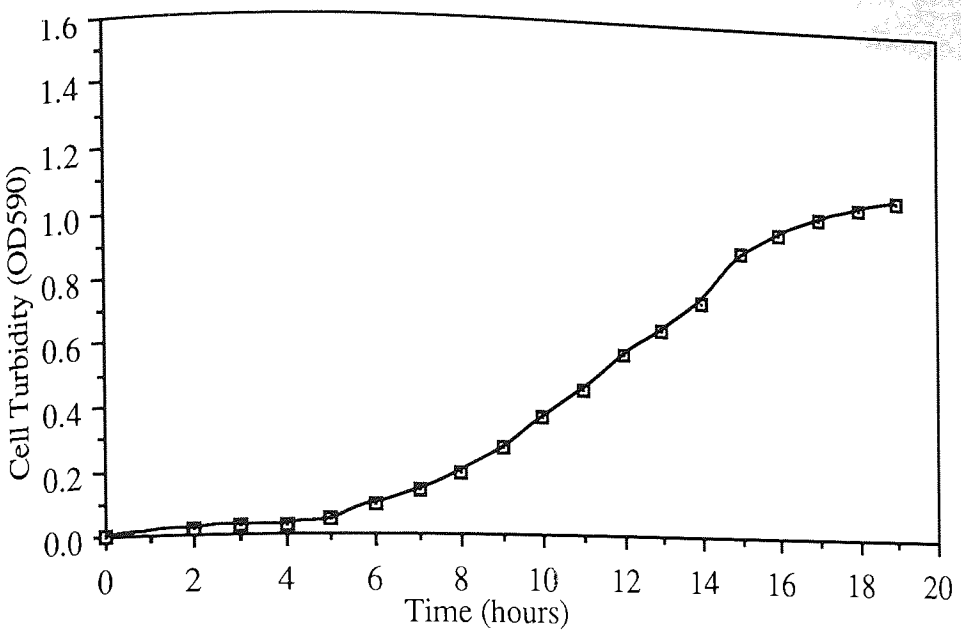


Figure 8.2: Cell growth during fermentation

8.1.1 Results and Discussion

Ajongwen[78] in his work showed that the type of yeast extract affected the enzyme activity produced during the fermentation. Before the large scale fermentation was performed, a laboratory scale fermentation (16 dm^3) was carried out to test the yeast extract. Enzyme activities in excess of 350 DSU/cm^3 were obtained during the laboratory scale fermentations. Using the same yeast extract and compositions, an enzyme activity of 200 DSU/cm^3 was obtained from the large scale fermentation. This lower enzyme activity was due to partial aeration[78], pH control and high temperature problems encountered.

8.2 PURIFICATION OF DEXTRANSUCRASE

To obtain the highly purified enzyme required for the bioreaction and separation in the SCCR-S, the enzyme was purified as shown in Figure 8.3.

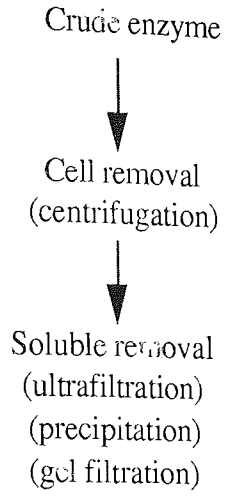


Figure 8.3: Purification route for dextransucrase.

8.2.1: Cell Removal by Centrifugation

In the experimental work a continuous clarifying bench type ultracentrifuge was used (Sharples Lab. T1). The temperature of the enzyme solution was maintained at between 4 and 8 ^{o}C with the aid of a chiller unit to minimise any enzyme activity loss. The cell concentration of the enzyme solution was measured by optical density determinations at 590nm after diluting the samples 25 times with saline. Enzyme activity was determined by the Hostettler method[107].

8.2.1.1: Results and Discussion

The throughput and centrifugation g-forces were varied during this experiment. As expected, the cell removal reduced drastically with an increase in throughput but increased with an increase in g-forces (Table 8.5). The enzyme recovery on the other hand, increased with throughput but was reduced with increasing centrifugal force. However, the degree of denaturation was not as much as the variation in cell removal, indicating that the shear created by the centrifugal forces used did not significantly affect the enzyme. Consequently, high centrifugal g-forces (28,000 to 30,000g) and throughputs of 100-150 cm^3/min were chosen as the optimum conditions for centrifugation producing broths with over 90% cell removal and enzyme recovery.

Table 8.5: Cell removal and enzyme recovery by centrifugation

Centrifugal Force (g)	Throughput (cm^3/min)	Residence Time (min)	% Cell Removal	% Enzyme Recovery
2,500	58	13.7	67	76.5
	100	8.0	45	–
	140	5.7	24	96.7
	220	3.7	12	97.6
5,600	58	13.7	91	70.3
	100	8.0	75	74.5
	140	5.7	54	88.5
	220	3.7	26	96.8
22,400	58	13.7	97	62.4
	100	8.0	98	–
	140	5.7	96	72.6
	220	3.7	75	95.4

2.2 Soluble Impurities Removal

2.2.1.1 Low Molecular Weights Removal

ıe low molecular impurities such as fructose, unused sucrose, leucrose, mannitol, gosaccharides, salts and colourings were removed by ultrafiltration using a Millipore TTK 000 05 flat cross-flow membrane having a 30,000 molecular weight cut off IWCO). HPLC was used to determine the degree of low molecular weight removal. The trafiltration was carried out at temperatures between 8 and 12 ^oC and a diafiltration mode operation was employed with at least four diafiltration stages.

2.2.1.2 Results and Discussion

he enzyme recovery and low molecular weight removal during the different stages of the trafiltration are shown in Figure 8.4. An overall enzyme recovery of 65.9% and 97.8% moval of the impurities were achieved after five diafiltrations. The enzyme losses were

possibly due to shear damage and adsorption of the enzyme (protein) on the membrane since the permeate did not contain any enzyme.

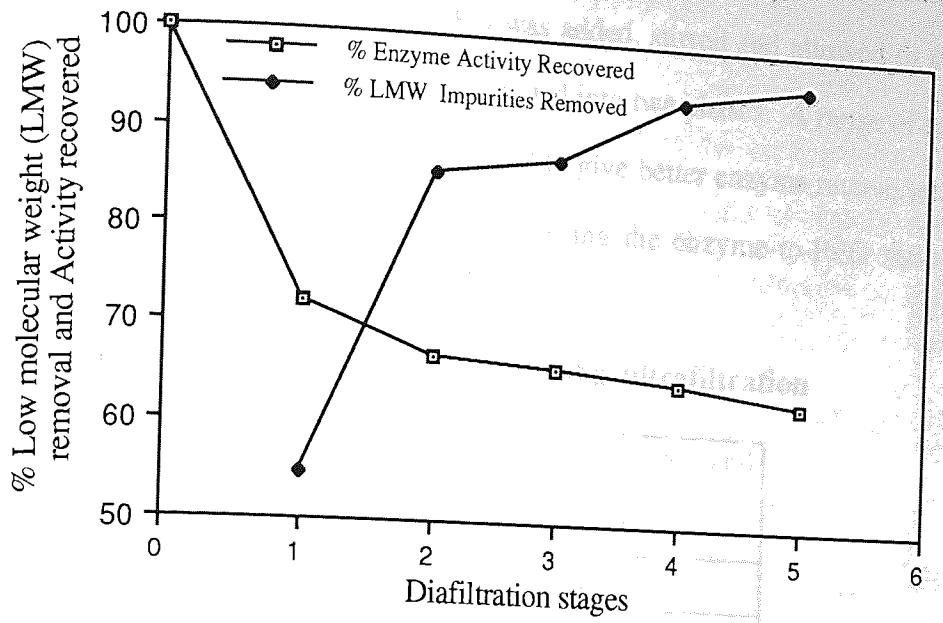


Figure 8.4: Results of Dextransucrase Ultrafiltration

8.2.2.2.1 Enzyme Desalting

The five stage ultrafiltration removed up to 99% of the soluble low molecular weight materials, but left substantial amounts of cations which affected the ion-exchange resin used in the chromatographic bioreaction and separation. Table 8.6 shows the amounts of the principal cations (Na^+ and K^+ from the fermentation medium) remaining in the enzyme broth at the end of each diafiltration stage. Determination of the concentration of ions in the broth was carried out by atomic absorption using a Perkin-Elmer Series 360 atomic absorption spectrophotometer. At the end of the fifth diafiltration, the purified broth was further concentrated and this resulted in solutions containing 1-2 g/dm^3 cations. It was therefore necessary to investigate ways of further reducing the ionic content of the enzyme before being contacted with the ion-exchange resin in the chromatographic system. Desalting methods that were investigated included precipitation with polyethylene glycol (PEG), the use of desalting gels, ultrafiltration at different pH values and ion-exchange chromatography.

8.2.2.2.2 Results and Discussion

Precipitation with PEG was initially considered as an alternative to ultrafiltration. The broth contained dextran and when the PEG was added, stirred and allowed to stand in a refrigerator for a few hours, the solution separated into two phases. A range of molecular weights of PEG and concentrations were found to give better enzyme recoveries, but did not improve the desalting of the enzyme. Varying the enzyme-to-PEG ratio did not improve the desalting (Figure 8.5).

Table 8.6: Ion removal during ultrafiltration

Number of diafiltrations	Total cations (Na^+ and K^+) g/dm^3
Initial broth	82.13
1	9.90
2	5.70
3	5.20
4	3.23
5	0.91

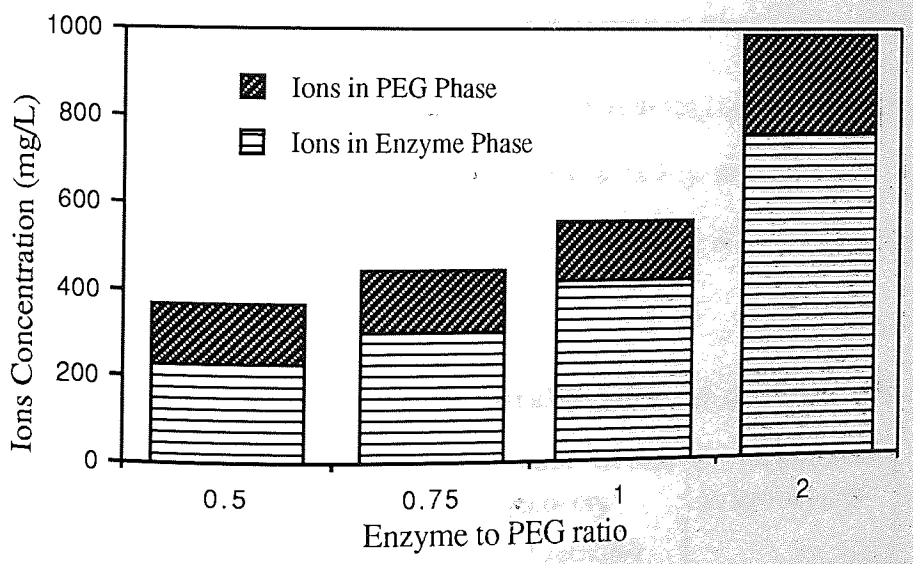


Figure 8.5: Desalting by PEG precipitation

Gel filtration was carried out using PD-10 Sephadex G-25 (Pharmacia-LKB) desalting columns. The columns were equilibrated with de-ionised water, loaded with the enzyme

samples and then eluted with more de-ionised water. The first elution samples were collected and the elution repeated three or four times. The solutions from the different elution stages were analysed for the presence of ions as well as dextransucrase activity. The first stage elution products contained about 54% of the total enzyme loaded into the columns but with less than 6% of the ions. Up to 8% of the enzyme came out in the second stage (Table 8.7), but contained over 70% of the ions eluted over the five stages. Apart from the low recovery of the enzyme, this process is very expensive in capital cost, slow and of limited throughput.

Table 8.7: Results of gel filtration using PD-10 Sephadex G-25M columns

Number of elutions	Concentration of ions in solution (mg/dm^3)	Enzyme activity	
		Total amount DSU	% Recovered
Initial broth	936	750	100
1	49	405.3	54
2	245	63.2	8
3	34	-	-
4	2.5	-	-
5	1.9	-	-

A third attempt at reducing the concentration of ions in the purified enzyme was to carry out the ultrafiltration at different pH values. Ultrafiltration at pH of 4.5 - 7.0 had no effect on the overall removal of ions, but at pH 4.5, severe losses of the enzyme activity occured (Table 8.8).

Table 8.8: Dextransucrase ultrafiltration at different pH values

pH	Enzyme recovery (% original activity)	% Ion removal
4.5	39.9	98.5
5.5	69.5	-
6.3	64.5	99.1
7.0	61.5	98.5

Finally, due to the fact that the ions in the enzyme solution displaced the calcium ions on the chromatographic resin, a single column (5.4 cm i.d. by 70 cm long) packed with calcium charged resin was used to remove the unwanted ions (Na^+ and K^+). Ultrafiltered enzyme was pumped through the column in a single pass. The stream exiting the column was collected and analysed for the degree of ions removal and activity loss (Table 8.9). Over 50% of the enzyme activity was lost in this single column and it was not thought possible to further reduce the ions concentration without total enzyme loss. Calcium ions have been reported to have an inhibitory effects on dextransucrase at concentrations greater than 1.0 mM[121]. It is possible that the displacement of these ions from the resin into solution, resulted in concentrations of over 2.5 mM and this could have been responsible for this loss in activity.

Table 8.9: Desalting by ion-exchange chromatography

		Solution in	Solution out
Ion Concentration (mg/L)	Ca^{2+}	35	101
	Na^+	239	105
	K^+	1904	766
Enzyme Activity (DSU/cm^3)		346	154

8.3 THE BIOSYNTHESIS OF DEXTRAN USING THE SCCR-S SYSTEM

After the production and purification of the dextransucrase required for the bioreaction and separation, the SCCR-S system was repacked with the cross-linked polystyrene resin in the Ca^{2+} form with a particle size range of 300-500μm (PUROLITE PCR 563). The columns in the system were then characterised as in Chapter 5. The physical separation of fructose and dextran in the SCCR-S system has been studied by previous researchers(16,115). The optimum flow rates for the separation of fructose and dextran determined by the previous researchers were chosen and used during the preliminary experiments.

The enzyme solution was prepared to the required strength from the purified enzyme and the sucrose feed was prepared by dissolving the required amount of the sucrose in de-ionised water.

It was shown in Chapters 6 and 7 that the SCCR-S system reaches steady state after 6-7 cycles of continuous operation. It was decided to start collecting samples from the SCCR-S after 6 cycles of continuous operation.

8.3.1 Preliminary Experiments on the SCCR-S system

The experimental conditions maintained during these experiments were as shown in Table 8.10.

Table 8.10: Experimental conditions

Run No	Average Flow Rates (cm^3/min)			Sucrose Conc (% w/v)	Enzyme Activity (DSU/min)	Switch Time (mins)	Temp (°C)
	Feed	Eluent	Purge				
SCCR-S/1	9	30	76	3	1500	30	25
SCCR-S/12	9	30	76	3	1500	30	25

8.3.1.1 Results and Discussion

The on-column profiles obtained during these runs are shown in Figures 8.6, 8.7 and 8.8. Comparing cycle 7 with cycle 11 (Figures 8.6 and 8.7), it can be seen that the separating performance of the SCCR-S system was lost. This was due to the amount of ions (Na^+ and K^+) present in the enzyme which slowly displaced the Ca^{2+} on the resin which complexes with the fructose to separate it from the dextran. The displacement of these ions also caused the formation of unwanted glucose as shown by Figure 8.7. It was from this experiment that it was then decided to start investigating methods of desalting the enzyme (Section 8.2).

Run SCCR-S/12 was designed to improve the separating performance of the system and inhibit the formation of glucose by the addition of 0.8mM calcium nitrate in the eluent stream.. As shown by Figure 8.8, the aim of this experiment was not achieved because there was still some glucose formation and the separation did not improve. From these results, it was decided to adopt a method of on-line regeneration of the resin in the SCCR-S system with calcium nitrate. This method had been used successfully by Joshi[14] when working with molasses which contained more Na^+, K^+ and other ions than the enzyme solution.

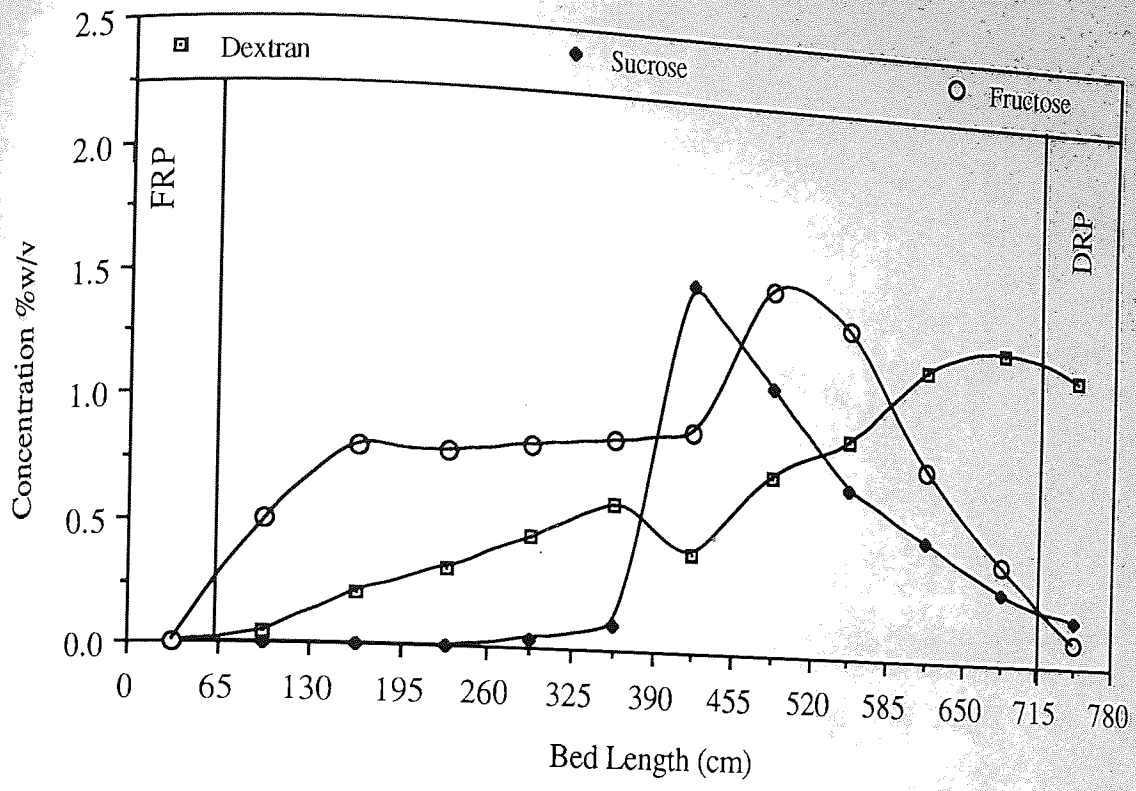


Figure 8.6 On-Column Concentration Profile for Run SCCR-S/1 Cycle 7

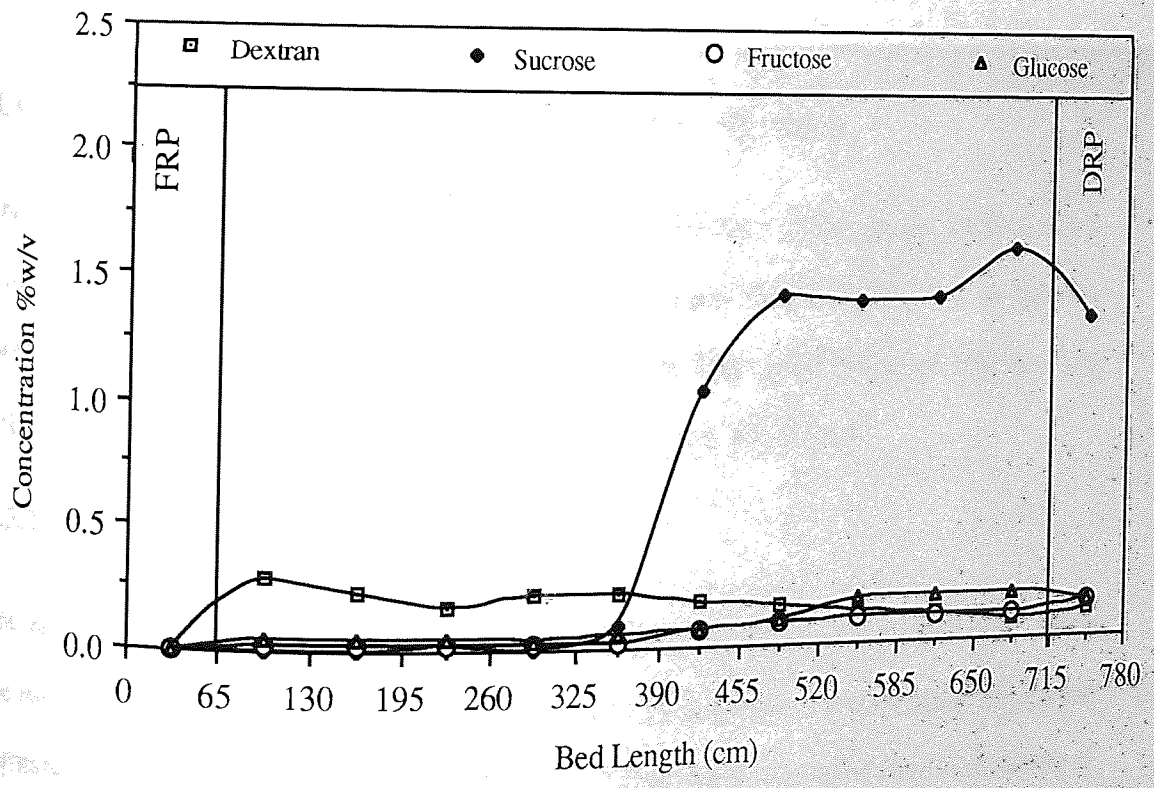


Figure 8.7: On-Column Concentration Profile for Run SCCR-S/1 Cycle 10

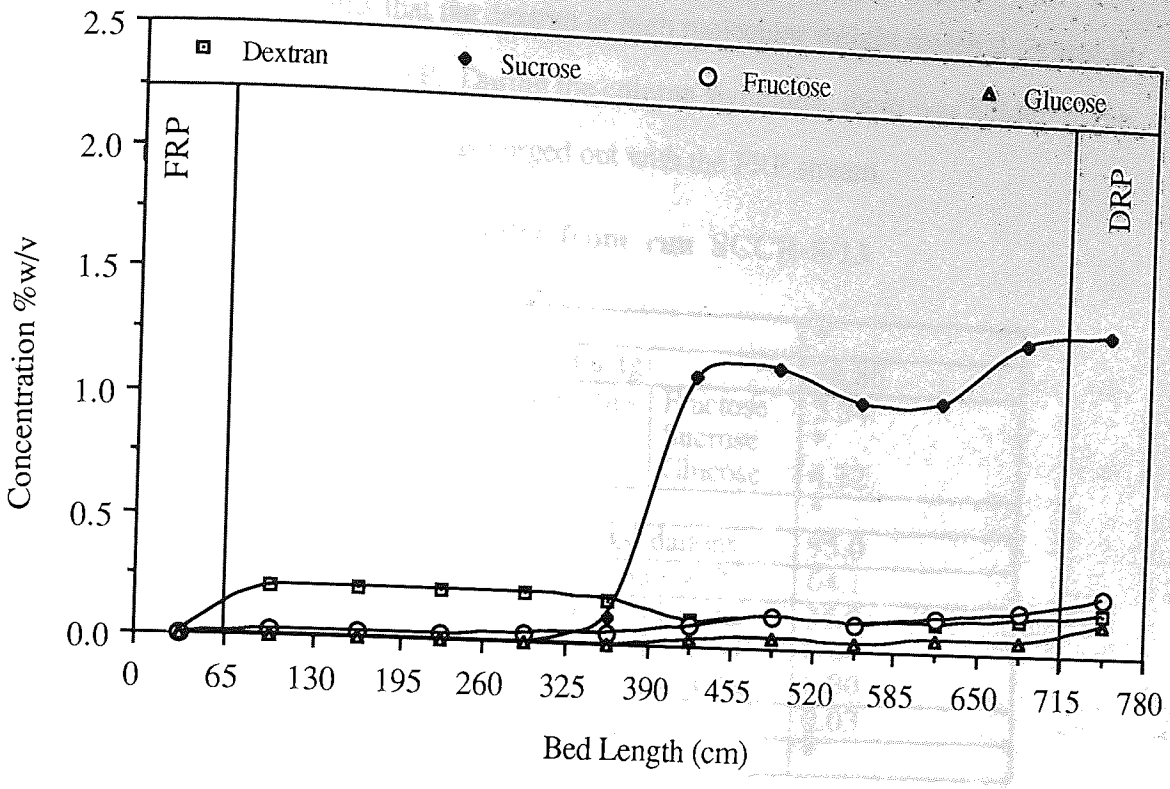


Figure 8.8: On-Column Concentration Profile for Run SCCR-S/12 Cycle 12

8.3.2 Experiment with on-line Regeneration of the Resin

During this experiment, the purge stream used was a 5%w/v calcium nitrate solution. Each column was regenerated in turn when it was being purged of FRP. Using this method, the twelve columns in the system were regenerated after each complete cycle. The experimental conditions were similar to those used in run SCCR-S/12.

8.3.2.1 Results and Discussion

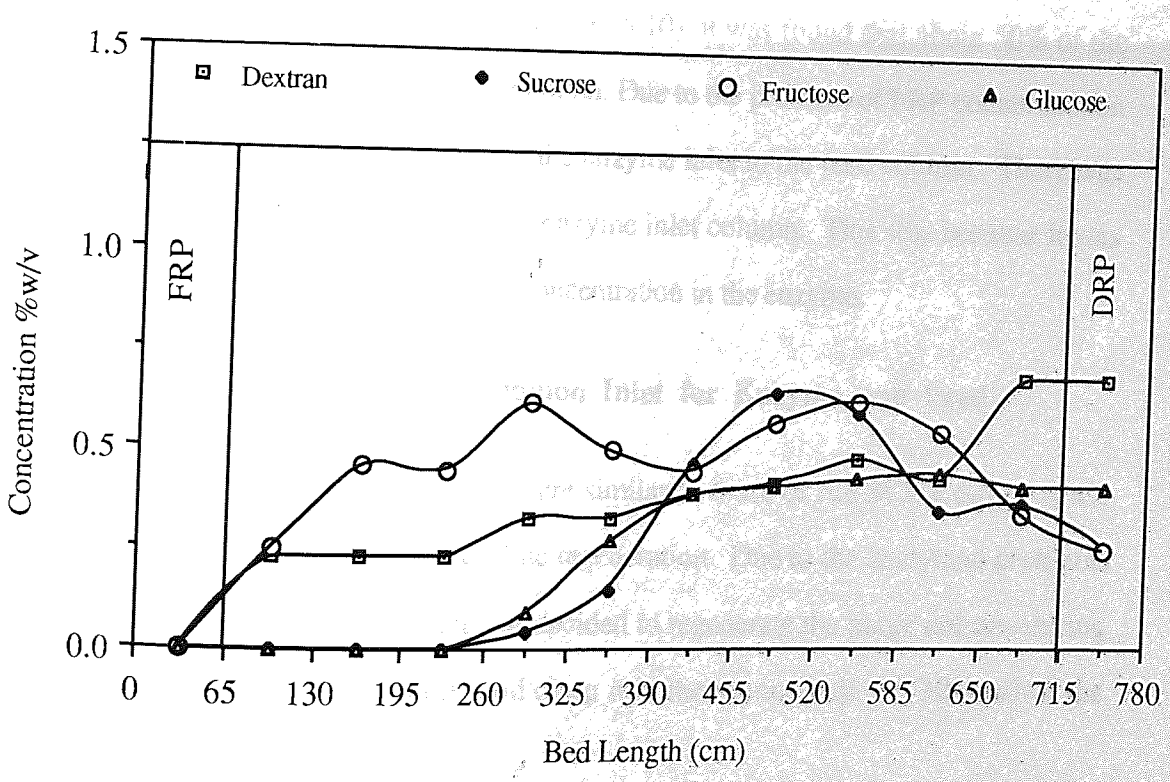
The results obtained during this run are summarised in Table 8.11 and Figure 8.9. From the results, it can be seen that the separation improved but there was an increase in glucose formation. This increase in glucose formation was possibly due to localised formation of nitric acid from the calcium nitrate used in the system causing acid hydrolysis of the sucrose. The pH of the streams through the system was observed to drop from 5.2 to 3.0-3.2.

It is also seen from the results that the dextran or high molecular weight material present in the enzyme contaminated the FRP. During the column switching, there was holdup in the column containing enzyme which was purged out with the FRP stream.

Table 8.11: Results from run SCCR-S/13

% Conversion			*
DRP	Dextran (g)		18.86
	Impurities (g)	Fructose	3.54
		Sucrose	*
		Glucose	4.72
DRP Concentration (%w/v)			*
% Dextran of MW > 160000 daltons			95.0
FRP	Fructose purity (%)		64.1
	Impurities (%)	Dextran	35.9
		Sucrose	0.00
		Glucose	0.00
FRP Concentration (%w/v)			0.03
% Mass Balance			*

* It was not possible to determine the amount of unreacted sucrose in the DRP because calcium nitrate and sucrose had the same elution times on the HPLC.



gure 8.9: On-Column Concentration Profile for Run SCCR-S/13 Cycle 11

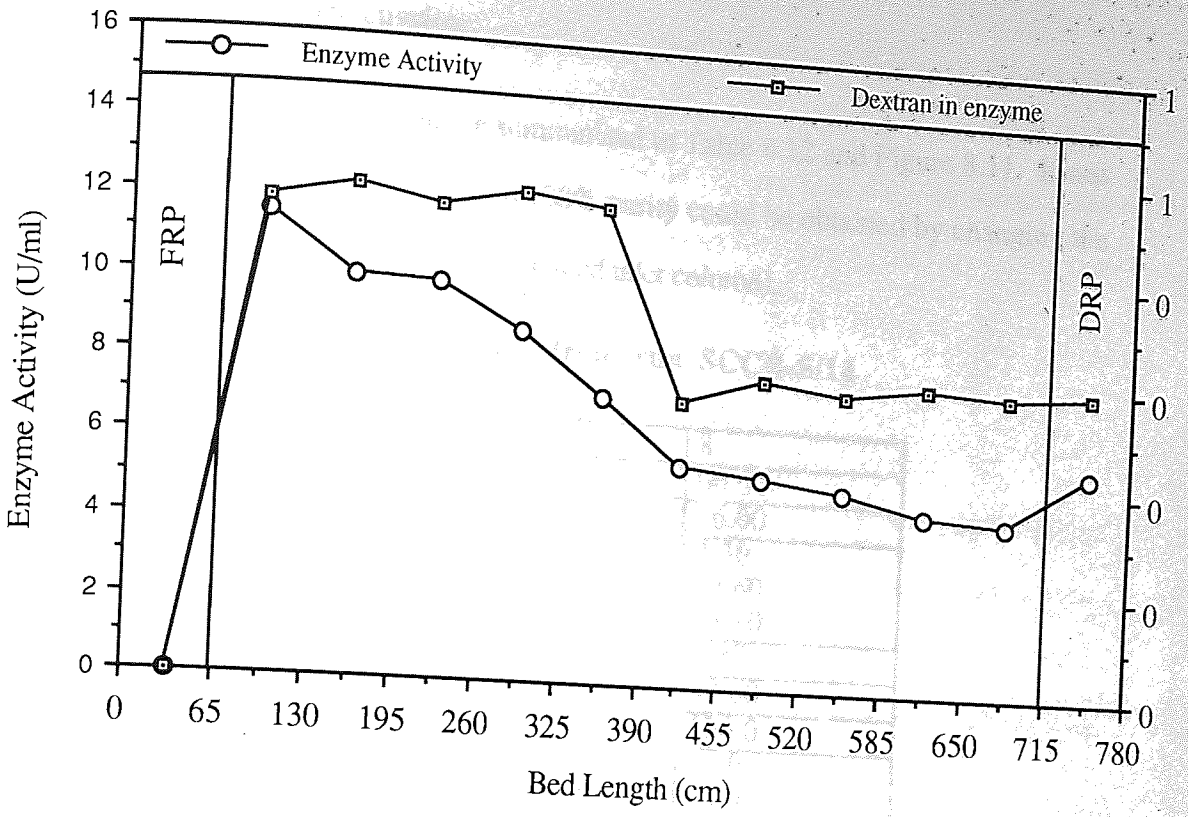


Figure 8.10: On-Column Enzyme Activity Profile

From an on-line enzyme activity profile (Figure 8.10), it was found that about 50% of the enzyme activity was lost before the feed column. Due to the problem of FRP contamination and activity loss, it was decided to change the enzyme inlet to the feed column. There was also a greater loss of Ca^{2+} from the eluent/enzyme inlet column. This was because it was the column in contact with the highest ion concentration in the enzyme.

.3.3 Experimental Run with a Common Inlet for Enzyme and Feed

he experimental conditions for this run were similar to those of run SCCR-S/12. A 4% lcium nitrate solution was used for on-line regeneration. Due to the analytical problems countered during run SCCR-S/13, it was decided to regenerate the purge column during e first half of the switch (0-15mins) and clean it in the second half (15-30mins) of the itch with de-ionised water.

8.3.3.1 Results and Discussion

The results obtained during this run are summarised in Table 8.12 and Figure 8.11. It was found that a fructose rich product of up to 100% purity could be obtained by changing the enzyme inlet from column 2 to column 7 (the feed inlet column).

Table 8.12: Results from run SCCR-S/14

Cycle Number			8
% Conversion			66.00
DRP	Dextran Purity (%)		46.00
	Impurities (%)	Fructose	2.30
		Sucrose	27.60
		Glucose	24.10
DRP Concentration (%w/v)			0.87
% Dextran of MW > 160,000 daltons			99.60
FRP	Fructose Purity (%)		100.0
	Impurities (%)	Dextran	0.00
		Sucrose	0.00
		Glucose	0.00
FRP Concentration (%w/v)			0.07
% Mass Balance			133.9

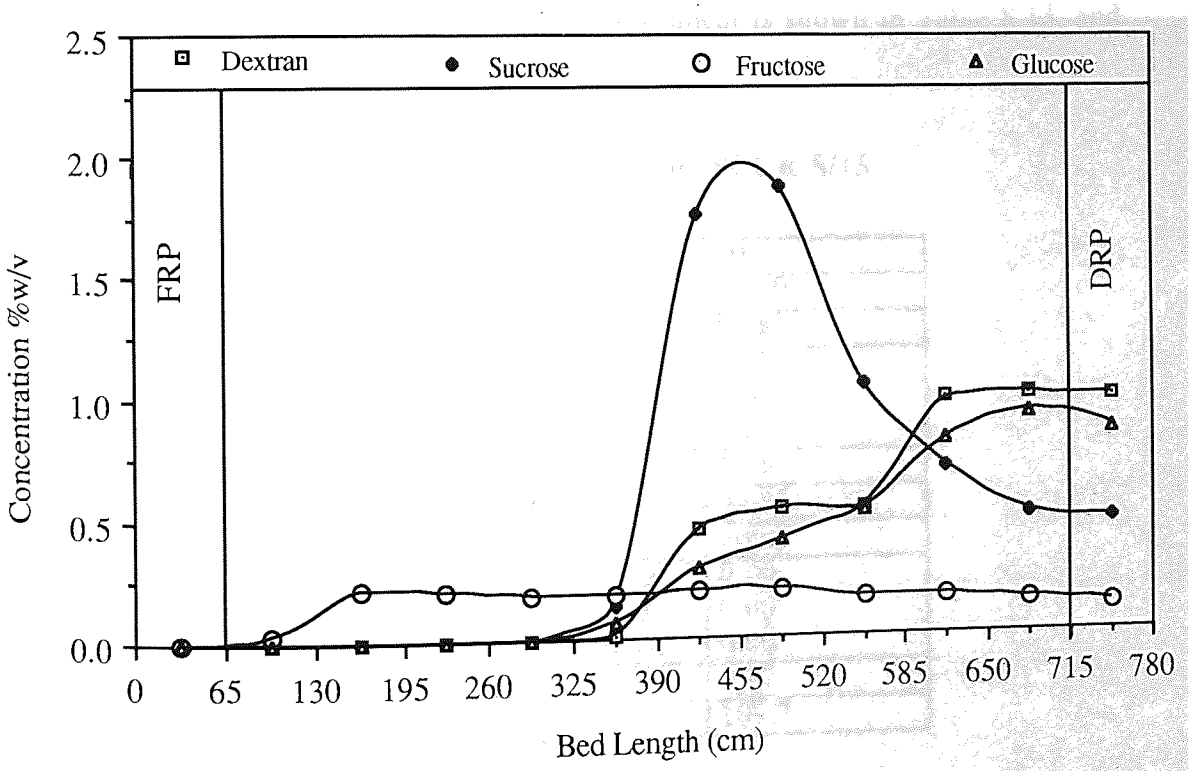


Figure 8.11: On-Column Concentration Profile for Run SCCR-S/14 Cycle 12

As shown in Figure 8.11, a 4%w/v calcium nitrate solution was not sufficient for on-line regeneration in 15 minutes since the separation was not maintained. The pH of the streams in and out of the system dropped from 5.2 to 3.9-4.0. It was then decided to do a run using a 10%w/v calcium nitrate solution for on-line regeneration during the first half of the switch period with the inlet streams to the system at very high pH (i.e about 10.0).

8.3.4 Experiment with High pH on-line Regeneration

Before this run was carried out, the system was regenerated with 10%w/v calcium nitrate at a pH of 12 until the inlet pH was equal to the outlet pH. Calcium hydroxide was used to increase the calcium nitrate pH to the required level. The experimental conditions were similar to those of run SCCR-S/12 except that the pH of the purge and eluent streams was 10.4. The enzyme and feed had the same inlet to the system.

8.3.4.1 Results and Discussion

A summary of the results obtained from this experiment is shown in Table 8.13, and Figures 8.12 and 8.13.

Table 8.13: Results from run SCCR-S/15

Cycle Number			10
% Conversion			100.0
DRP	Dextran Purity (%)		91.80
	Impurities (%)	Fructose	0.00
		Sucrose	0.00
		Glucose	8.20
DRP Concentration (%w/v)			1.09
% Dextran of MW > 160,000 daltons			94.85
FRP	Fructose Purity (%)		100.0
	Impurities (%)	Dextran	0.00
		Sucrose	0.00
		Glucose	0.00
FRP Concentration (%w/v)			0.17
% Mass Balance			107.7

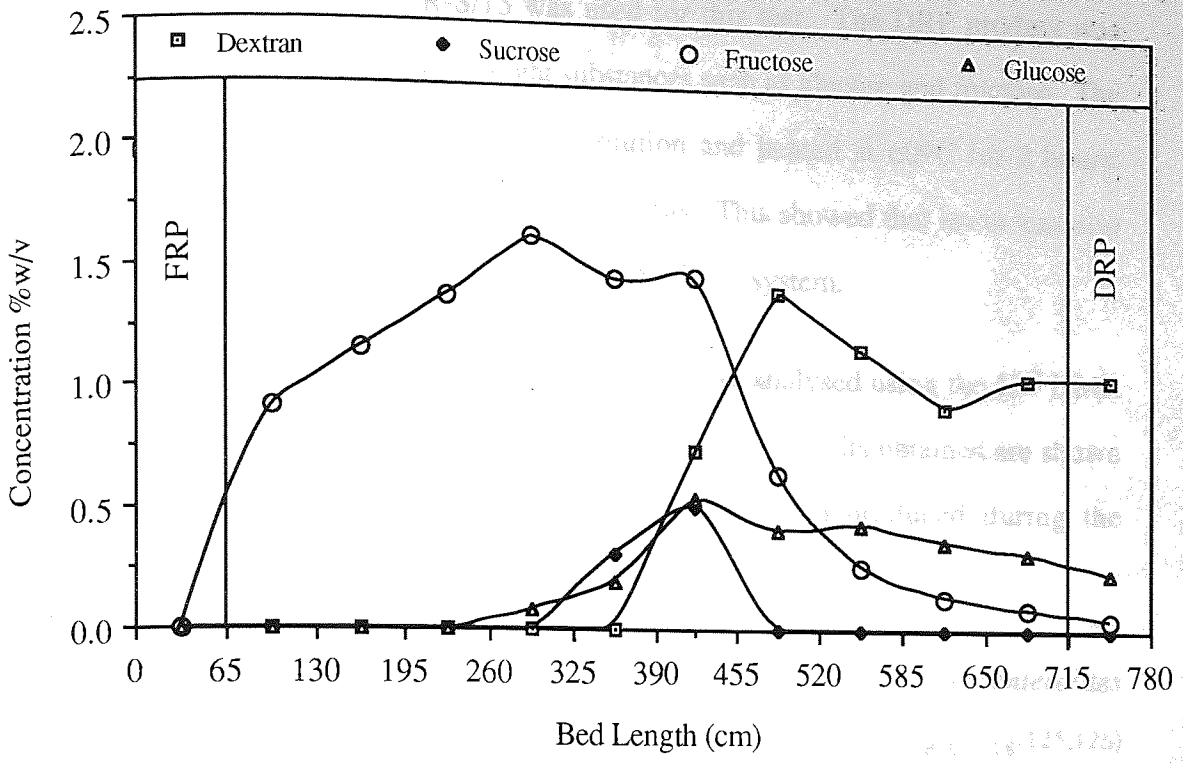


Figure 8.12: On-Column Concentration Profile for Run SCCR-S/15 Cycle 10

These results showed an improvement in the separating power of the system and there was complete conversion of the sucrose. Glucose was still formed but at a lower concentration than in the previous runs. The pH of the streams changed from 10.4 to 6.3 (DRP) and 4.5 (FRP). It was therefore decided to carry out a series of tests to find out the cause of the glucose formation.

8.3.5 Tests for the Cause of Glucose Formation

The formation of glucose even at high eluent and purge pH indicated that acid hydrolysis was not the only or main cause of formation of the glucose observed. After private communications with Professor SA Barker[122] and Dr. I Blackburn[123], it was decided to carry out an acid hydrolysis test to find out whether levan (a poly-fructose) was also produced in the SCCR-S system. Levan production releases glucose as byproduct. Dextran is more stable than levan which is hydrolysed to fructose under acidic conditions at a temperature of 60°C.

The DRP product from run SCCR-S/15 was used for this test. The DRP product was dialysed to remove the low molecular weight substances such as glucose and fructose. The dialysed sample was added to a 0.5N HCL solution and heated at 60°C for 2 hours. Fructose was found to be formed during the hydrolysis. This showed that levan was also produced during the biosynthesis of dextran on the SCCR-S system.

The same sample used for the acid hydrolysis test was also analysed using the 13^C NMR technique by Dr. Baggett[124] of Birmingham university. The results obtained are shown in Appendix C. The results confirmed that levan was also produced during the biosynthesis of dextran on the SCCR-S system.

The production of levan by the enzyme dextransucrase from *leuconostoc mesenteroides* strain B512F under certain conditions has been reported by some researchers[125,126] working with this enzyme. Small amounts of levan are also known to be formed in the conventional dextran batch fermentation process. This levan is removed in the ethanol fractionation downstream process[125].

Further studies need to be carried out to determine if it is possible to minimise or eliminate the formation of unwanted levan and thus glucose during the bioreaction and separation on the SCCR-S system.

CHAPTER 9

MATHEMATICAL MODELLING AND COMPUTER SIMULATION OF THE SCCR-S SYSTEM

9.1 INTRODUCTION

The mathematical modelling and computer simulation of chromatographic reactors have been reported[10,41,48,49,127]. Most of these models were for reversible chemical reactions with the aim of achieving conversions higher than the equilibrium conversions.

Petroulas *et al.*[41] developed an ideal reactor model and simulated a counter-current moving bed chromatographic reactor for a chemical reversible reaction, namely, the hydrogenation of mesitylene (MES) with excess hydrogen to 1,3,5-trimethylcyclohexane (TMC) over a platinium on alumina catalyst. The model was tested for different feed concentrations and reactor lengths. They showed that reaction and separation can be achieved simultaneously in a chromatographic reactor and that under appropriate operating conditions, a reactor fed at the bottom with the species more favoured by thermodynamic equilibrium can lead to 100% product purity with overall conversions lower than with a conventional fixed bed reactor.

Zafar *et al.*[10] studying the biosynthesis of dextran from sucrose using the enzyme dextransucrase developed a model to simulate a batch chromatographic bioreactor-separator. This model based on Langer's work[128], assumed linear adsorption of components and that the reaction mainly takes place in the mobile phase. The model used finite difference analysis to solve a set of partial differential equations derived from mass balances. The results obtained from the simulation showed reasonably good agreement with the experimental results at low pulse sizes.

Ching *et al.*[127] developed an axial dispersion model to simulate a fluidised bed reactor-separator for the isomerisation of glucose to fructose in the presence of immobilised isomerase. The multiple shooting method was used to solve a set of non-linear ordinary

differential equations derived from mass balances. A good fit of the experimental and predicted concentration profiles was obtained.

Akintoye[16,49] developed a mathematical model based on the theoretical plate concept to simulate the SCCR-S system for the inversion of sucrose and simultaneous separation of the products. This model assumed linear adsorption of the components with reaction taking place only in the mobile phase.

Akintoye's approach was modified and used during this research work to simulate the SCCR-S system for the simultaneous hydrolysis of lactose and separation of the products, galactose and glucose when the enzyme and the feed are introduced through the same port into the system.

9.2 MATHEMATICAL MODEL

The model was based on the theoretical plate concept where a chromatographic column is considered to consist of a series of idealised mixing stages. To develop a material balance for each component in the SCCR-S system over a plate, the following assumptions were made:

(1) Steady state

(2) Adsorption of components are linear and can be defined as Kd = P/C where P and C are concentrations of the components in the stationary and mobile phases respectively;

(3) Constant fluid density and viscosity (i.e constant velocity);

(4) Negligible axial dispersion;

(5) Flat velocity profile;

(6) Plugflow (flat concentration profile);

(7) The hydrolysis of lactose to galactose and glucose follows the Michaelis-Menten model

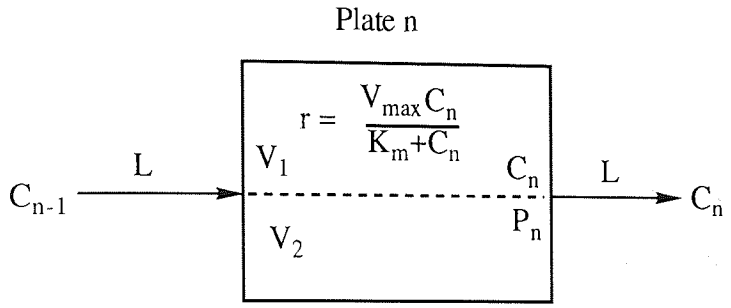
(8) The reaction takes place only in the mobile phase;

(9) Equimolar amounts of galactose and glucose are produced from each mole of lactose hydrolysed;

(10) The mobile phase leaving each plate is at equilibrium with the stationary phase in the plate.

9.2.1 Mass Balance

Consider a mobile phase flowrate L, passing through the plate "n" having an initial solute concentration of C_{n-1} and an exit solute concentration of C_n, with the bioreaction, r, taking place only in the mobile phase, a mass balance for the substrate (lactose) over plate "n' gives:



$$V_1\frac{dC_n}{dt} + V_2\frac{dP_n}{dt} = LC_{n-1} - LC_n - r\,V_1 \qquad 9.1$$

Where, V_1 = plate volume of the mobile phase

V_2 = plate volume of the stationary phase

C_n = concentration of solute in the mobile phase

P_n = concentration of solute on the stationary phase

r = reaction rate (Michaelis-Menten model)

$$= \frac{V_{max}C_n}{Km+C_n} \qquad 9.2$$

Equilibrium on the plate is represented by the distribution coefficient Kd, where :-

$$Kd_n = \frac{P_n}{C_n} \qquad 9.3$$

Substituting Equations 9.2 and 9.3 into Equation 9.1 gives:-

$$(V_1 + V_2Kd_L)\frac{dCn}{dt} = LC_{n-1} - LC_n - \frac{V_1VmaxC_n}{Km + C_n} \qquad 9.4$$

Similar mass balance for galactose (F) and glucose (G) gives:

$$(V_1 + V_2Kd_F)\frac{dFn}{dt} = LF_{n-1} - LF_n + \frac{0.526V_1VmaxC_n}{Km + C_n} \qquad 9.5$$

$$(V_1 + V_2Kd_G)\frac{dGn}{dt} = LG_{n-1} - LG_n + \frac{0.526V_1VmaxC_n}{Km + C_n} \qquad 9.6$$

9.3 METHODS OF SOLUTION

9.3.1 Akintoye's Approach[16,49] (Analytical Solution)

Akintoye in his approach to solve Equations 9.4, 9.5 and 9.6 analytically, simplified these equations by making an assumption regarding the Michaelis-Menten equation (Equation 9.2). He assumed a pseudo-first order reaction for all feed concentrations i.e Equation 9.2 becomes:

$$r = \frac{VmaxC_n}{Km} \qquad 9.10$$

Substituting Equation 9.10 into 9.4, 9.5 and 9.6 give:

$$(V_1 + V_2Kd_L)\frac{dCn}{dt} = LC_{n-1} - LC_n - \frac{V_1VmaxC_n}{Km} \qquad 9.11$$

$$(V_1 + V_2Kd_F)\frac{dFn}{dt} = LF_{n-1} - LF_n + \frac{0.526V_1VmaxC_n}{Km} \qquad 9.12$$

$$(V_1 + V_2Kd_G)\frac{dGn}{dt} = LG_{n-1} - LG_n + \frac{0.526V_1VmaxC_n}{Km} \qquad 9.13$$

Equations 9.11, 9.12 and 9.13 are linear first order differential equations and were solved analytically using Laplace transforms to give Equations 9.14, 9.15 and 9.16 respectively.

$$Cn(t) = \beta C_{n-1} - \beta\exp-[(\alpha_S + y_S)\Delta t] + C_{no}\exp-[(\alpha_S + y_S)\Delta t] \qquad 9.14$$

$$Fn(t) = F_{n-1}[1 - \exp(-\alpha_F\Delta t)] + y_F\, C_n\exp(-\alpha_F\Delta t) + F_{no}\exp(-\alpha_F\Delta t) \qquad 9.15$$

$$Gn(t) = G_{n-1}[1 - \exp(-\alpha_G\Delta t)] + y_G\, C_n\exp(-\alpha_G\Delta t) + G_{no}\exp(-\alpha_G\Delta t) \qquad 9.15$$

where,

$$ß = \frac{L}{L + \frac{V_1 Vmax}{Km}}$$

$$\alpha_i = \frac{L}{V_1 + V_2 Kd_i}$$

$$y_i = \frac{L}{Km(V_1 + V_2 Kd_i)}$$

When Equation 9.14 was applied to the feed plate, the term C_{n-1} was replaced by the ratio:

$$\frac{LC_{n-1} + FC_f}{L + F}$$

where C_f is the feed concentration and F the feed flowrate.

For the purging column where no reaction takes place, Akintoye[16] modified Equations 9.4 to 9.6 without the reaction term and solved analytically to give:-

$$An = A_{n-1}[1-\exp(-\alpha_i \Delta t)] + A_{no}\exp(-\alpha_i \Delta t) \qquad 9.18$$

The computer simulation work carried out using this method of solution did not agree with the experimental results. The model predicted concentrations higher than the concentrations obtained during the experiments. Values of distribution coefficients for fructose and glucose were selected on a trial and error basis to fit the simulation results to the experimental results.

The simulation results obtained using the same distribution coefficients for other feed concentrations did not fit the experimental results. This was due to the assumption made by Akintoye simplifying the Michaelis-Menten model (Equation 9.2) to Equation 9.10 to linearize Equations 9.4, 9.5 and 9.6. The simulation did not account for the effects of flowrates and background concentrations on the distribution coefficients of the components and the simulation also did not account for the change in flowrate from the last plate of the pre-feed Section to the first plate (feed plate) in the post-feed Section during the calculation of the concentration of the products in the system.

9.3.2 Numerical Solution

During this work, the Fourth-order Runge-Kutta Method[129] was used to solve Equations 9.4, 9.5 and 9.6. These equations are non-linear ordinary differential equations. Using this method, the solution to Equation 9.4 is given by:

$$C_n = C_n + \frac{1}{6}(K_1 + 2K_2 + 2K_3 + K_4)$$

where, $K_1 = h \times f(C_n,t)$

$K_2 = h \times f(C_n+1/2(K_1),t+1/2(h))$

$K_3 = h \times f(C_n+1/2(K_2),t+1/2(h))$

$K_4 = h \times f(C_n+K_3,t+h)$

h = stepsize

Similar solutions can be obtained for Equations 9.5 and 9.6.

When Equations 9.4, 9.5 and 9.6 are applied to the feed plate, the term C_{n-1} is replaced by the ratio:

$$\frac{LC_{n-1} + FC_f}{L + F + E}$$

where C_f is the feed concentration, F the feed flowrate and E the enzyme flowrate when the enzyme is introduced with the feed into the system.

This method of solution does not require simplification of the Michaelis-Menten equation (Equation 9.2) as was required by the analytical solution. By using this method, it is possible to incorporate the enzyme inhibition constant by using the modified Michaelis-Menten model (Equation 3.2).

9.4 THE COMPUTER SIMULATION PROGRAMME

The simulation programme developed by Akintoye was modified and used during this work. The programme was modified by incorporating a subroutine in which the Fourth-Order Runge-Kutta method is used to solve Equations 9.4, 9.5 and 9.6. This subroutine unlike Akintoye's programme takes account of the change in flowrate from the last plate in the pre-feed section to the first plate (feed plate) in the post-feed section of the SCCR-S system.

The main section of the programme was modified to take into account the effect of flowrates and background concentrations on the distribution coefficients of galactose and glucose using the correlations developed in Chapter 5. The program was also modified to include the introduction of enzyme and the feed through the same port into the system.

The computer programme is shown in Appendix D and the flowchart is provided in Figure 9.1.

The programme was designed to predict the on-column concentration profiles for lactose, glucose and galactose during the hydrolysis of lactose in the SCCR-S system. The Michaelis-Menten constant, Km, used was a constant value which was calculated during the kinetic studies (Chapter 4). The maximum initial reaction velocity, Vmax, was a variable depending on the enzyme activity. The distribution coefficients (Kds) and the number of theoretical plates used were obtained during the system characterisation (Chapter 5).

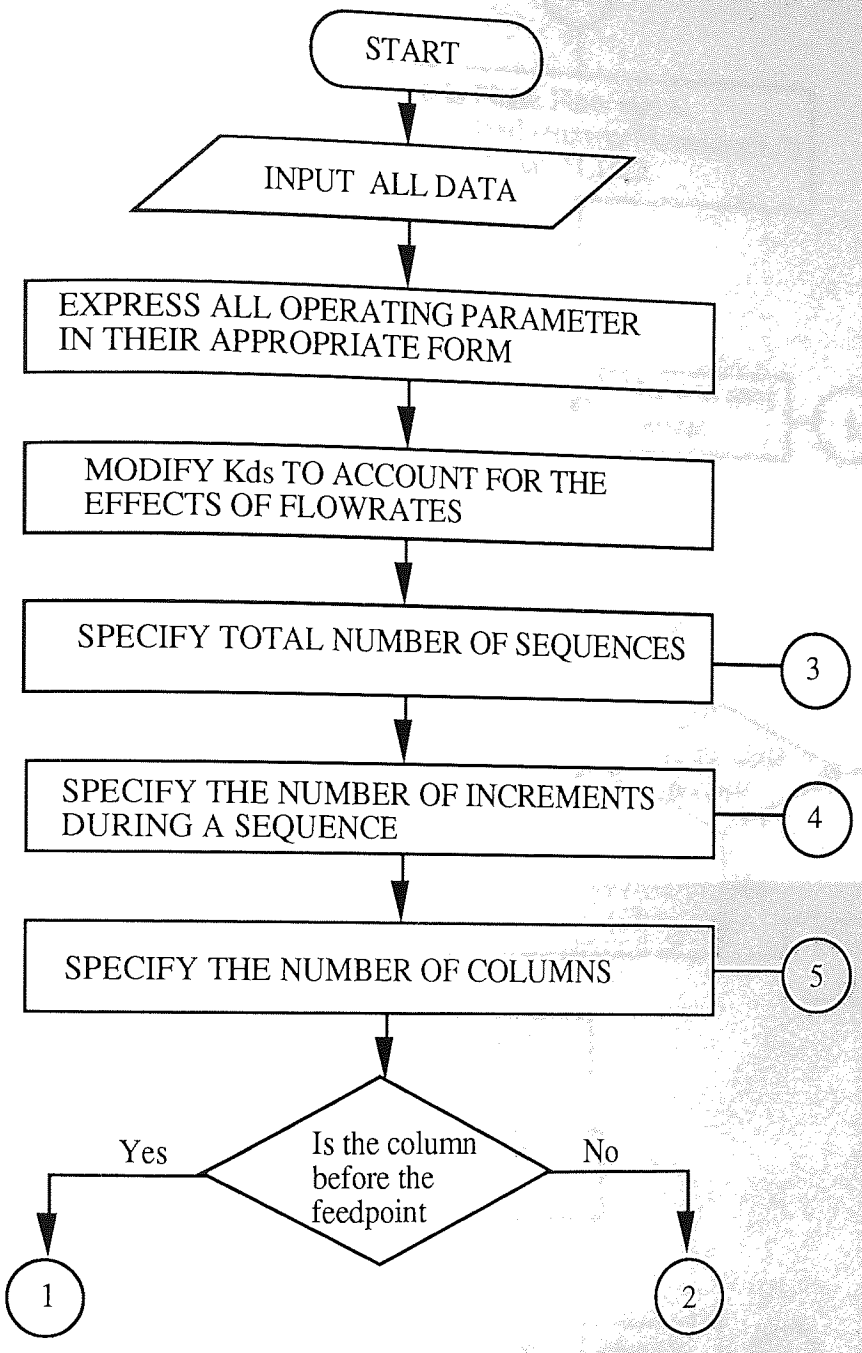


Figure 9.1: Computer programme flowchart for the simulation of the SCCR-S system for the continuous hydrolysis of lactose

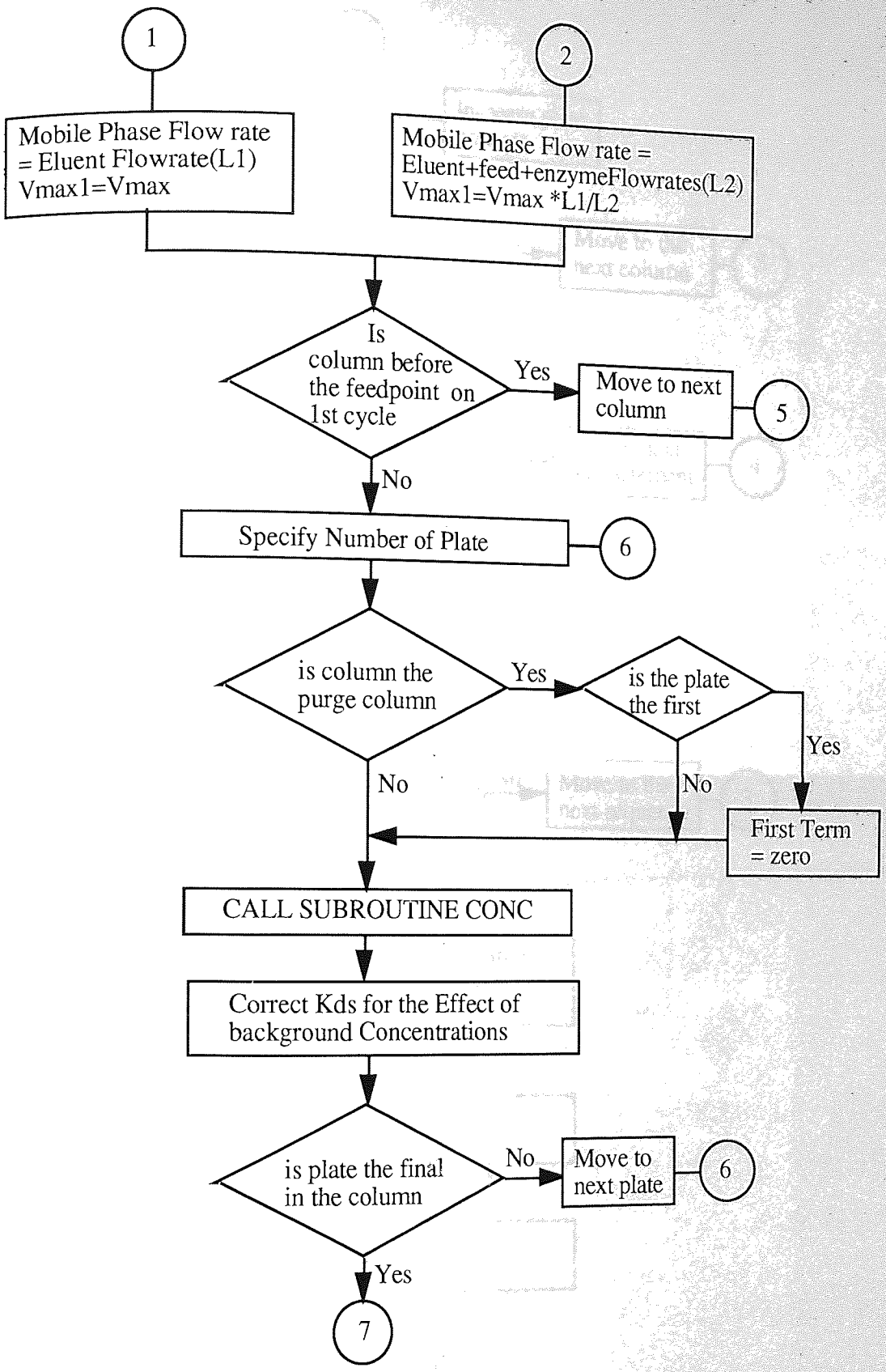


Figure 9.1: Continued

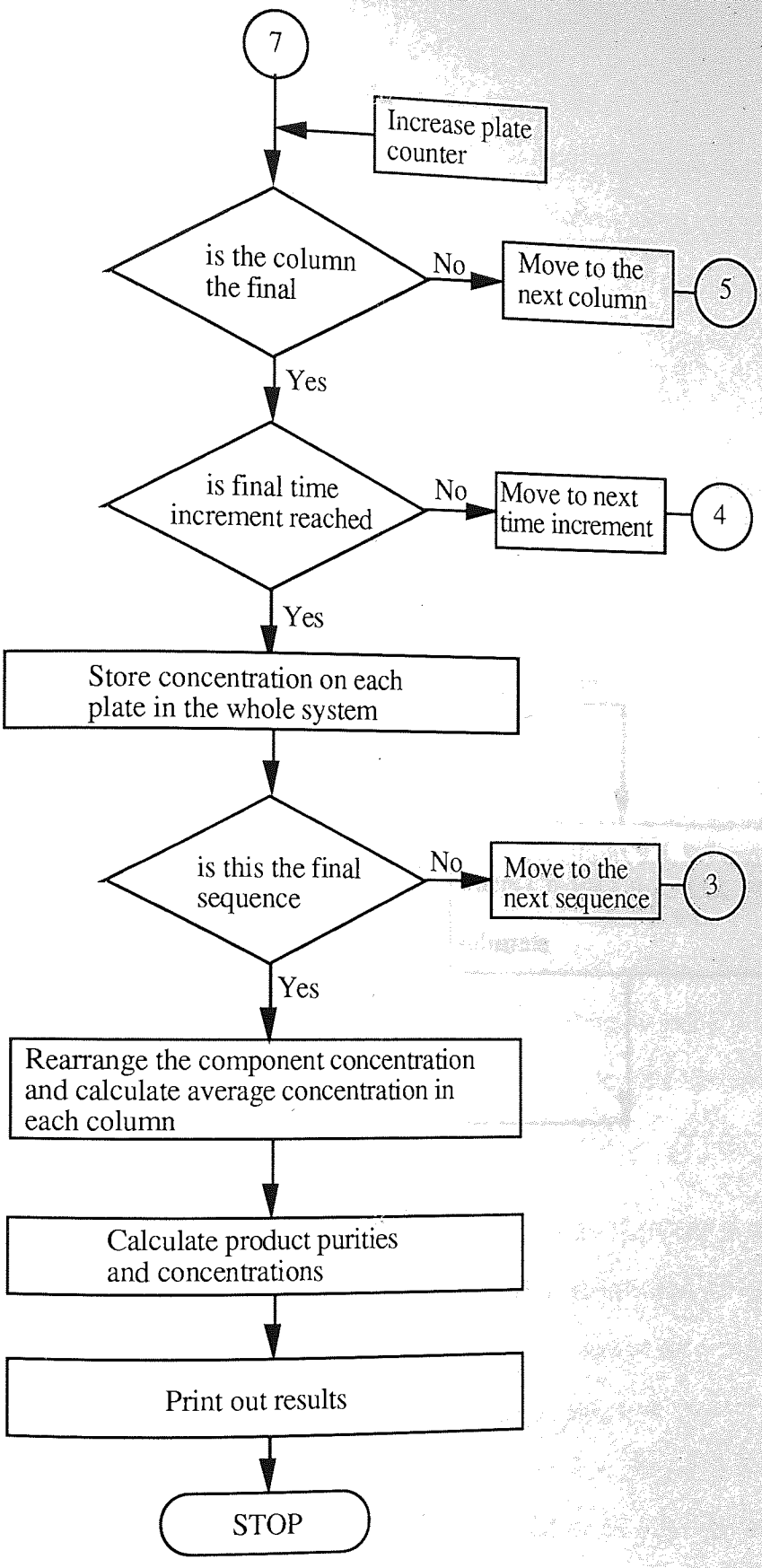


Figure 9.1: Continued

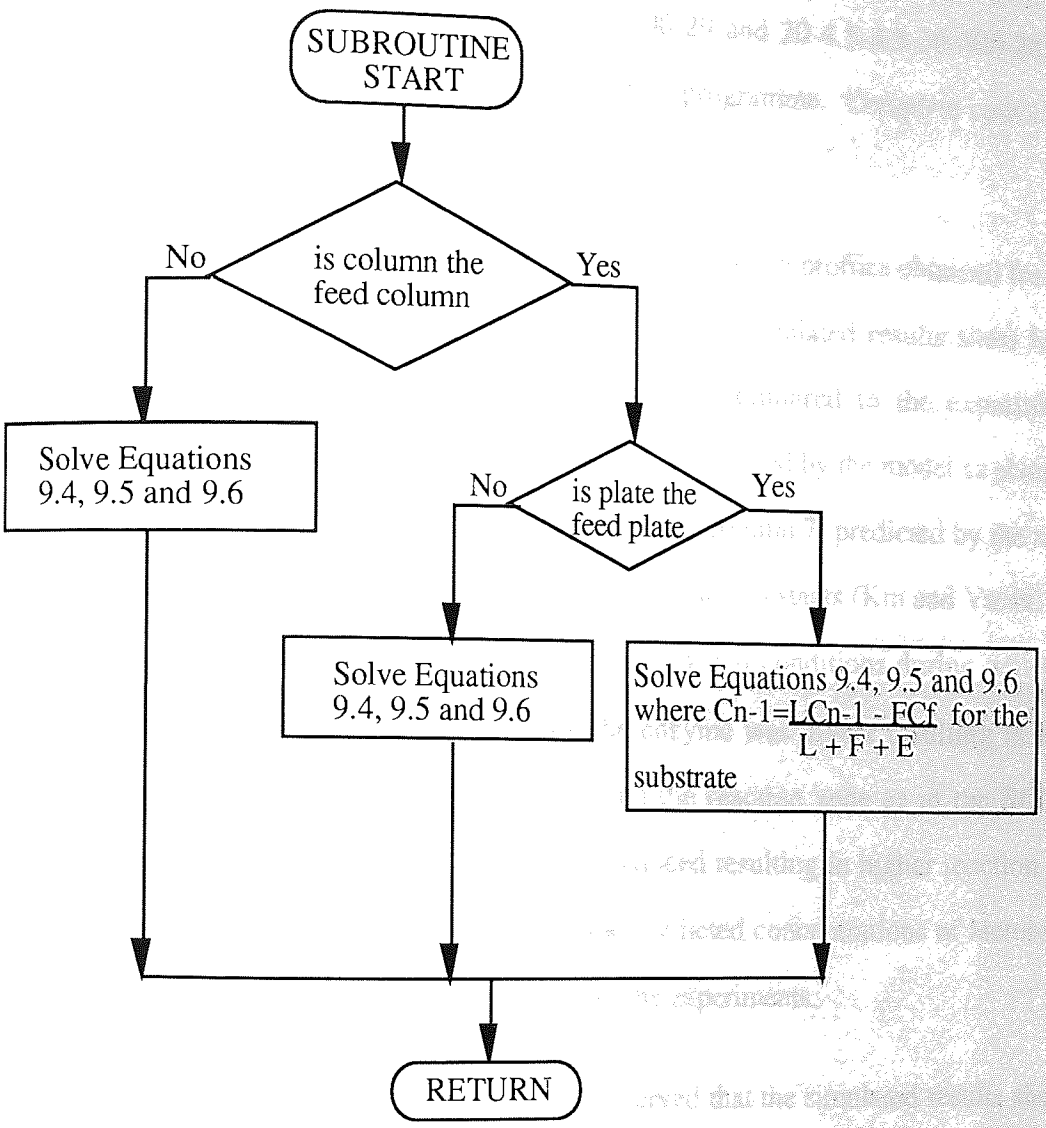


Figure 9.1: Continued

9.5 SIMULATION RESULTS AND DISCUSSION

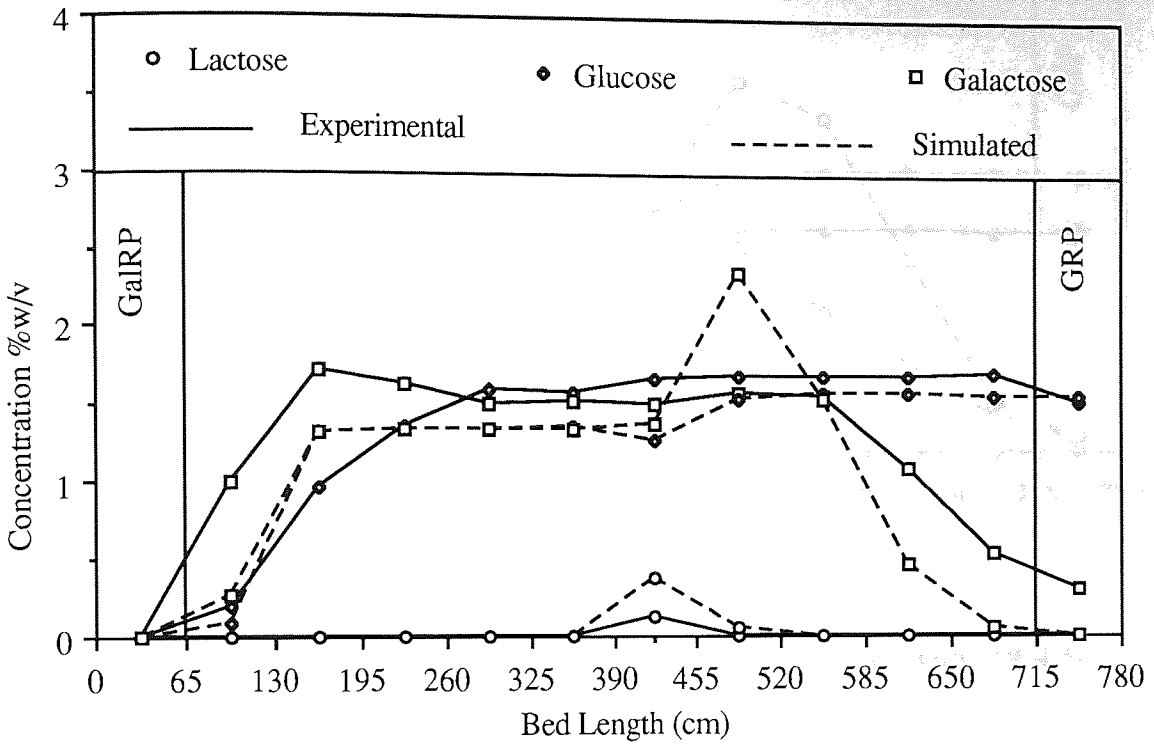
The simulation results were compared to the experimental on-column concentration profiles under various operating conditions. The experimental conditions for runs 5.7-4.5-4.5-30-200-27.5, 10-4.5-4.5-30-300-28, 15-4.5-4.5-28-300-29 and 20-4.5-4.5-30-300-28 have been used to examine the accuracy of the simulation programme. Complete conversions were achieved during all these runs.

Figures 9.2, 9.3, 9.4 and 9.5 show the on-column concentration profiles obtained from the simulation compared to the experimental profiles. The simulated results show higher concentrations of galactose in column 8 (552.5cm) compared to the experimental concentrations. This is due to the lower reaction rates predicted by the model as shown by the higher concentration of lactose in the feed columns (column 7) predicted by the model compared to the experimental concentrations. The kinetic constants (Km and Vmax) used in the simulation were obtained under batch experimental conditions during which the inhibition effect of the galactose product on the enzyme was higher resulting in lower reaction rates. By separating the galactose from the reaction zone as in the SCCR-S system, the inhibition effect of the galactose is reduced resulting in higher reaction rates than for a batch reaction. This explains why the predicted concentrations of lactose and galactose in columns 7 and 8 are higher than from the experiments.

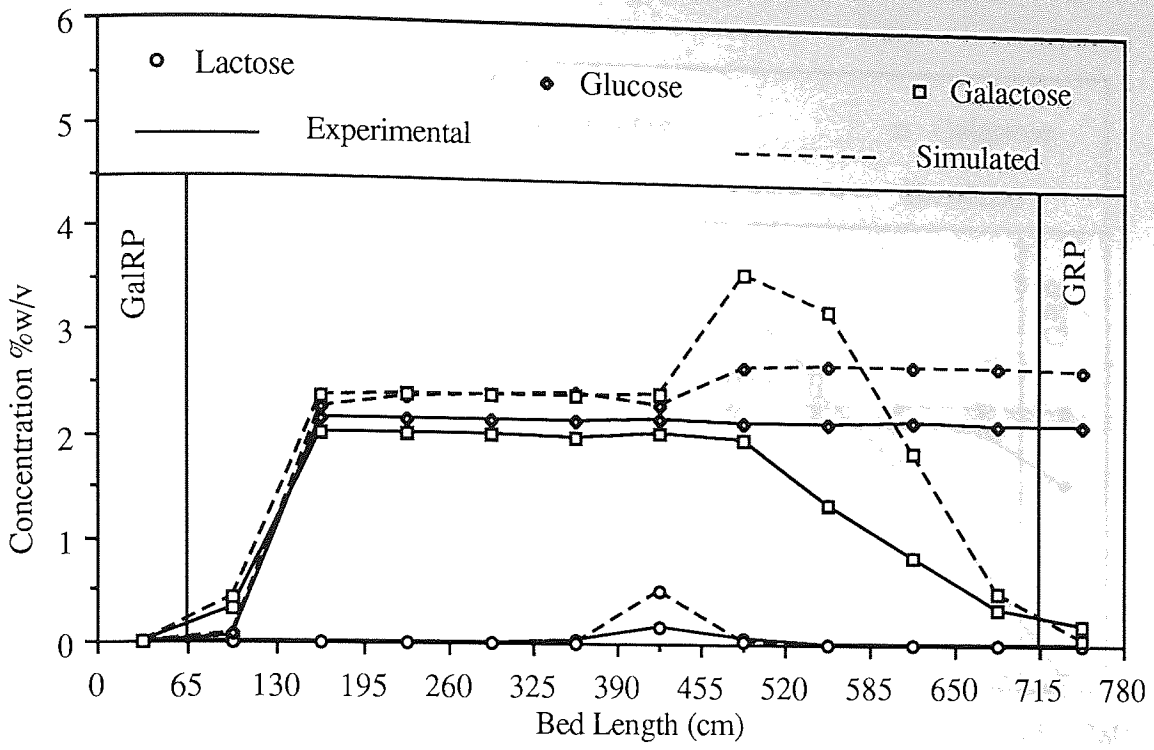
From Figures 9.2, 9.3, 9.4 and 9.5, it was also observed that the simulated results showed better separation performance in the post-feed Section of the system than the experimental results indicated. These differences can be attributed to the effect of the protein (enzyme) background concentration on the distribution coefficients of the components in the system which is not accounted for in the simulation programme. It is also open to discussion whether the assumption that the number of plates calculated for the individual components during the batch mode characterisation is identical to that in the continuous mode.

The simulation programme has been shown to predict the trend of the on-column concentration profile quite well, however, the accuracy of the model could be improved if

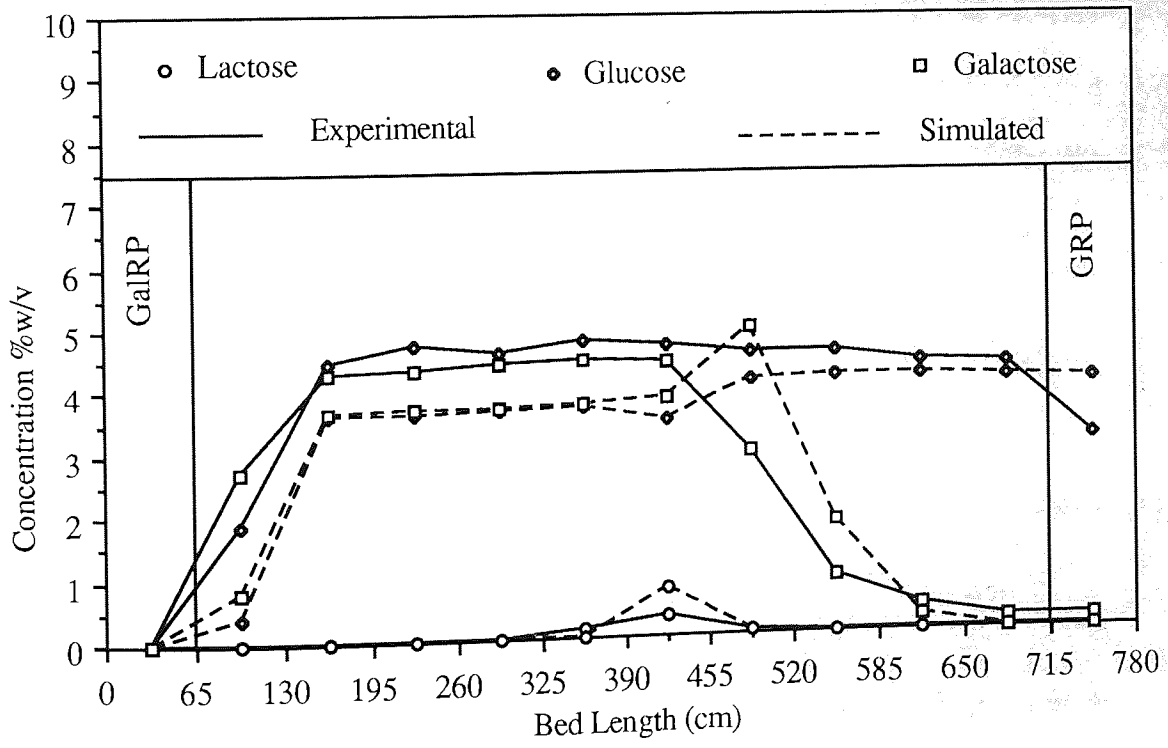
e actual kinetic constants in the system are used and the effect of the enzyme background ɔncentration on the distribution coefficients accounted for in the simulation.



igure 9.2: Simulated On-Column Concentration Profile for Run 5.7-4.5-.5-30-200-27.5 Cycle 8



gure 9.3: Simulated On-Column Concentration Profile for Run 10-4.5-5-30-300-28 Cycle 7



'igure 9.4: Simulated On-Column Concentration Profile for Run 15-4.5-.5-28-300-29 Cycle 14

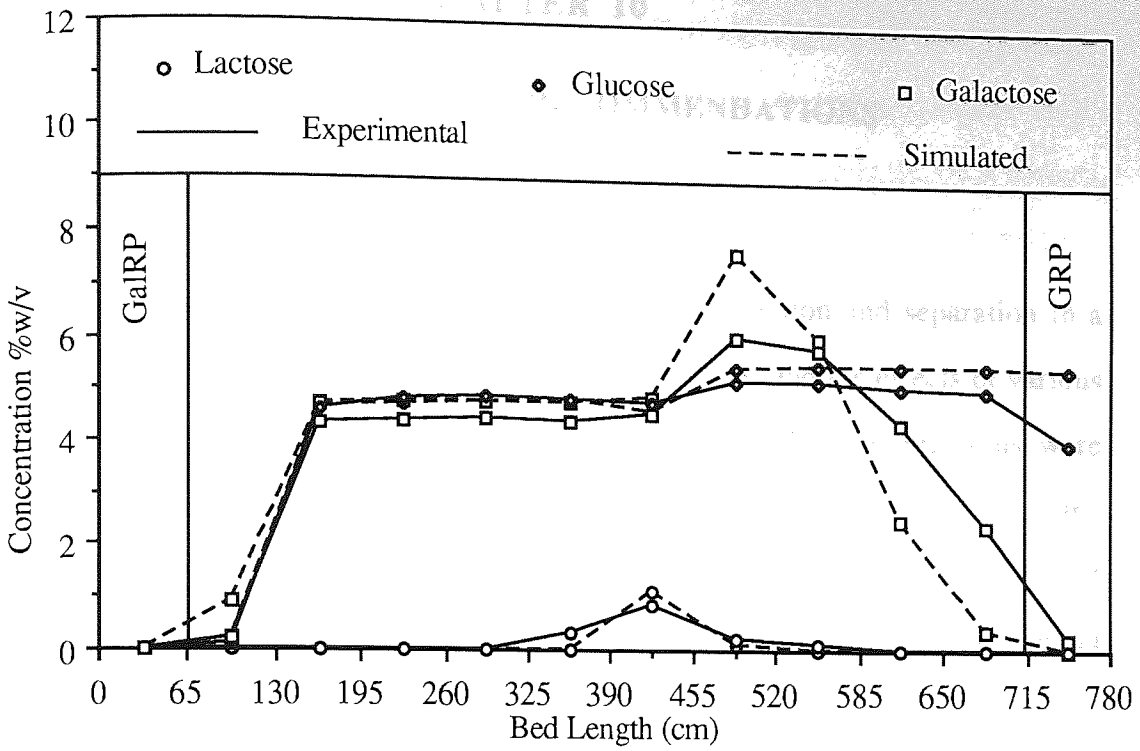


Figure 9.5: Simulated On-Column Concentration Profile for Run 20-4.5-.5-30-300-28 Cycle 8

CHAPTER 10

CONCLUSIONS AND RECOMMENDATIONS

10.1 CONCLUSIONS

The aim of this work was to study the integration of bioreaction and separation in a simulated counter-current chromatographic system, (SCCR-S) and the effects of various operating parameters on the performance of the system. Three bioreactions were considered, namely, the saccharification of modified starch to maltose using the enzyme maltogenase, the hydrolysis of lactose in the presence of lactase and the biosynthesis of dextran using dextransucrase. The main conclusions from this work are summarised in this chapter.

10.1.1 The Saccharification of Modified Starch to Maltose Using the Enzyme Maltogenase

1) The bioreaction and separation results of the continuous saccharification of modified starch to maltose have shown that it is possible to carry out combined bioreaction and separation in a SCCR-S system. The eluent was a dilute enzyme solution. The maltose and small amounts of glucose produced were retarded and moved with the stationary phase to be purged as the maltose rich product (MRP) while the unconverted starch, dextrin, moved with the mobile phase to be collected as the dextrin rich product (DRP).

2) The switch time was found to affect the performance of the SCCR-S system. Changes in the switch time affected the residence time and the concentration profiles of the components in the system. An increase in switch time increased the residence time and the maltose concentration profile was higher in the post-feed section. This led to an increase in the MRP purities and a reduction in the DRP purities.

(3) The eluent flowrate was found to have similar effects on the performance of the SCCR-S as the switch time. Increasing the eluent flowrate reduced the residence time resulting in a reduction in substrate conversion. At higher eluent flowrates, the MRP purities were improved and the product concentration reduced while the DRP purities were reduced and the product concentration increased. The eluent flowrate required was found to depend on the switch time related by the ratio Lm/P (where Lm and P are the mobile and stationary phase flowrates respectively).

(4) Increasing the feed concentration was found to significantly increase the operating pressure due to increased viscosity and this caused a reduction in the MRP purities. The increaseed viscosity in the system caused more dextrin to be retained in the pre-feed Section and also resulted in lower conversions due to poor mixing of the enzyme with the starch substrate. These effects coupled with the larger amounts of glucose produced at higher feed concentrations resulted in poorer MRP purities.

(5) The dextrose equivalent (DE) of the starch feed was found to affect the conversion of starch to maltose in the system. A starch with a lower DE was found to be more suitable for the SCCR-S system in terms of conversion although with a feed of lower DE the operating pressure was higher.

(6) The addition of pullulanase, a debranching enzyme was found to improve the conversion with more glucose being produced, which resulted in a reduction of the MRP purities.

(7) The amounts of enzyme required by the SCCR-S system was found to be lower than the theoretical amounts of enzyme required by a conventional bioreactor over the same time period. During runs 9.5-9-24-30-19.5 and 20.9-9-24-60-19, the SCCR-S required only 46.0% and 42.8% respectively of the theoretical amount of enzyme required by a conventional bioreactor. This confirms the advantages of the SCCR-S system when used as a combined bioreactor-separator in minimising substrate or product inhibition.

8) A product splitting technique was used to improve the MRP concentrations. The dilute fractions of the MRP stream were recycled as the purge stream. Employing this technique, the MRP product concentrations were increased. During run 14.78-9-25.5-60-21, the MRP concentration was increased from 0.88%w/v to 1.88%w/v.

9) The SCCR-S system was compared to the continuous annular chromatograph (CRAC) system used for the saccharification of modified starch using the enzyme maltogenase. The SCCR-S required only 34.6-47.3% of the amount of enzyme required by the CRAC system. Higher conversions were also obtained with the SCCR-S system. The ability of the CRAC system to handle multicomponent mixtures made it possible for the CRAC to produce maltose of higher purities by separating the maltose from the small amounts of glucose produced.

10.1.2 Continuous and Batch Operation of the SCCR-S System for the Hydrolysis of Lactose

(10) The SCCR-S system was successfully used for the continuous hydrolysis of lactose using the enzyme, lactase, from *Aspergillus oryzae* and simultaneously separating the products, galactose and glucose. Unlike for the saccharification of modified starch, it was found to be more efficient to introduce the enzyme and the feed through the same port into the system during the hydrolysis of lactose. Complete conversions were achieved at feed throughputs of 4.5Kg/h/m^3 and feed concentrations of 30%w/v. Galactose rich (GalRP) and glucose rich product purities (GRP) of 77.1% and 99.9% respectively were obtained.

(11) The SCCR-S system required less enzyme compared to the theoretical amount of enzyme that is required by a conventional reactor to hydrolyse the same amount of lactose over the same time period. This again confirmed the advantage of the SCCR-S system to minimise substrate or product inhibition when used as a combined bioreactor-separator. During runs 5.1-4.5-4.5-30-100-26, 15-4.5-4.5-28-300-29, 20-

4.5-4.5-30-300-28 and 32-4.5-4.5-30-300-28 the SCCR-S required only 71%, 72%, 54% and 34% respectively of the theoretical amount of enzyme required to achieve complete hydrolysis of the same amount of lactose over the same time period.

12) The operating parameters, eluent flowrate, switch time, feed concentration and enzyme activity were shown to affect the performance of the SCCR-S system when used for the continuous hydrolysis of lactose. Increasing the eluent flowrate and switch time had positive effects on the galactose rich product (GalRP) at the expense of the glucose rich product (GRP) while increasing the feed concentration had a negative effect on the separation capability of the SCCR-S system.

13) The SCCR-S system was modified and operated in the batch mode as a combined bioreactor-separator for the hydrolysis of lactose. The operating parameters such as eluent flowrate, feed concentration and pulse size were shown to affect the performance of the SCCR-S system operating in the batch mode. An increase in the amount of enzyme used and a reduction in the separation of the products were observed at high eluent flowrates, feed concentrations and pulse sizes.

(14) In comparing batch and continuous operation of the SCCR-S system as a combined bioreactor-separator, the throughputs in the continuous mode were 2.3, 2.6 and 5 times of those in the batch mode at feed concentrations of 10, 20 and 30%w/v respectively.

(15) The SCCR-S system operating in the continuous mode required only between 32-51% of the amount of enzyme required by the system operating in the batch mode. This confirms the effective use of the chromatographic bed when the system is operated in the continuous mode.

(16) The SCCR-S system operating in the batch mode was found to produce products of higher concentrations. However, carrying out product splitting and recycling the dilute fractions could improve the product concentrations when the SCCR-S is operated in the continuous mode.

17) Notable advantages of the SCCR-S system operating in the continuous mode are the reproducibility of the product quality, the flexibility and the fact that no recycle is necessary.

18) The computer simulation for the SCCR-S system operating in the continuous mode as a combined bioreactor-separator for the hydrolysis of lactose has been successfully carried out. The simulation was based on the theoretical plate model incorporating the Michaelis-Menten enzyme kinetic model. The non-linear ordinary differential equations obtained from mass balances were solved by the Fourth-Order Runge-Kutta method. Correlations were incorporated in the simulation programme to account for the effects of flowrates and background concentrations on the distribution coefficients. The model was capable of predicting the on-column concentration profiles of lactose, glucose and galactose and the effects of various operating parameters on the concentration profiles and product purities.

10.1.3 The Biosynthesis of Dextran Using The SCCR-S System

(19) The production and purification of the enzyme dextransucrase from *Leuconostoc mesenteroides* strain NRRL B512F required for the biosynthesis of dextran experiments was successfully carried out. The enzyme purification results showed that it was possible to produce high purity dextransucrase on a large scale with an overall recovery of 65.9% by centrifugation and ultrafiltration techniques. Several methods of desalting the enzyme including PEG precipitation and gel filtration were investigated but were not deemed to be suitable due to the high enzyme losses.

(20) Initial results obtained using the SCCR-S system for the biosynthesis of dextran showed that it was not possible to operate continuously. This was due to loss of separation caused by displacement of the calcium ions on the resin by potassium and sodium ions in the enzyme solution and the formation of unwanted glucose. The fructose rich product (FRP) obtained was also contaminated with dextran and other

high molecular weight material in the enzyme solution when the eluent used was a dilute enzyme solution.

21) By employing a method of on-line regeneration of the resin with a 10%w/v calcium nitrate at high pH and introducing the enzyme into the system through the same port as the feed while using deionised water as the eluent, it was possible to operate continuously with fructose rich products of up to 100% being produced, although the formation of the unwanted glucose was not inhibited.

22) Acid hydrolysis tests and NMR analysis showed that the cause of glucose formation during the biosynthesis of dextran in the SCCR-S system was due to the formation of levan (a poly-fructose) releasing glucose as the byproduct.

10.2 RECOMMENDATIONS

The application of the SCCR-S system as a combined bioreactor-separator looks promising and therefore further research is required to ensure that this process is commercially viable. From the results obtained during this project, the following recommendations have been made:

(1) Carry out more bioreaction-separation experiments using a larger particle size resin with better selectivity between maltose and dextrin. This is required to reduce the operating pressure encountered at high starch feed concentrations.

(2) Carry out more bioreaction-separation work on the SCCR-S system for the hydrolysis of lactose using a stationary phase with higher selectivity between glucose and galactose. This will improve the product purities and concentrations.

(3) Further studies should be carried out to try and minimise or eliminate the formation of unwanted levan and glucose during the biosynthesis of dextran in the SCCR-S system.

4) The possibility of using an immobilised enzyme in the SCCR-S system should be investigated to reduce the consumption of flowing enzyme. This could be done by either immobilising the enzyme directly on to the stationary phase or on to a suitable carrier.

5) The purification of dextransucrase by affinity chromatography needs to be investigated on a large scale. This might aid in desalting the enzyme, remove the dextran in the enzyme solution and also eliminate side reactions.

6) More experimental work should be carried out to investigate the effects of background protein concentration on the distribution coefficients of the components used in this work.

7) The mathematical model and computer simulation programme should be modified to include non-linear adsorption isotherms and the effects of background protein concentration on the distribution coefficients.

8) A feasibility study and an economic evaluation needs to be carried out to estimate the commercial viability of the SCCR-S system as a bioreactor-separator.

9) A universal performance criterion should be developed as a basis of comparing chromatographic bioreactor-separators and conventional bioreactors with a subsequent separation step.

NOMENCLATURE

b	calibration constant for the GPC
Cm'	resistance to mass transfer in the mobile phase
Cs'	resistance to mass transfer in the stationary phase
D	polydispersity
d	dilution factor
DE	dextrose equivalent
DSU	dextransucrase unit
e	natural logarithm
FRP	fructose rich product
GalRP	galactose rich product
GRP	glucose rich product
h	peak height (cm)
HETP	height equivalent to a theoretical plate
Kdi	distribution coefficient of component i
Ki	enzyme inhibition constant
Km	Michaelis-Menten constant (g/cm^3)
$K^{\infty}di$	distribution coefficient of component i at infinite dilution
L1	eluent flowrate (cm^3/min)
L3	purging flowrate
Le	mobile phase flowrate (cm^3/min)
Mn	number average molecular weight
MRP	maltose rich product
Mw	weight average molecular weight
N	number of theoretical plates
N*	apparent number of theoretical plates
P	stationary phase effective flowrate (cm^3/min)
r	reaction rate
Rs	Resolution of a chromatographic column
S	switch time (min)
t	time (min)
t_{Ri}	retention time of component i (min)
U	enzyme activity unit
V_1	plate volume of mobile phase (cm^3)
V_2	plate volume of stationary phase (cm^3)
Vmax	maximum initial reaction velocity ($g/cm^3/min$)
Vo	void volume (cm^3)

V_{Ri}	retention volume of component i (cm^3)
V_S	volume of stationary phase (cm^3)
V_T	total empty column volume (cm^3)
$W^{h/e}$	peak width at height h/e
W_i	peak width at base of elution curve of component i
X_i	component i concentration on stationary phase (g/cm^3)
Y_i	component i concentration in the mobile phase (g/cm^3)

Greek letters

ε	voidage
ω	rotation rate
θ	angular coordinate
μ	viscosity
$\delta 1$	fractional changes due to effect of background concentration on distribution coefficient
$\delta 2$	fractional changes due to effect of system characterisation on distribution coefficient

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APPENDIX A

Data Aquisition Programme for the GPC Columns

```
1Ø   DIM A(4ØØ),Z(5)
6ØØØ I=1:T1=TI:Y=1
6Ø1Ø GO SUB 7ØØØ
6Ø2Ø E=TI-T1
6Ø3Ø IF E<12Ø THEN 6Ø2Ø
6Ø4Ø IF Y=5 THEN 6Ø9Ø
6Ø5Ø Y=Y+1:T1=TI
6Ø6Ø GO TO 6Ø1Ø
6Ø9Ø T1=TI
61ØØ A(I)=Z(I)+Z(2)+Z(3)+Z(4)+Z(5))/5
61Ø5 PRINT"READING";I/6;"=";A(I),A(I)/4
651Ø IF I<36Ø THEN 675Ø
655Ø FOR Q=156 TO I
656Ø OPEN 1,4,1
657Ø OPEN 2,4,2
658Ø FS="9999.99      S9999.99         S9999.99"
659Ø PRINT#2,FS
66ØØ PRINT#1,Q/6,A(Q),A(Q)/4
661Ø CLOSE1:CLOSE2
662Ø NEXT Q
665Ø END
675Ø Y=1.Ø:I=I+1
676Ø GO TO 6Ø1Ø
7ØØØ OPEN 1,9,1
7Ø1Ø GET#1,J$,K$
7Ø2Ø IF K$="" THEN K=-224:GO TO 7Ø4Ø
7Ø3Ø K=ASC(K$)-224
7Ø4Ø IF K<Ø THEN D=(K+32)*-1
7Ø5Ø IF K>=Ø THEN D=K
7Ø6Ø D=D*256
7Ø7Ø IF J$="" THEN J=Ø:GO TO 7Ø9Ø
7Ø8Ø J=ASC(J$)
7Ø9Ø IF K<Ø THEN J=J*-1
71ØØ Z(Y)=J+D
71Ø5 PRINT Z(Y),Z(Y)/4
711Ø CLOSE1
712Ø RETURN
```

Computer Programme for Calculating the Average Molecular Weights and Molecular Weight Distributions from GPC Data

```
5 REM PROGRAM TO CALCULATE THE MOL
10 REM WEIGHT DISTRIBUTION OF A DEXTRAN SAMPLE
12 REM
15 REM
17 REM
20 DIM A(400),Z(5),B(170),WIN(170),
   S(170),SS(170),MAX(2),AMIN(170)
40 X=0.0
60 PRINT "RUN A VO-VT...YES OR NO"
80 INPUT ANS$
100 IF ANS$="YES" THEN X=1:GOTO 320
120 PRINT "THEN INPUT TO AND TT"
140 INPUT MAX(1),MAX(2)
150 X=2.0
160 PRINT "INPUT THE FLOW RATE"
180 INPUT RATE
200 PRINT "TYPE IN THE BATCH NO"
220 INPUT KR$
240 PRINT "TYPE IN DATE"
260 INPUT KP$
280 PRINT "READY TO START TYPE...GO"
300 INPUT NUM$
320 I=1:T1=TI:Y=1
340 GOSUB 1570
360 E=TI-T1
380 IF E<120 THEN 360
400 IF Y=5 THEN 460
420 Y=Y+1:T1=TI
440 GOTO 340
460 T1=TI
480 A(I)=(Z(1)+Z(2)+Z(3)+Z(4)+Z(5))/5
500 PRINT "READING AT";I/6;"MINS =";A(I)/4
520 IF I>=210 THEN 580
540 Y=1.0:I=I+1
560 GOTO 340
580 IF X=1.0 THEN GOSUB 1380
600 IF X=0.0 THEN 150
610 GOSUB 3140
620 GOSUB 2140:GOSUB 2520
640 OPEN1,4
660 OPEN2,4,1
680 OPEN3,4,2
700 F$="999  9.999  9999999  9999.99  99.99  99.99"
720 PRINT#3,F$
740 PRINT#1,CHR$(1)"BATCH NUMBER:";KR$
760 PRINT#1:PRINT#1
780 PRINT#1,CHR$(1)"DATE OF ANALYSIS:";KP$
800 PRINT#1:PRINT#1
820 PRINT#1,"FLOWRATE=";RATE;"ML/MIN"
840 PRINT#1,"     VO=";MAX(1)
860 PRINT#1,"     VT=";MAX(2)
880 PRINT#1,"     VS=";BEGIN
900 PRINT#1,"     VF=";FINISH
920 PRINT#1
```

```
940 PRINT#1,"WEIGHT AVERAGE MOL WT="AAAW
960 PRINT#1,"NUMBER AVERAGE MOL WT="AVMN
1000 PRINT#1,"            MW/MN RATIO="SPR
1020 PRINT#1:PRINT#1:PRINT#1
1040 HD$="POINT"+"       "+"KD VALUE"+"       "
1060 GH$="MOL WEIGHT"+"      "
1080 FH$="HEIGHT"+"       "+" WT FRAC "+"    CUMM%"
1100 EH$=HD$+GH$+FH$
1120 PRINT#1,EH$
1140 FOR I=NMAX TO 1 STEP -1
1160 PRINT#2,I,VIN(I),AMIN(I),B(I),S(I),SS(I)
1180 NEXT I
1200 PRINT#1:PRINT#1:PRINT#1
1220 PRINT#1,"ASTON GPC CONSTANTS"
1240 PRINT#1,"          B1=";B1
1260 PRINT#1,"          B2=";B2
1280 PRINT#1,"          B3=";B3
1300 PRINT#1,"          B4=";B4
1320 PRINT#1,"          B5=";B5
1340 CLOSE 1:CLOSE 2:CLOSE 3
1345 GOSUB 3300
1355 GOTO 200
1360 END
1380 REM  TO FIND TO AND TT TIMES
1400 Z=1.0
1420 FOR I=30 TO 210
1440 IF A(I)<200 THEN 1500
1460 IF A(I)>=A(I-1) THEN MAX(Z)=I/6:GO TO 1500
1480 Z=2.0
1500 NEXT I
1510 PRINT "   "
1520 PRINT "TO=";MAX(1);"TT=";MAX(2)
1530 PRINT "   "
1535 GOTO 3140
1540 X=0.0
1560 RETURN
1570 REM HEIGHTS FROM REFRACTOMETER
1580 OPEN1,9,15
1600 GET#1,J$,K$
1620 IF K$="" THEN K=-224:GOTO 1660
1640 K=ASC(K$)-224
1660 IF K<0 THEN D=(K+32)*-1
1680 IF K>=0 THEN D=K
1700 D=D*256
1720 IF J$="" THEN J=0:GOTO 1760
1740 J=ASC(J$)
1760 IF K<0 THEN J=J*-1
1780 Z(Y)=J+D
1800 PRINT Z(Y),Z(Y)/4
1820 CLOSE 1
1840 RETURN
2130 REM CALC OF ELUTION VOLUMES
2145 VIE=RATE*BEGIN
2160 VFE=RATE*FINISH
2180 VI=(VIE-(RATE*MAX(1)))/(RATE*(MAX(2)-MAX(1)))
2200 VF=(VFE-(RATE*MAX(1)))/(RATE*(MAX(2)-MAX(1)))
2220 VH=(VF-VI)/(NMAX-1)
```

```
2240 RETURN
2520 REM CALC OF MWD
2540 B1=-8.971:B2=5.122:B3=-3.637:B4=14.481:B5=-911.858
2560 S1=0.0:S2=0.0:S3=0.0
2580 FOR I=1 TO NMAX
2600 VIN(I)=VI+VH*(I-1)
2620 IF VIN(I)>1.0 THEN 2680
2640 IF VIN(I)<0.0 THEN 2720
2660 AMIN(I)=B5+EXP(B4+B1*VIN(I)+B2*(VIN(I)↑2)
     +B3*(VIN(I)↑3)):GOTO 2740
2680 AMIN(I)=B5+EXP(B4+B1+B2+B3)
2700 GOTO 2740
2720 AMIN(I)=B5+EXP(B4)
2740 S1=S1+B(I):S2=S2+(AMIN(I)*B(I))
2760 S3=S3+(B(I)/AMIN(I)):NEXT I
2780 AAAW=S2/S1
2800 AVMN=S1/S3
2820 SPR=AAAW/AVMN
2840 PRINT "WEIGHT AVERAGE MOL WT=";AAAM
2860 PRINT "NUMBER AVERAGE MOL WT=";AVMN
2880 PRINT "              MW/MN RATIO";SPR
2900 S4=0.0
2920 FOR I=1 TO NMAX:S4=S4+B(I):NEXT I
2940 FOR I=1 TO NMAX:S(I)=B(I)*100/S4:NEXT I
2960 S5(NMAX+1)=0.0
2980 FOR I=NMAX TO 1 STEP -1
3000 S5(I)=S5(I+1)+S(I)
3020 NEXT I
3040 RETURN
3140 FOR I=30 TO 210
3160 OPEN 1,4,1
3180 OPEN 2,4,2
3200 F$="9999.99     $9999.99      $9999.99"
3220 PRINT#2,F$
3240 PRINT#1,I/6,A(I),A(I)/4
3260 CLOSE1:CLOSE2
3280 NEXT I
3290 IF X=1.0 GOTO 1540
3300 PRINT " IF DATA IS GOOD, TYPE Y"
3320 PRINT " ELSE TYPE ANY OTHER"
3340 PRINT " LETTER"
3360 INPUT ROP$
3380 IF ROP$="Y" THEN 3640
3400 REM
3420 PRINT "TYPE IN THE START AND FINISH TIMES"
3440 INPUT AB,BC
3445 PRINT "INPUT BASELINE"
3450 INPUT SJS
3460 NMAX=0.0:SAP=0.0
3535 IF A(BC)<0.0 THEN A(BC)=0.0
3540 FOR I=AB TO BC
3550 NMAX=NMAX+1
3560 IF 78>=I THEN B(NMAX)=(A(I)-A(48))/4:GOTO 3600
3580 B(NMAX)=(A(I)-SJS)/4
3600 NEXT I
3620 BEGIN=AB/6:FINISH=BC/6
3630 GOTO 620
3640 RETURN
```

APPENDIX B

B1 Calculation of Enzyme Usage During a Standard Run for the Production of Maltose on the SCCR-S System.

1 Unit of the enzyme maltogenase is defined as the amount of enzyme required to produce 1μmol of maltose in 1 minute at pH 5.3 and a temperature of 60°C.

I.e I Unit ———> 3.42×10^{-6}g maltose in 1 minute

Experimental Conditions:- Run 9.5-9-27-30-19.5

Enzyme activity used = 30 U/cm^3
Volumetric rate of enzyme fed into the system = 27 cm^3/min.
Actual amount of enzyme used = 30 x 27 = 810 U/min.

Concentration of maltose in the maltose rich product = 0.0057g/cm^3
Volume of the maltose rich product collected in 1 minute = 76cm^3
Concentration of maltose in the dextrin rich product = 0.0018g/cm^3
Volume of the dextrin rich product collected in 1 minute = 36cm^3

Total amount of maltose produced = 0.0057 x 76 + 0.0018 x 36 = 0.498g/min.

Therefore, theoretical amount of enzyme required in 1 minute $= \frac{0.498}{3.42 \times 10^{-4}}$

$= 1456.14$ U

$$\text{ENZYME USAGE} = \frac{\text{Actual amount of enzyme used}}{\text{Theoretical amount of enzyme required}}$$

$$= \frac{810}{1456.14} = 0.556$$

B2 Calculation of Enzyme Usage During Continuous Hydrolysis of Lactose in the SCCR-S System

1 Unit of the lactase enzyme is defined as the amount of enzyme required to produce 1μmol of glucose in a minute at pH 5.0 and a temperature of 55°C.

I.e 1Unit ———> 1.8×10^{-4} g of glucose in a minute

Experimental Conditions:- 30-4.5-4.5-30-400-28

Enzyme activity used = 400U/cm^3

Volumetric rate of enzyme fed into the system = 4.5cm^3/min.

Actual amount of enzyme used = 4.5 x 400 = 1800U

Concentration of glucose in the glucose rich product = 0.0122g/cm^3

Volume of glucose rich product collected in 1 minute = 39cm^3

Concentration of glucose in the galactose rich product = 0.0026g/cm^3

Volume of galactose rich product collected in 1 minute = 76cm^3

Total amount of glucose produced = 0.0122 x 39 + 0.0026 x 76 = 0.6734g/min.

Therefore, theoretical amount of enzyme required in 1 minute = $\frac{0.6734}{1.80 \times 10^{-4}}$

= 3741.1 U

$$\text{ENZYME USAGE} = \frac{\text{Actual amount of enzyme used}}{\text{Theoretical amount of enzyme required}}$$

$$= \frac{1800}{3741.1} = 0.48$$

B3 Calculation of Enzyme Usage During the Hydrolysis of Lactose in the SCCR-S System Operating in the Batch mode

Experimental Conditions:- 20-2-25-45

Enzyme activity used = 45U/cm^3

Volumetric rate of enzyme fed into the system = 25cm^3/min.

Elution time (T1-T0) = 210 mins.

Actual amount of enzyme of enzyme used = 210 x 25 x 45 = 236250U

Total amount of lactose injected = 360 x 0.20 = 72g

For a complete conversion, amount of glucose produced = 36g = 2 x 10^5 µmols

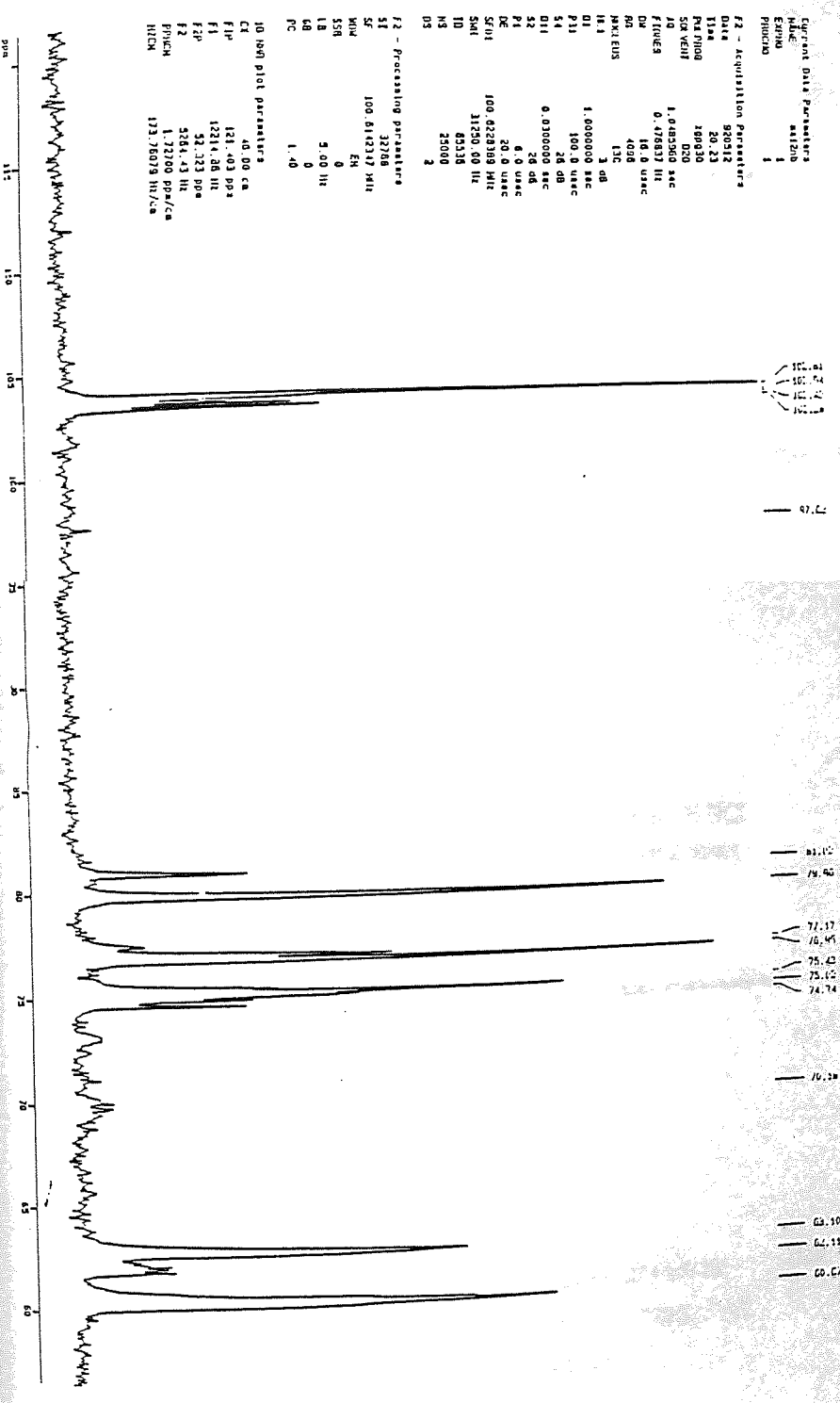
From the enzyme unit defination, theoretical amount of enzyme required = 2 x 10^5 U

$$\text{ENZYME USAGE} = \frac{\text{Actual amount of enzyme used}}{\text{Theoretical amount of enzyme required}}$$

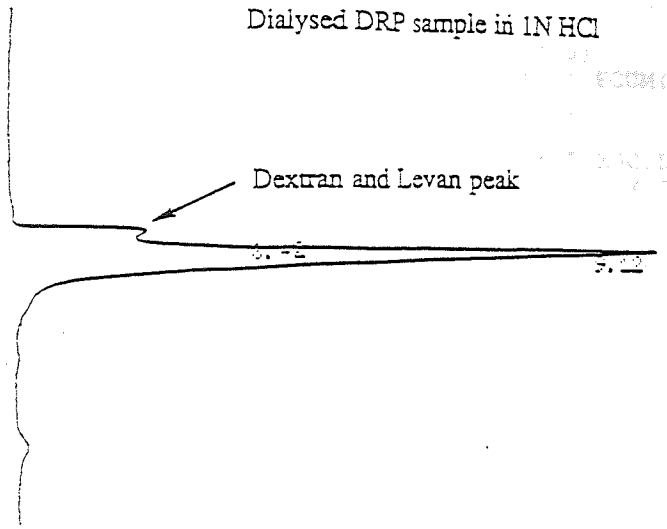
$$= \frac{236250}{2 \times 10^5} = 1.18$$

APPENDIX C

C1 Results of the NMR Analysis Carried out by Dr N Baggett of Birmingham University Department of Chemistry on the Dextran Rich Product used for the Test of Glucose Formation During the Biosynthesis of Dextran in the SCCR-S System



C2 Results of the Acid Hydrolysis Test Carried out on the Dextran Rich Product

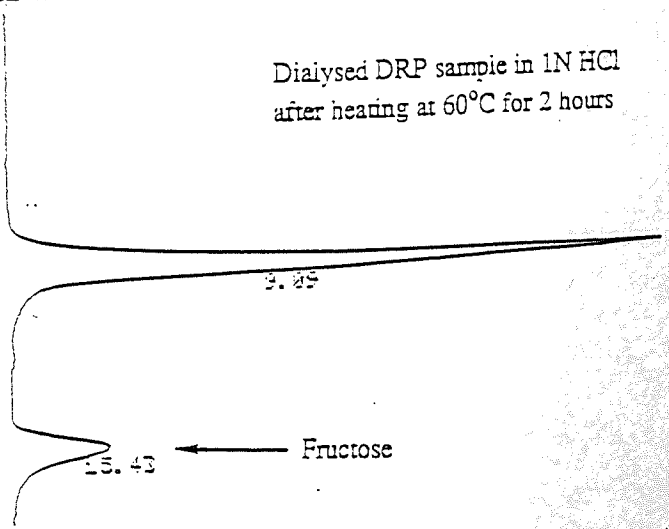


01/28/92 15:58:04 CH= "A" PS= 1.

FILE 1. METHOD 0. RUN 362 INDEX 362

PEAK#	AREA%	RT	AREA BC
1	16.934	8.42	137610 02
2	83.066	9.12	675024 02
TOTAL	100.		812634

CHANNEL A INJECT 01/28/92 17:16:55



01/28/92 17:16:55 CH= "A" PS= 1.

FILE 1. METHOD 0. RUN 363 INDEX 363

PEAK#	AREA%	RT	AREA BC
1	81.921	9.09	748260 01
2	18.079	15.43	165131 01

APPENDIX D

Simulation Programme of the SCCR-S System Used for the Continuous Hydrolysis of Lactose

```
C     SIMULATION OF THE SCCR-S SYSTEM FOR THE CONTINUOUS
C     HYDROLYSIS OF LACTOSE
C     BY MARTIN TANTOH SHIEH
      DIMENSION AS(2500),AF(2500),AD(2500)
     1,SMASS(2500),SCUM(2500),FMASS(2500),FCUM(2500),DMASS(2500)
     2,DCUM(2500),SCONC(2500),FCONC(2500)
     3,DCONC(2500),VS1(20),VF1(20),VD1(20)
      REAL KD1,KD2,KD3,KM,VMAX,VMAX1,KC2,KSC,LVT,KD23,KD22,V1,V2,
     4 KD32,KD33,KDF,KDD,TCONC,SNT,GNT,FNT,KK,ISTKK,LSTKK,DT
      INTEGER KNL(20),M,NTCLS,MP
      EXTERNAL SNT,GNT,FNT
      COMMON KD1,KD2,KD3,CFLOWC,CFLOW,V1,V2,KM,SFEED,FFLOW,VMAX1
      COMMON/PARAM/NN,NNFEED
      COMMON/PARAM1/S(2500)
      COMMON/PARAM2/D(2500)
      COMMON/PARAM3/F(2500)
      OPEN (15,FILE='SIMRESULT',STATUS='NEW')
C     INPUT ALL OPERATING PARAMETERS
      CFLOW=0.4667
      FFLOW=0.075
      ENFLOW=0.075
      TFLOW=FFLOW+ENFLOW
      SFLOW=1.267
      SFEED=0.15
      ENACTI=300.0
      ACTI=0.0
      TACTI=ACTI+(ENACTI*ENFLOW/CFLOW)
      ACT=TACTI*5.7E-6
      DT=0.1
      TEMP=55
      NTCLS=12
      NFEED=7
      KCYC=7
      KTOTAL=KCYC*NTCLS
      SWT=1740.0
      KM=5.0E-2
      NNBED=97
      KC2=0.625
      VMAX=KC2*ACT
      DIA=5.4
      XSA=(3.1416*(DIA**2))/4.0
      KD1=0.28
      KD2=0.52
      KD3=0.38
      ENFLOWM=ENFLOW*60.0
      TFLOWM=TFLOW*60.0
      CFLOWM=CFLOW*60.0
      SFLOWM=SFLOW*60.0
      FFLOWM=FFLOW*60.0
      TFEED=SFEED
      CFEED=TFEED*100.0
      V1=625.0/NNBED
      V2=865.0/NNBED
      SWP=SWT/60.0
      LVT=(CFLOWM*2+TFLOWM)/(XSA*2)
C     FLOWRATE CORRECTION OF DISTRIBUTION COEFFICIENTS
      KD1=(116.79-15.327*LVT)*KD1/100.0
      KDF=(114.20-14.327*LVT)*KD2/100.0
      KDD=(132.60-26.577*LVT)*KD3/100.0
      KD2=KDF
      KD3=KDD
C     PRINT OUT ALL OPERATING PARAMETERS
      WRITE (15,5)
      WRITE (15,6)
      WRITE (15,7)
      WRITE (15,8)
```

```
      WRITE (15,51) CFEED,CFLOWM,FFLOWM,SFLOWM,SWP,ENFLOWM,ENACTI,ACTI
      WRITE (15,9)
      WRITE (15,12) KCYC
      WRITE (15,10)
      WRITE (15,34)
    5 FORMAT(1H1,///,6X,'FEED',1X,'ELUENT',1X,'FEED',3X,'PURGE',2X,
     1'SWITCH',1X,'ENZYME',2X,'ACTIVITY',2X,'ACTIVITY')
    6 FORMAT(6X,'CONC',1X,'FLOW',3X,'FLOW',3X,'FLOW',3X,'PERIOD',1X,
     1'FLOW',2X,'IN FEED',6X,'IN ELUENT')
    7 FORMAT(8X,'%',2X,'RATE',3X,'RATE',3X,'RATE',3X,'MINS',3X,
     1'RATE')
    8 FORMAT(11X,'ML/MIN',1X,'ML/MIN',1X,'ML/MIN',6X,'ML/MIN',13X,
     1'DSU/ML'//)
    9 FORMAT(//,10X,'AVERAGE CONCENTRATION OF SUGARS ON EACH',
     11X,'COLUMN')
   12 FORMAT(10X,'AFTER',I3,' CYCLES')
   10 FORMAT(//,9X,'COL NO',3X,'AV LACTOSE CONCN',4X,'AV',1X,
     1'GALACTOSE CONCN',4X,'AV GLUCOSE CONCN'/)
   34 FORMAT(23X,'%W/V',18X,'%W/V',16X,'%W/V'/)
   51 FORMAT(6X,F4.1,1X,F5.1,2X,F4.1,3X,F5.1,2X,F4.1,3X,F5.2,5X,F5.1,
     15X,F6.2)
      DO 99 I=1,2500
      S(I)=0.0
      F(I)=0.0
      D(I)=0.0
      AS(I)=0.0
      AF(I)=0.0
      AD(I)=0.0
   99 CONTINUE
      NNTOT=NNBED*NTCLS
      NNNINE=NNBED*(NTCLS-1)+1
      NNFEED=(NFEED-1)*NNBED+1
      DO 100 K=1,KTOTAL
      IF (K.EQ.1) ISTKK=0
      ISTKK=SWT*(K-1)+1
      LSTKK=SWT*K
      DO 200 KK=ISTKK,LSTKK,DT
      DO 300 N=1,NTCLS
      IF (N.LE.(NFEED-1))CFLOWC=CFLOW
      VMAX1=VMAX*(CFLOW/CFLOWC)
      IF (N.GE.NFEED)CFLOWC=CFLOW+TFLOW
      VMAX1=VMAX*(CFLOW/CFLOWC)
      IF (N.LE.(NFEED-K))GO TO 300
      NNFST=NNBED*(N-1)+1
      NNLST=NNBED*N
      DO 400 NN=NNFST,NNLST
      IF (N.EQ.1)GO TO 90
      IF ((N.EQ.2).AND.(NN.EQ.NNFST))GO TO 40
      IF (NN.EQ.NNFEED)GO TO 50
      GO TO 70
   40 S(NN-1)=S(NNBED)
      F(NN-1)=F(NNBED)
      D(NN-1)=D(NNBED)
      GO TO 75
   70 IF(S(NN-1).LT.0.1E-20)S(NN-1)=0.0
      IF(F(NN-1).LT.0.1E-20)F(NN-1)=0.0
      IF(D(NN-1).LT.0.1E-20)D(NN-1)=0.0
   75 CALL CONC(DT)
C     BACKGROUND CONCENTRATION CORRECTION OF DISTRIBUTION COEFFICIENTS
      TCONC=F(NN)+D(NN)+S(NN)
      KD22=(101.8+48.667*F(NN))*KDF/100.0
      KD23=(100.0+95.333*D(NN))*KDF/100.0
      KD32=(98.25+63.667*F(NN))*KDD/100.0
      KD33=(92.017+211.67*D(NN))*KDD/100.0
      IF(TCONC.EQ.0.0)THEN
      KD2=KDF
```

```
      KD3=KDD
      ELSE
      KD2=(F(NN)*KD22+D(NN)*KD23+S(NN)*KDF)/TCONC
      KD3=(F(NN)*KD32+D(NN)*KD33+S(NN)*KDD)/TCONC
      END IF
      GO TO 150
   50 IF (S(NN-1).LT.0.1E-20)S(NN-1)=0.0
      IF (F(NN-1).LT.0.1E-20)F(NN-1)=0.0
      IF (D(NN-1).LT.0.1E-20)D(NN-1)=0.0
      CALL CONC(DT)
C     BACKGROUND CONCENTRATION CORRECTION OF DISTRIBUTION COEFFICIENTS
      TCONC=F(NN)+D(NN)+S(NN)
      KD22=(101.8+48.667*F(NN))*KDF/100.0
      KD23=(100.0+95.333*D(NN))*KDF/100.0
      KD32=(98.25+63.667*F(NN))*KDD/100.0
      KD33=(92.017+211.67*D(NN))*KDD/100.0
      IF(TCONC.EQ.0.0)THEN
      KD2=KDF
      KD3=KDD
      ELSE
      KD2=(F(NN)*KD22+D(NN)*KD23+S(NN)*KDF)/TCONC
      KD3=(F(NN)*KD32+D(NN)*KD33+S(NN)*KDD)/TCONC
      END IF
      GO TO 150
   90 IF(NN.EQ.NNFST)GO TO 95
      IF(S(NN-1).LT.0.1E-20)S(NN-1)=0.0
      IF(F(NN-1).LT.0.1E-20)F(NN-1)=0.0
      IF(D(NN-1).LT.0.1E-20)D(NN-1)=0.0
      GO TO 97
   95 S(NN)=0.0
      F(NN)=0.0
      D(NN)=0.0
      GO TO 400
   97 CALL CONC(DT)
C     BACKGROUND CONCENTRATION CORRECTION OF DISTRIBUTION COEFFICIENTS
      TCONC=F(NN)+D(NN)+S(NN)
      KD22=(101.8+48.667*F(NN))*KDF/100.0
      KD23=(100.0+95.333*D(NN))*KDF/100.0
      KD32=(98.25+63.667*F(NN))*KDD/100.0
      KD33=(92.017+211.67*D(NN))*KDD/100.0
      IF(TCONC.EQ.0.0)THEN
      KD2=KDF
      KD3=KDD
      ELSE
      KD2=(F(NN)*KD22+D(NN)*KD23+S(NN)*KDF)/TCONC
      KD3=(F(NN)*KD32+D(NN)*KD33+S(NN)*KDD)/TCONC
      END IF
  150 IF(K.EQ.KTOTAL.AND.KK.EQ.(LSTKK*SWT))GO TO 160
      GO TO 400
  400 CONTINUE
  300 CONTINUE
  200 CONTINUE
  160 DO 500 NN=1,NNBED
      AS(NN)=S(NN)
      AF(NN)=F(NN)
      AD(NN)=D(NN)
  500 CONTINUE
      DO 600 NN=1,NNTOT
      IF(NN.GE.NNNINE)GO TO 2010
      NNADJ=NN+NNBED
      S(NN)=S(NNADJ)
      F(NN)=F(NNADJ)
      D(NN)=D(NNADJ)
      GO TO 600
 2010 NNADJ=NN+1-NNNINE
      S(NN)=AS(NNADJ)
```

```
      F(NN)=AF(NNADJ)
      D(NN)=AD(NNADJ)
  600 CONTINUE
  100 CONTINUE
      SCUM(1)=0.0
      FCUM(1)=0.0
      DCUM(1)=0.0
      DO 11 I=1,NNTOT
      SMASS(I)=S(I)*V1
      FMASS(I)=F(I)*V1
      DMASS(I)=D(I)*V1
   11 CONTINUE
      L=NNBED
   27 DO 14 I=1,NNTOT+1
      IF(I.EQ.(L+1))SCUM(I)=SMASS(I)
      IF(I.EQ.(L+1))FCUM(I)=FMASS(I)
      IF(I.EQ.(L+1))DCUM(I)=DMASS(I)
   14 CONTINUE
      L=L+NNBED
      IF(L.EQ.(NNTOT+NNBED))GO TO 15
      GO TO 27
   15 L=NNBED
      I=2
      Z=0
      SCUM(1)=0.0
      FCUM(1)=0.0
      DCUM(1)=0.0
   13 DO 16 M=I,L
      SCUM(M)=SMASS(M)+SCUM(M-1)
      FCUM(M)=FMASS(M)+FCUM(M-1)
      DCUM(M)=DMASS(M)+DCUM(M-1)
      SCONC(M)=SCUM(M)/625.0
      FCONC(M)=FCUM(M)/625.0
      DCONC(M)=DCUM(M)/625.0
      KOLNO=M/NNBED
      IF(M.EQ.L) GO TO 18
      GO TO 16
   18 Z=Z+1
      KNL(Z)=KOLNO
      VS1(Z)=SCONC(M)*100.0
      VF1(Z)=FCONC(M)*100.0
      VD1(Z)=DCONC(M)*100.0
      VS1(1)=VS1(NTCLS)
      VF1(1)=VF1(NTCLS)
      VD1(1)=VD1(NTCLS)
      S(1)=0.0
      F(1)=0.0
      D(1)=0.0
   16 CONTINUE
      I=L+2
      L=L+NNBED
      IF(L.EQ.(NNTOT+NNBED)) GO TO 355
      GO TO 13
  355 WRITE(15,4)KNL(1),S(1),F(1),D(1)
      FINLST=NTCLS-1
      DO 886 Z=1,FINLST
      TST=Z+1
      WRITE(15,4)KNL(TST),VS1(Z),VF1(Z),VD1(Z)
  886 CONTINUE
    4 FORMAT(11X,I2,9X,F6.3,15X,F6.3,14X,F6.3)
      GO TO 17
   17 P=SWT
      FRU=(VF1(1)+VF1(1)*KD2*V2)/100.0
      DLU=(VD1(1)+VD1(1)*KD3*V2)/100.0
      SCU=(VS1(1)+VS1(1)*KD1*V2)/100.0
      FRPC=FRU*625.0/(P*SFLOW)
```

```
      DLPC=DLU*625.0/(P*SFLOW)
      SLPC=SCU*625.0/(P*SFLOW)
      MP=11*NNBED
      SU=S(MP)
      DU=D(MP)
      FU=F(MP)
      PUF=(FRPC/(FRPC+DLPC+SLPC))*100
      PUD=(DU/(DU+FU+SU))*100
      PUS=(SU/(DU+FU+SU))*100
      WRITE(15,80)
   80 FORMAT(//24X,'GAL PRO',22X,'GLUC PRO',/,13X,'GACONC',2X,'GCONC',
     13X,'PURITY',8X,'GACONC',2X,'GCONC',8X,'PURITY',/,60X,'GLUC',
     26X,'LAC')
      WRITE(15,66)FRPC,DLPC,PUF,FU,DU,PUD,PUS
   66 FORMAT(13X,F5.3,2X,F6.3,2X,F5.1,8X,F6.3,2x,F6.3,5X,F6.2,2X,F6.2)
      CLOSE (15)
      STOP
      END

C     EXTERNAL FUNCTIONS USED FOR SOLVING THE NON LINEAR ORDINARY
C     DIFFERENTIAL EQUATIONS IN THE SUBROUTINE

          REAL FUNCTION SNT(x,y)
      REAL KD1,KD2,KD3,KM
      COMMON KD1,KD2,KD3,CFLOWC,CFLOW,V1,V2,KM,SFEED,FFLOW,VMAX1
      COMMON/PARAM/NN,NNFEED
      common/param1/s(2500)
      common/param2/d(2500)
      common/param3/f(2500)
      FTOT=SFEED*FFLOW
      FINA=CFLOWC*S(NN)
      VOLM=V1+KD1*V2
      IF(NN.EQ.NNFEED)THEN
      CINI=CFLOW*S(NN-1)
      ELSE
      CINI=CFLOWC*S(NN-1)
      FTOT=0.0
      END IF
      REAC=V1*VMAX1*S(NN)/(KM+S(NN))
      SNT=(CINI+FTOT-FINA-REAC)/VOLM
      RETURN
      END

         REAL FUNCTION GNT(x,y)
      REAL KD1,KD2,KD3,KM
      COMMON KD1,KD2,KD3,CFLOWC,CFLOW,V1,V2,KM,SFEED,FFLOW,VMAX1
      COMMON/PARAM/NN,NNFEED
      COMMON/PARAM1/S(2500)
      common/param2/d(2500)
      common/param3/f(2500)
      IF(NN.EQ.NNFEED)THEN
      GNT=(CFLOW*D(NN-1)-CFLOWC*D(NN)+0.526*V1*VMAX1*S(NN)/(KM+S(NN)))
     1/(V1+V2*KD3)
      ELSE
      GNT=(CFLOWC*(D(NN-1)-D(NN))+0.526*V1*VMAX1*S(NN)/(KM+S(NN)))
     2/(V1+V2*KD3)
      END IF
      RETURN
      END

         REAL FUNCTION FNT(x,y)
      REAL KD1,KD2,KD3,KM
      COMMON/PARAM1/S(2500)
      common/param2/d(2500)
      common/param3/f(2500)
      COMMON KD1,KD2,KD3,CFLOWC,CFLOW,V1,V2,KM,SFEED,FFLOW,VMAX1
```

```
      COMMON/PARAM/NN,NNFEED
      IF(NN.EQ.NNFEED)THEN
      FNT=(CFLOW*F(NN-1)-CFLOWC*F(NN)+0.526*V1*VMAX1*S(NN)/(KM+S(NN)))
     3/(V1+KD2*V2)
      ELSE
      FNT=(CFLOWC*(F(NN-1)-F(NN))+0.526*V1*VMAX1*S(NN)/(KM+S(NN)))
     4/(V1+KD2*V2)
      END IF
      RETURN
      END

C     SUBROUTINE FOR CALCULATING THE CONCENTRATION OF EACH COMPONENT
C     ON A PLATE

            SUBROUTINE CONC(DT)
      COMMON/PARAM1/S(2500)
      COMMON/PARAM2/D(2500)
      COMMON/PARAM3/F(2500)
      COMMON KD1,KD2,KD3,CFLOWC,CFLOW,V1,V2,KM,SFEED,FFLOW,VMAX1
      COMMON/PARAM/NN,NNFEED
      S1=DT*S(NN)
      S5=DT*SNT(S(NN),DT)
      S2=DT*(S(NN)+S5/2.0)
      S6=DT*SNT(S(NN)+S5/2.0,DT+S1/2.0)
      S3=DT*(S(NN)+S6/2.0)
      S7=DT*SNT(S(NN)+S6/2.0,DT+S2/2.0)
      S4=DT*(S(NN)+S7)
      S8=DT*SNT(S(NN)+S7,DT+S3)
      S(NN)=S(NN)+(S5+2.0*S6+2.0*S7+S8)/6.0

      G1=DT*D(NN)
      G5=DT*GNT(D(NN),DT)
      G2=DT*(D(NN)+G5/2.0)
      G6=DT*GNT(D(NN)+G5/2.0,DT+G1/2.0)
      G3=DT*(D(NN)+G6/2.0)
      G7=DT*GNT(D(NN)+G6/2.0,DT+G2/2.0)
      G4=DT*(D(NN)+G7)
      G8=DT*GNT(D(NN)+G7,DT+G3)
      D(NN)=D(NN)+(G5+2.0*G6+2.0*G7+G8)/6.0

      F1=DT*F(NN)
      F5=DT*FNT(F(NN),DT)
      F2=DT*(F(NN)+F5/2.0)
      F6=DT*FNT(F(NN)+F5/2.0,DT+F1/2.0)
      F3=DT*(F(NN)+F6/2.0)
      F7=DT*FNT(F(NN)+F5/2.0,DT+F2/2.0)
      F4=DT*(F(NN)+F7)
      F8=DT*FNT(F(NN)+F7,DT+F3)
      F(NN)=F(NN)+(F5+2.0*F6+2.0*F7+F8)/6.0

      RETURN
      END
```

Result From the Simulation Programme

FEED CONC %	ELUENT FLOW RATE ML/MIN	FEED FLOW RATE ML/MIN	PURGE FLOW RATE ML/MIN	SWITCH PERIOD MINS	ENZYME FLOW RATE ML/MIN	ACTIVITY IN FEED	ACTIVITY IN ELUENT DSU/ML
10.0	30.0	4.5	76.0	28.0	4.50	300.0	0.00

AVERAGE CONCENTRATION OF SUGARS ON EACH COLUMN
AFTER 7 CYCLES

COL NO	AV LACTOSE CONCN %W/V	AV GALACTOSE CONCN %W/V	AV GLUCOSE CONCN %W/V
1	0.000	0.000	0.000
2	0.000	0.441	0.078
3	0.000	2.372	2.251
4	0.000	2.382	2.381
5	0.000	2.390	2.401
6	0.001	2.401	2.420
7	0.499	2.411	2.324
8	0.033	3.595	2.697
9	0.000	3.252	2.723
10	0.000	1.887	2.716
11	0.000	0.499	2.707
12	0.000	0.045	2.693

GAL PRO			GLUC PRO			
					PURITY	
GACONC	GCONC	PURITY	GACONC	GCONC	GLUC	LAC
0.007	0.001	88.2	0.000	0.026	99.84	0.00

List of Symbols Used in the Simulation Program

ACTI	enzyme activity in the eluent (U/cm^3)
Ai	array containing concentrations of component i (g/cm^3)
CFLOW	eluent flowrate (cm^3/sec)
D	array containing glucose concentration on each plate (g/cm^3)
DT	time increment (mins)
ENACTI	enzyme activity entering the system with the feed (U/cm^3)
ENFLOW	enzyme flowrate (cm^3/sec)
F	array containing galatose concentration on each plate (g/cm^3)
FFLOW	feed flowrate (cm^3/sec)
iCONC	average concentration of component i in each column (g/cm^3)
iCUM	cumulative mass of component i (g)
iMASS	array containing the mass of component i on each plate (g)
ISTKK	first time increment in a sequence
KC2	reaction rate constant
KCYC	total number of cycles
KD1	distribution coefficient of lactose
KD2	distribution coefficient of galactose
KD3	distribution coefficient of glucose
KKINK	number of time increments in a sequence
KM	Michaelis-Menten constant
KTOTAL	total number of sequence
LSTKK	last time increment in a sequence
N	number of column
NFEED	number of feed column
NN	plate counter
NNBED	number of plates in each column (based on galactose)
NNFEED	number of feed plate
NNFST	first plate in a column
NNLST	last plate in a column
NNTOT	total number of plates in the system
NTCLS	total number of columns in the system
S	array containing the lactose concentration on each plate
SFEED	lactose feed concentration (g/cm^3)
SFLOW	purge flowrate (cm^3/min)
SWP	switch time (mins)
V1	mobile phase plate volume (cm^3)

V2	stationary phase plate volume (cm^3)
VMAX	maximun initial reaction velocity
XSA	column cross sectional area (cm^2)

PUBLICATIONS AND PROPOSED PUBLICATIONS

(1) Ajongwen NJ, Akintoye A, Barker PE , Ganetsos G and Shieh MT
Large scale Purification of Leuconostoc mesenteroides NRRL B512F Dextransucrase for use in the biosynthesis of dextran by Batch and Continuous Chromatography
The Chemical Engineering Journal, 51, (1993), B43-B50.

(2) Barker PE, Ajongwen NJ, Shieh MT, Ganetsos G
Simulated Counter-Current Chromatographic Bioreactor-Separators.
4th International Conference on the Fundamentals of Adsorption, Tokyo, Japan, May 1992
Proceedings published in "Fundamentals of adsorption", pp35-44. Edited by M. Suzuki, Published by Kodansha, Japan, (1993).

(3) Shieh MT, Barker PE, Ajongwen NJ and Ganetsos G
Production of dextran by Continuous Chromatographic Reaction-Separation.
The Proceeding of the 1992 IChemE Research Event, UMIST, Manchester, January 9-10, 1992. Published by the Institution of Chemical Engineers, Rugby, U.K.

(4) Shieh MT and Barker PE
Simulated Counter-Current Chromatographic Bioreactor-Separator.
The Proceeding of the 1993 IChemE Research Event, Birmingham University, Published by the Institution of Chemical Engineers, Rugby, U.K., 1993.

(5) Shieh MT and Barker PE
The Study of a Preparative Scale Simulated Counter-Current Chromatographic Bioreactor-Separator. To be Published in the Proceeding of the 1994 IChemE Research Event at University College London and will be presented orally at this event .

(6) Shieh MT and Barker PE
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