# Bioreaction and Separation in Preparative Batch Chromatographic Columns

## *The Hydrolysis of Lactose to Yield Glucose, Galactose and Oligosaccharides*

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**Christopher Michael West** **PhD** **1997**

## SUMMARY

The initial aim of this project was to improve the performance of a chromatographic bioreactor-separator (CBRS). In such a system, a dilute enzyme solution is pumped continuously through a preparative chromatographic column, while pulses of substrate are periodically injected on to the column. Enzymic reaction and separation are therefore performed in a single unit operation.

The chromatographic columns used were jacketed glass columns ranging from 1 to 2 metres long with an internal diameter of 1.5 cm. Linking these columns allowed 1, 2, 3 and 4 metre long CBRS systems to be constructed. The hydrolysis of lactose in the presence of β-galactosidase was the reaction of study. From previous work at Aston University, there appeared to be no difficulties in achieving complete lactose hydrolysis in a CBRS. There did, however, appear to be scope for improving the separative performance, so this was adopted as an initial goal. Reducing the particle size of the stationary phase was identified as a way of achieving this improvement. A cation exchange resin was selected which had an average particle size of around half that previously used when studying this reaction. A CBRS system was developed which overcame the operational problems (such as high pressure drop development) associated with use of such a particle size. A significant improvement in separative power was achieved. This was shown by an increase in the number of theoretical plates (N) from about 500 to about 3000 for a 2 metre long CBRS, coupled with higher resolution. A simple experiment with the 1 metre column showed that combined bioreaction and separation was achievable in this system.

Having improved the separative performance of the system, the factors affecting enzymic reaction in a CBRS were investigated; including pulse volume and the degree of mixing between enzyme and substrate. The progress of reaction in a CBRS was then studied. This information was related to the interaction of reaction and separation over the reaction zone.

The effect of injecting a pulse over a length of time as in CBRS operation was simulated by fed batch experiments. These experiments were performed in parallel with normal batch experiments where the substrate is mixed almost instantly with the enzyme. The batch experiments enabled samples to be taken every minute and revealed that reaction is very rapid. The hydrodynamic characteristics of the two injector configurations used in CBRS construction were studied using Magnetic Resonance Imaging, combined with hydrodynamic calculations.

During the optimisation studies, galactooligosaccharides (GOS) were detected as intermediates in the hydrolysis process. GOS are valuable products with potential and existing applications in food manufacture (as nutraceuticals), medicine and drug targeting. The focus of the research was therefore turned to GOS production. A means of controlling reaction to arrest break down of GOS was required. Raising temperature was identified as a possible means of achieving this within a CBRS. Studies were undertaken to optimise the yield of oligosaccharides, culminating in the design, construction and evaluation of a Dithermal Chromatographic Bioreactor-separator.

**KEY WORDS:** Dithermal Chromatographic Bioreactor-separator, Magnetic Resonance Imaging, β-galactosidase, Chromatography, Galactooligosaccharides.

*To my wife Jackie for her support and encouragement,*

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# Introduction

## 1.1 Origins of the Research

The term *chromatography* was first used by Tswett, who, in 1903, used column chromatography to separate plant pigments, eluting with petroleum ether from a calcium carbonate bed. Tswett's work is described briefly by M$^{c}$Murray (1992). Although gas and liquid chromatography were being used on the laboratory scale, it was not until the 1940s that Martin and Synge (1941) elucidated the fundamental theories of chromatography. In their work, a theoretical interpretation of the processes involved in a separation was proposed, based on the operating parameters of the column; this expressed the efficiency of the separation in terms of the theoretical plate concept. Briefly, the greater the number of theoretical plates (N), the better is the separation. The number of theoretical plates has been used as the principal indicator of chromatographic efficiency in this thesis. This and other chromatographic terms are described in greater detail in Appendix A-1.

Following development of chromatography as an analytical tool, it was realised that if the process was scaled up, it could be used as a means of purifying or concentrating products of commercial value. By 1956, the scale-up of gas chromatography had been initiated, the 1950s and 60s seeing much activity in this area. Since the 1970s however, liquid chromatography has become the dominant area of development. This upsurge in interest in liquid systems was, in part, fuelled by the restructuring of the U.S.A.'s sweetener industry.

Traditionally, reaction and chromatographic separation have been carried out as separate unit operations. The late 1950s and early 1960s saw attempts to marry the processes of reaction and separation in a single unit operation with the development of chromatographic chemical reactors. Most of the work published in this field was confined to gas chromatography. According to Schweich and Villermaux (1982), one of the first industrial reactions to be performed in a chromatographic reactor was the dehydrogenation of butane and butenes. However, some liquid chromatographic applications were beginning to emerge. The mid 1980s saw the emergence of chromatographic bioreactor-separator (CBRS) systems. In these systems, a dilute enzyme

solution is pumped continuously through a chromatographic column, while substrate is introduced to the column in pulses. Barker and Zafar (1987) were the first to use a chromatographic reactor for performing an enzymic reaction, in this case, the synthesis of dextran polymer. In this way, the chromatographic bioreactor-separator (CBRS) was conceived and further explored at Aston University.

Combining enzymic reaction and chromatographic separation as a single unit operation not only reduces plant and operational costs, but also results in greater yields than can be obtained when compared to conventional batch reaction in a stirred tank (Zafar, 1986). There have since been further developments in the field of simultaneous biochemical reaction and separation. Chromatographic bioreactor-separators have been developed which not only operate in the conventional batch mode (Zafar, 1986), but also semi-continuously (Shieh and Barker (1993), Shieh (1994)) or continuously (Sarmidi and Barker, 1993).

The scale-up of chromatographic columns from the sizes utilised in analytical applications is not without problems. There is strong commercial pressure to maximise throughput, but if this is at the expense of separation efficiency, the value of the product is reduced. On scale-up, there has to be a balance between throughput and efficiency, while considering operational aspects such as the maintenance of the separation characteristics of the bed.

The ease by which a chromatographic process can be scaled up may be dictated by the mode of operation required. The three main operational modes are continuous, semi-continuous and batch.

Continuous Operation

Continuous operation offers many advantages, such as increased flexibility and constant product quality with minimal or no recycling. Continuous operation also permits more effective utilisation of the available mass-transfer area. In order to increase throughput, high feed concentrations are used (Ganetsos and Barker 1993). An important development has been that of annular chromatography. Here, the bed results from packing the annular space formed by two concentric cylinders (Bridges and Barker, 1993). Such a system has been used to purify mixtures of carbohydrates (Bridges, 1990). In this

system, the annular bed rotated continuously about its axis, while feed was introduced continuously through a fixed feed pipe immersed in the bed. A mobile phase is passed downwards through the bed, passing uniformly through all parts. Sarmidi (1993) used a continuous rotating annular chromatograph as a bioreactor-separator, for the inversion of sucrose to glucose and fructose.

Semi Continuous Operation

Semi Continuous Chromatographic Processes offer similar benefits to the continuous systems. An example of a semi-continuous system is the semi-continuous counter-current chromatographic reactor-separator (SCCR-S), as used by Akintoye (1989) and Shieh (1994). In this type of system, a moving bed system is simulated; the bed is held stationary while periodically altering the positions at which feed and product streams enter or leave the system (Akintoye, 1989). A series of valves enables the simulation of counter current movement of the mobile and stationary phases.

Batch Operation

The batch mode of operation involves the pulsed input of mixtures to the column while continuously flowing a mobile liquid phase. This mode of chromatography has received the most attention in terms of scale-up, mainly due to simplicity of operation and relative simplicity in model construction. During the 1960s ABCOR, an American company, successfully scaled up both liquid and gas chromatographic systems, focusing on the separation of chemicals such as flavours, fragrances and petrochemicals (Ganetsos and Barker 1993). They selected systems where conventional purification methods, such as distillation, were either inadequate or uneconomical. The 1970s saw much development and application of batch processes, especially in the area of petrochemicals.

In the field of large-scale carbohydrate separation, the Finnish Sugar Co. Ltd. were considered to be the leaders (Ganetsos and Barker 1993), having installed plants throughout the world. Batch chromatographic bioreactor-separators were first studied at Aston University by Barker and Zafar (1988); they have since been studied by Taddei (1994), Shieh (1994) and West (1995). Batch operation may be criticised for leading to high product dilution and being difficult to automate (Lameloise and Viard, 1993). It was also pointed out by Barker and Thawait (1986) that such systems have restricted column

utilisation and limited throughput. However, batch chromatography has the advantages of simple operation and ease of scale-up (Ganetsos and Barker, 1993).

Despite the flexibility and high throughputs offered by semi-continuous or continuous operation, there are still applications suited to batch operation, such as the production of small quantities of high value biochemicals. It was for this reason that the batch mode of operation was selected for use in the author's work. A potential advantage of performing enzymic reactions within a chromatographic column is the reduction of competitive inhibition effects (Taddei and Barker, 1993). This is achieved by chromatographically separating inhibitory products from the substrate. Since it can be assumed that reaction takes place only where there is substrate, the location of the substrate pulse is regarded as the reaction zone. This was why Taddei (1994) used a batch chromatographic bioreactor-separator to study the hydrolysis of lactose to glucose and galactose by the enzyme β–galactosidase (lactase) from Aspergillus oryzae:

$$\text{LACTOSE} \xrightarrow{\beta\text{–galactosidase}} \text{GLUCOSE} + \text{GALACTOSE},$$

it being reported in the literature that galactose has an inhibitory effect on lactase activity (Friend and Shahani, 1982).

Taddei (1994) studied the effects of pulse size, pulse concentration, eluant flow rate and enzyme activity in terms of their influence on separation of the products. Glucose and galactose have a low separation factor, i.e. 1.2-1.4, so Taddei reported difficulty in separating these products in a 4 metres long batch chromatographic bioreactor-separator. However, Shieh and Barker (1994) did report some success in performing this reaction in a Simulated Counter-Current Chromatographic Bioreactor-Separator (SCCR-S); the difficulty in separating glucose and galactose was approached by operating in batch mode, connecting the twelve columns of the SCCR-S in series to increase the column length to 7.8 metres.

The enzymic hydrolysis of lactose was selected as the system for initial study in this research project because of the chromatographic challenge presented by the lactose-lactase system, as well as the commercial importance of the process. Lactose is a non-sweet sugar and has a low solubility in aqueous solution, so lactose syrups tend to be granular and therefore unpalatable. Lactose is hydrolysed by lactase to produce the monosaccharides D-glucose and D-galactose, which have a sweetening power of about 0.8 relative to sucrose and are 3-4 times more soluble than lactose (Nijpels, 1980). This conversion results in a sweeter syrup which has a more attractive texture, making it a high value product with many applications, such as in sweetening dairy products.

Another application of lactase is in the production of low lactose milk for persons suffering from lactose intolerance. The incorporation of lactase-treated milk into flavoured milk drinks means that addition of sugar can be reduced without reducing the sweetness of the product. Lactose cannot be absorbed directly from the intestine, unlike its hydrolysis products glucose and galactose.

## 1.2 Modeling of Chromatographic Processes

The movement of bands through a chromatographic column is commonly simulated by producing a mass balance equation which is then integrated numerically over time and space to give an elution profile. This calculation can be performed by using finite difference methods to extrapolate from the concentration value at a given time and position in the column to the next value (Czok and Guiochon 1990).

This approach has been followed in modeling the behaviour of Batch Chromatographic Bioreactor-separators, generally with the incorporation of an enzymic reaction rate term of the standard Michaelis-Menten form:

$$\left[1+\frac{(1-\varepsilon)\,Kd_i}{\varepsilon}\right]\frac{dC_i}{dt}+u\frac{dC_i}{dz}-\frac{C_i Vmax}{C_i+Km}=0$$

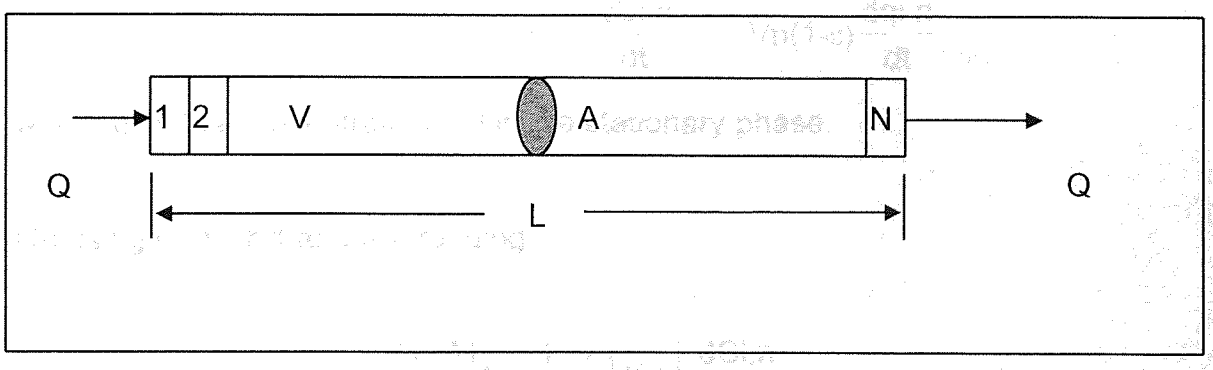
representing the conversion of a substrate (i) occurring in an element dz of the chromatographic bed, where C = concentration, $Kd_i$ = distribution coefficient,

$\varepsilon$ = bed voidage, Vmax = maximum reaction velocity, Km = Michaelis-Menten constant, t = time, z = abscissa along the column and u = linear velocity of eluent.

Similar equations may be written to describe the generation of each product involved in the process (Taddei and Barker, 1994). Taddei (1994) used a FORTRAN program for the algorithm. The two main methods used in modelling batch chromatographic bioreactor-separators are the theoretical plate model and the differential model (Jeng and Langer, 1992).

The Theoretical Plate Model

This theory treats the chromatographic bed as a series of well mixed equilibrium stages. Drawing an analogy to distillation theory, Martin and Synge (1941) defined HETP (height equivalent to one theoretical plate) as the thickness of a layer (in a chromatographic column) over which the solute issuing from it is in equilibrium with the mean concentration of solute in the stationary phase throughout the layer. They also said that the value of HETP could be taken as a constant through a given column, except when the ratio of concentrations of the solution entering and leaving the plate differs greatly from unity. It was assumed that the diffusion of solute from one plate to another must be negligible. Also, it was assumed that at equilibrium, the distribution ratio of one solute between the two phases must be independent of its absolute concentration and the presence of other solutes. The column packing is visualised as a series of well-mixed equilibrium stages. The column is divided into sections, each one being the length of a theoretical plate, as shown below.




Here L = column length, A = Cross-sectional area, N = Number of theoretical plates and V = Column volume.

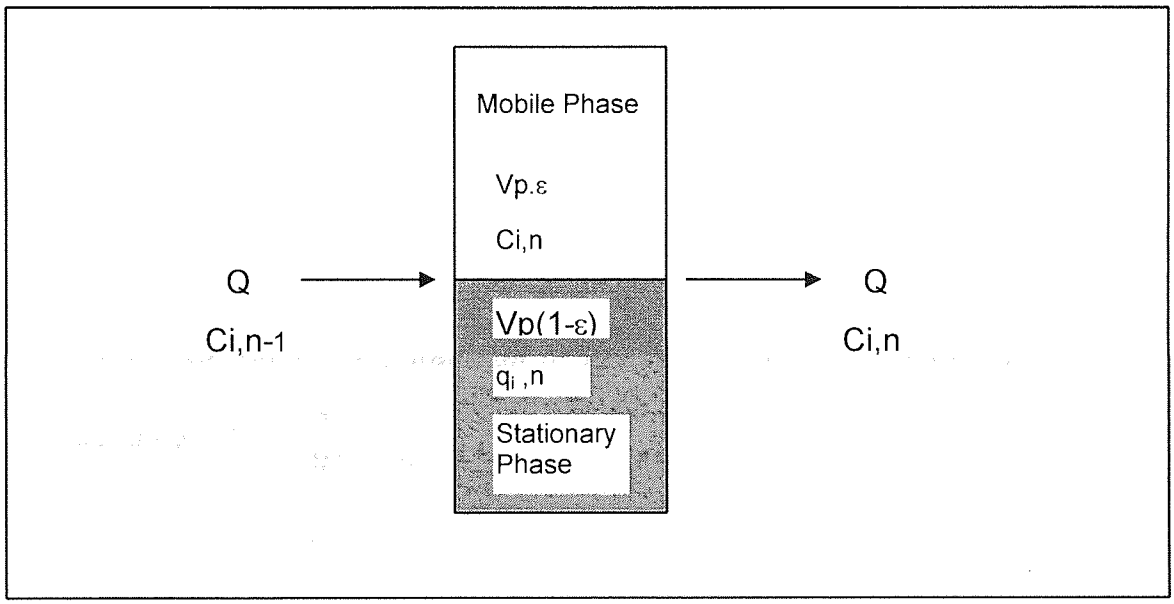
Now, V = A.L = N.Vp where Vp = plate volume and N = Number of plates

Dividing through by N and A gives: $\frac{L}{N}$ = HETP, the height equivalent to a theoretical plate. In each section, or plate, the concentration of the component of interest, component i, is assumed to be in equilibrium between the stationary phase and mobile phase. Consequently,

$$q_i = C_i . Kd_i$$

where $Kd_i$ is the equilibrium distribution coefficient and $q_i$ is the concentration of component i adsorbed on to the stationary phase.

Plate n




A mass balance on i leads to:

Input Rate - Output Rate= Accumulation Rate

$$Q.C_{i,\,n-1} - Q.C_{i,\,n} = (Vp.\varepsilon)\frac{dCi,n}{dt} + Vp(1-\varepsilon)\frac{dqi,n}{dt}$$

where qi is the concentration of I in the stationary phase.

On using $q_i = C_i . Kd_i$ and rearranging,

$$(C_{i,\,n-1} - C_{i,\,n}) = \left(\frac{Vp.\varepsilon}{Q}\right)\left\{1+\left(\frac{1-\varepsilon}{\varepsilon}\right).Kd_i\right\}.\frac{dCi,n}{dt}$$

where $\left(\frac{Vp.\varepsilon}{Q}\right)$ is $\tau_p$ , the residence time of an unadsorbed component in the plate.

and

$\tau_p \left\{1+\left(\frac{1-\varepsilon}{\varepsilon}\right).Kd_i\right\} = \tau_{p,i}$ , the residence time of an adsorbed component, i, in the plate.

For a column with N plates,

$$tr_0 = N.\ \tau_p$$

and

$$tr_i = N.\ \tau_{p,i}$$

$tr_i - tr_0$ gives a measure of the resolution of the two components.

Since

$$tr_i = tr_o \left\{1+\left(\frac{1-\varepsilon}{\varepsilon}\right).Kd_i\right\},$$

if $tr_i$, $tr_o$ and $\varepsilon$ are known, Kdi can be evaluated. The other feature of a chromatogram is the broadening or spread. The variance or $\sigma^2$, where $\sigma$ is the standard deviation, is the parameter used to measure the broadening.

Theory shows that $\frac{\sigma^2}{tr^2} = \frac{1}{N}$

or $N = \frac{tr^2}{\sigma^2}$

The latter is used in practice to estimate N having evaluated $\sigma^2$.

$\sigma^2 = \frac{\left(\frac{W_h}{e}\right)^2}{8}$ is the approximation used in the thesis.

The number of theoretical plates is calculated using the following equation, which was developed by Gleukauf (1955):

$$N = 8\left(\frac{tr}{W_{h/e}}\right)^2$$

where: N = total number of theoretical plates with regard to a specified solute
tr = retention time of specified solute

h = solute peak height

e = base of the natural logarithm

The peak width (W) is measured at $^{h}/_{e}$ to reduce inaccuracies due to baseline height fluctuation. N is a useful measure of column performance, indicating the degree of peak broadening. In chromatography, narrow peaks are desired, since this reduces the likelihood of overlap between neighbouring peaks. Overlap of peaks should be minimised to give maximum resolution. Resolution is important in analytical systems for qualitative and qualitative analysis. In preparative systems good resolution is needed to maximise yields of desired products. Resolution (Rs) is a measure of the degree of separation between two adjacent peaks and is given by:

$$Rs = 2\left(\frac{tr2 - tr1}{W1 + W2}\right)$$

where: tr2 = retention time of peak with higher retention time

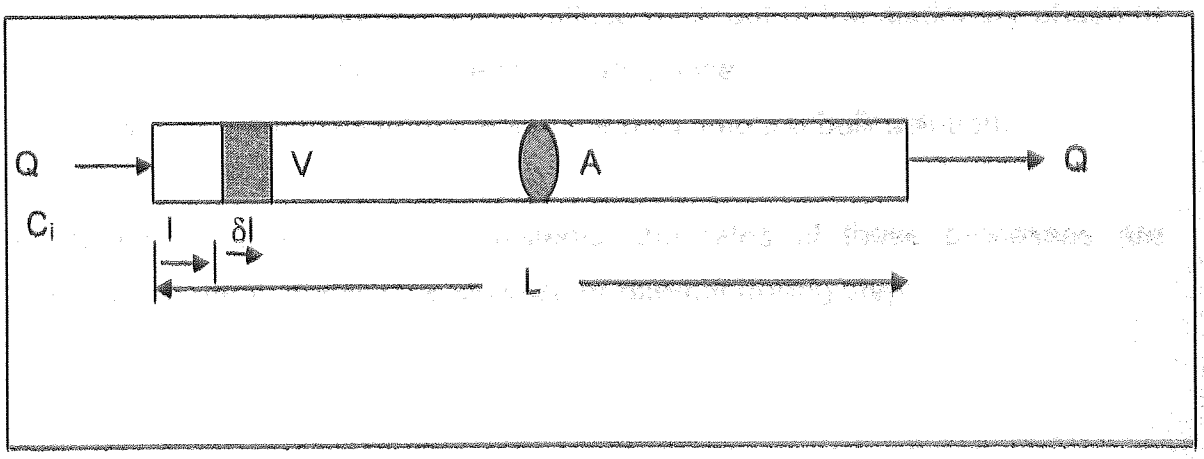
tr1 = retention time of peak with lower retention time

W1& W2 = width (at base) of peaks

The larger the value of Rs, the better the separation. A value of between 1.2 and 1.5 is considered ideal (Braithwaite and Smith 1996), whereas a value of 0.8 or less indicates a poor separation.

The Dispersed Plug Flow Model

This model is based on a mass balance performed across an infinitely thin slice of the column $\delta l$.



$$-D_{L,i}\frac{d^2Ci}{dl^2}+u\left(\frac{dCi}{dl}\right)+\left\{1+\left(\frac{1-\varepsilon}{\varepsilon}\right).Kd_i\right\}\left(\frac{dCi}{dt}\right)=0$$

Here C = concentration, $Kd_i$ = distribution coefficient, $\varepsilon$ = bed voidage, $D_L$ is the axial diffusion coefficient and u = linear velocity of eluant.

This model, as described by Ruthven and Ching (1993), is based on the Thomas model with the addition of the term $D_{L,i}$ which accounts for axial dispersion. Under normal chromatographic conditions, both the plate and dispersed plug flow models describe the characteristic chromatograms equally well, on the basis of the condition:

$$Pe = 2N$$

where $$Pe = \frac{U\,L}{D_L}\ .$$

A qualitative description of the chromatographic process is given by Belter *et al* (1988). They describe a five stage mechanism accounting for diffusion and chemical reaction between the solute and the stationary phase:

(i) The solute is transferred from the bulk solution to the surface of the stationary phase.

(ii) It diffuses into the stationary phase.

(iii) It reacts reversibly with the stationary phase, onto which it may be adsorbed followed by desorption.

(iv) The desorbed solute diffuse back out of the stationary phase to the surface of the stationary phase.

(v) It diffuses from the surface back into the bulk solution.

In modelling chromatographic systems, the rates of these processes are studied in order to identify the slowest, or rate-controlling step.

Mass Transfer Effects

The mass transfer process can be thought of as including the following steps:

(i) External mass transfer from the bulk liquid to the external surface of the particle.

(ii) Diffusion into the gel-like particle. (Styrene-Divinyl Benzene Cation Exchange Resin).

(iii) Adsorption / desorption.

Villermaux et al (1993) has shown that a characteristic time can be associated with each of these steps. The total mass transfer time, $t_m$, is calculated by addition of the characteristic times taken for external mass transfer ($t_e$), diffusion into the particle ($t_d$) and the adsorption / desorption process ($t_a$).

So that: $t_m = t_e + t_d + t_a$

It can then be shown that

$$\frac{\sigma^2}{tr_i^2} = \frac{1}{N} + \left(\frac{2K^I}{1+K^I}\right).\frac{t_{m,i}}{tr_{,i}}$$

where

$$K^I = \left\{\left(\frac{1-\varepsilon}{\varepsilon}\right).Kd_i\right\}.$$

So, as $Kd_i$ increases, the peak spreads more, this effect being enhanced by external mass transfer. Simulation work shows that as $t_{m,i}$ increases, the broadening is increased.

In general,

$$HETP = \left(\frac{\sigma^2}{tr_i^2}\right).L$$

$$= \frac{L}{N} + \left(\frac{2K^I}{1+K^I}\right).\frac{t_{m,i}}{tr_i i}.L$$

Because of the link between the theoretical plate model and the axially dispersed model

$$\frac{1}{N} \approx \frac{2}{Pe} = \frac{2D_{L,i}}{UL}$$

The relationship for HETP becomes:

$$HETP = \frac{2\,D_{L,i}}{U} + \left(\frac{2\,K^{I}}{1+K^{I}}\right) \cdot \frac{t_{m,i}}{tr,\,i} L$$

Also, $tr_{,i} = tr_o\,(1+K^{I})$

and $$U = \frac{L}{tr_o}$$

giving

$$HETP = \frac{2\,D_{L,i}}{U} + \frac{2\,K^{I}}{\left(1+K^{I}\right)^{2}} \cdot t_{m,i} \cdot u$$

This relationship has the same form as the van Deemter relationship.

Van Deemter *et al* (1956) developed an expression for plate height which takes into account the relative importance of physical factors involved in separation, such as the influence of the particle diameter of the column packing and its impact on axial diffusion.

$$H = A + \frac{B}{U} + C_m U + C_s U$$

The A term is due to eddy diffusion:

$$A = \lambda dp$$

where $\lambda$ is a constant taking into account the particle size range, packing uniformity, column dimensions and geometry; dp is the packing particle diameter.

The term B is due to molecular diffusion:

$$B = 2\gamma D_m$$

where $\gamma$ is the hindrance factor dependent on the characteristics of the packing. $D_m$ is the diffusion coefficient of the component in the mobile phase.

The C terms are due to mass transfer:

The $C_m$ term describes diffusion of component molecules in the mobile phase as they move to the stationary phase.

$$C_m = \frac{dp^2}{D_m}$$

$D_m$ is the diffusion coefficient of the component in the mobile phase.

The $C_s$ term relates to diffusion in the stationary phase and is directly related to the stationary phase film thickness, $d_f$ . $D_s$ is the diffusion coefficient of the component in the stationary phase.

$$C_s = \frac{d_f^2}{D_s}$$

Normal operation is in the region where pore diffusion controls, so the C term has the most effect on HETP.

External Mass Transfer

External resistance to mass transfer depends on the hydrodynamic conditions around the stationary phase particles. The rate of mass transfer (Belter *et al* 1988) is generally estimated from a relationship of the type:

$$(1-\varepsilon)\frac{dq}{dt} = k_L a\left(C - C^*\right)$$

where $k_L$ is the mass transfer coefficient, q is the concentration of the component on the stationary phase, a is the surface area to volume ratio of the

stationary phase, C is the concentration of the component in the mobile phase, $C^*$ is the concentration of the component in solution which would be in equilibrium with that on the stationary phase.

The mass transfer coefficient, $K_L$, can be obtained from the following relationship (Storti *et al* 1993):

$$Sh = \frac{1.09}{\varepsilon} Re^{0.33} Sc^{0.33} \qquad \text{for Re 0.0015 to Re 55}$$

and

$$Sh = \frac{0.25}{\varepsilon} Re^{0.69} Sc^{0.33} \qquad \text{for Re 55 to Re 1050}$$

where $Sh = \frac{k_L dp}{D_m}$

$D_m$ being the molecular diffusion coefficient of the mobile phase.

$$Re = \frac{U dp \rho}{\mu}$$

$$Sc = \frac{\mu}{\rho D_m}$$

where u is superficial velocity, dp is particle diameter of stationary phase, $\rho$ is mobile phase density and $\mu$ is mobile phase viscosity.

For example a factor of 10 increase in velocity gives therefore a factor of $10^{0.33}$ = 2.14 increase in $k_La$ where Re is between 0.0015 and 55.

Rates of Adsorption and Desorption

In chromatographic operations both the rate of adsorption and the shape of the equilibrium isotherm influence separative performance. During a chromatographic run, the adsorption zone broadens and acquires a specific shape. The front or rear boundary of that zone can be sharp or diffuse according to the type of adsorption isotherm describing the system (Gosling *et*

*al* 1989). Although dispersion effects will also contribute to the zone spreading, the strength of adsorption has the greater effect (Arnold *et al* 1985).

The Thomas model ignores both axial dispersion and mass transfer kinetics (Golshan-Shirazi and Guiochon, 1992). It assumes the rates of adsorption and desorption are finite and given by second order Langmuir kinetics:

$$\left(\frac{dCi}{dt}\right) = k_a\ (q_s - C_s)\ C - k_d C_s$$

where $k_a$ and $k_d$ are the rate constants of adsorption and desorption respectively and $q_s$ is the specific adsorption capacity of the adsorbent.

Modelling of liquid chromatographic systems is reviewed in detail by Bellot and Condoret (1991). They state that the plate theory is limited to linear adsorption cases while the differential model is more useful, allowing the incorporation of various physical and thermodynamic phenomena.

Golshan-Shirazi *et al* (1988) modelled non-linear chromatography, describing methods of measuring adsorption equilibrium isotherms. One technique described was frontal analysis. A stream of solution of the compound studied in the mobile phase, at concentration $C_i$, is equilibrated with the column until the detector baseline is stable. The stream is then abruptly replaced with another one, at the same flow rate, with a higher concentration, $C_b$. For concave (Langmuir type) isotherms, the resulting breakthrough curve is a self-sharpening front. An integral mass balance equation is then written to relate the amount sorbed by the stationary phase to the concentration in the new stream. On-column procedures such as this give more meaningful results than stirred batch adsorption studies since hydrodynamic conditions will be different, resulting in different external mass transfer characteristics.

Gosling *et al* (1989) describe a typical stirred batch method for producing adsorption isotherms where a batch of resin was agitated with the eluant which contained the adsorbate, aspartic acid. Samples were then withdrawn and analysed.

Chromatographic models developed (e.g. Taddei 1994) for bioreactor-separators such as that studied in this thesis have been modelled with the assumption that adsorption of a compound is linear and defined:

$$K_d = \frac{q}{C}$$

where q is the concentration of solute in the stationary phase and C is the concentration of solute in the mobile phase. Also it was assumed that the adsorption equilibrium is instantaneous. Linearity is applicable only to very dilute solutions. At higher concentrations, a marked departure from linearity occurs and peak position depends on sample size. This condition is known as overloading and results in non-linear chromatography, a characteristic of non-linear chromatography being that concave Langmuir isotherms apply (Taddei (1994). The solute concentrations studied in this thesis, typically ranging from 5-20% w/v, mean that some overloading is likely.

## 1.3 Kinetics of Lactose Hydrolysis

Elucidation of the kinetics of enzymic lactose hydrolysis is hindered by the complexity of the reaction. The reaction is complex due to the formation of oligosaccharides. These oligomers are formed as intermediates which eventually degrade to glucose and galactose. Higher concentrations of oligosaccharides are formed when higher initial lactose concentrations are used (Lopez-Leiva and Guzman, 1995). Iwasaki *et al* (1996) observed that complete hydrolysis of lactose to glucose and galactose is favoured by low initial lactose concentrations

The hydrolysis of lactose and the synthesis of oligosaccharide take place simultaneously, making the reaction mechanism highly complicated (Iwasaki *et al*, 1996) and, consequently, difficult to model. This complexity is exacerbated by the fact that the reaction undergoes competitive product inhibition by galactose (Friend and Shahani, 1982). Flaschel *et al* (1982), working with Aspergillus niger lactase, found that mutarotation of galactose is important. The α-form has 12 times the inhibitory effect of the β-form. It is possible that lactases from other sources also exhibit anomeric specificity. This highlights a problem encountered when searching for literature data, caused by the wide range of lactase sources studied. However, Aspergillus oryzae and Aspergillus

niger are the most common source organisms. The following appears to be the simplest plausible reaction scheme and is based on the reviews of Prenosil *et al* (1987) and Yang and Tang (1988).

$$E + L \Leftrightarrow [EL]$$

$$H_2O + [EL] \Leftrightarrow [E.Gal] + Gluc$$

$$[E.Gal] \Leftrightarrow E + Gal$$

$$[E.Gal] + L \Leftrightarrow E + Tris + H_2O$$

$$Tris + [E.Gal] \Leftrightarrow E + Tets + H_2O$$

where Tris refers to trimers and Tets to tetramers.

Here, mutarotation is ignored and only trisaccharides and tetrasaccharides are considered. Flaschel *et al* considered mutarotation while ignoring oligosaccharide formation. Inclusion of oligosaccharide formation and mutarotation would render the model too complex to be of practical use (Prenosil *et al* , 1987). In terms of modelling lactase kinetics in plug-flow reactors, such as batch chromatographic bioreactor-separators of the type used in this thesis, the classic Michaelis-Menten model for competitive product inhibition is used:

$$\frac{-d[L]}{dt} = \frac{Vmax[L]}{[L] + Km\left(1 + \frac{[Gal]}{Ki}\right)}$$

This expression is derived as follows, considering the following mechanism which ignores oligosaccharide formation:

$$(1) \qquad E + L \underset{k-1}{\overset{k1}{\Leftrightarrow}} [E.L]$$

$$(2) \qquad [E.L] + H_2O \overset{k2}{\Rightarrow} [E.Gal] + Gluc$$

$$(3) \qquad [E.Gal] \underset{k-3}{\overset{k3}{\Leftrightarrow}} E + Gal$$

Assuming (2) is the slowest and rate-controlling step:

Rate of Lactose consumption (in a well-mixed batch reactor)

$$= -\frac{d[L]}{dt} = k\,[E.L]\,[H_2O]$$

For (1) and (3), using the pseudo-steady-state approach:

$$k_1[E][L] \approx k_{-1}[E.L]$$

$$k_3[E.Gal] \approx k_{-3}[E][Gal]$$

Also, total enzyme concentration,

$$E_0 = [E] + [E.L] + [E.Gal]$$

These relationships can now be used to show that:

$$\frac{-d[L]}{dt} = \frac{\left(k_2[E]_0[H_2O]\right)[L]}{\left(\frac{k_{-1}}{k_1}\right) + [L] + \left(\frac{k_{-1}}{k_1}\right)\left(\frac{k_{-3}}{k_3}\right)[Gal]}$$

Introducing the usual symbols:

$$\frac{-d[L]}{dt} = \frac{Vmax[L]}{[L] + Km\left(1 + \frac{[Gal]}{Ki}\right)}$$

where $Vmax = k_2[E]_0[H_2O]$

$$Km = \frac{k_{-1}}{k_1}$$

$$Ki = \frac{k_3}{k_{-3}}$$

This approach mirrors that of Yang and Okos (1998) who went on to incorporate the kinetic expression into a mathematical model to study the effect of temperature on lactose hydrolysis in an immobilised enzyme reactor. Ignoring oligosaccharide formation resulted in only a small degree of lack of fit between this model and the data. This kinetic model can and has been (Taddei

1994) incorporated into a mathematical model describing a batch chromatographic bioreactor separator of the type used in the thesis.

**Integration of the Rate Equation**

Integration of the Rate Equation results in the following expression:

$$\frac{-d[L]}{dt} = \frac{Vmax\,[L]}{[L] + Km\left(1 + \frac{[Gal]}{Ki}\right)}$$

assuming $[L]_0 - [L] = [Gal]$

and $[L] = [L]_0$ at $t = 0$.

Separating the variables, [L] and t, substituting for [Gal] and then integrating:

$$Vmax \int_0^t dt = -\int_{[L]_0}^{[L]} \left(1 + \frac{Km}{[L]} + \frac{Km}{Ki} \cdot \frac{[L]_0}{[L]} - \frac{Km}{Ki}\right) . d[L]$$

gives:

$$Vmax\,t = \left(1 - \frac{Km}{Ki}\right).([L]_0 - [L]) + \left(\frac{Km}{Ki}[L]_0 + Km\right) \ln \frac{[L]_0}{[L]}$$

This expression can be used to estimate the kinetic constants or to estimate conversion in a plug-flow reactor of residence time t, having set the enzyme concentration $[E]_0$.

## 1.4 Research Objectives

The preliminary aim of this work was to improve a chromatographic bioreactor-separator system which was able to effectively separate glucose and galactose. Once this was achieved, the plan was to study other enzymic reactions. To improve separation efficiency, an increase in the number of theoretical plates was required. This can be achieved by a reduction in resin particle size, and this was the approach followed, while simultaneously overcoming the operational problems associated with the use of a resin of small particle size.

The aim was then to investigate the factors which affect enzymic reaction in a chromatographic bioreactor-separator (CBRS), such as injector design, before selecting a different enzyme reaction for study. It was during these studies that oligosaccharides were detected. Consequently, the same enzyme system was retained, but emphasis was changed from the end products of lactose hydrolysis, glucose and galactose, to maximising the yield of oligosaccharides, which form as intermediates in this reaction.

# Chapter Two

## The Experimental Programme and Thesis Structure

### 2.1 Evolution of the Experimental Programme

The initial aim of this project was to improve the performance of a chromatographic bioreactor-separator, with the enzymic hydrolysis of lactose as the reaction of study. In such a system, a dilute enzyme solution is pumped continuously through a preparative chromatographic column, while pulses of substrate are periodically injected on to the column. Enzymic reaction and separation are therefore performed in a single unit operation.

From previous work in this department, there appeared to be scope for improving the separative performance, so this was adopted as an initial goal. Reducing the particle size of the stationary phase was identified as a way of achieving this improvement. A chromatographic bioreactor-separator (CBRS) system was developed which overcame the operational problems (such as high pressure drop development) associated with use of such a particle size. For example, HPLC pumps were adapted for use in pulse and eluant delivery.

To enable quantitative evaluation of any performance improvements, the CBRS used was geometrically similar to that used by Taddei (1994) and an operating flow rate selected so as to give the same interstitial velocity. A significant improvement in separative power was achieved.

A simple experiment with the 1 metre column that combined bioreaction and separation was achievable in this system. It was realised that separative performance is poorer under combined bioreaction and separation conditions than when the column is operated purely as a separator. It was thought likely that this is because a finite time is needed for enzymic reaction, leaving the remainder of the column residence time for product separation. Thus, having improved the separative performance of the system, it was decided to investigate the reaction characteristics. The system studied so far, the enzymic hydrolysis of lactose in the presence of β-galactosidase, was chosen for these experiments. The approach taken was to progressively decrease column residence time while monitoring

degree of lactose conversion. This enabled the progress of reaction to be measured and related to the degree of separation observed during the corresponding residence time. In this way, the relative time scales of reaction and separation were examined. This gave an indication of the likelihood of interaction between these two processes.

The effect of varying pulse volume was shown to be significant, leading to a study of mixing between enzyme and substrate at the point of injection. The hydrodynamic characteristics of the two injector configurations used in CBRS construction: sinter type and needle type were first investigated. The technique of Magnetic Resonance Imaging was used to supplement hydrodynamic calculations. A study was then made of the chromatographic bioreaction and separation process, considering the relative contributions of bulk liquid mixing, internal and external mass transfer and adsorption to the rate of the overall process. The effect of injecting a pulse over a length of time, as in chromatographic bioreaction and separation, was then simulated by fed batch experiments. These experiments were performed in parallel with normal batch experiments where the substrate is mixed almost instantly with the enzyme. The fed batch experiments provided a crude simulation of a pulse being injected into the CBRS where, due to the expected plug-like flow conditions, gradual mixing of enzyme and lactose solution would be anticipated.

An interesting observation made during the earlier experiments was the production of an unknown carbohydrate, which was tentatively identified as galactooligosaccharide (GOS). Reference to the literature supported this theory as such sugars are commonly produced under conditions favouring incomplete lactose conversion, such as higher initial lactose concentrations (Yang and Tang, 1988). It was also discovered that GOS are valuable products with potential and existing applications, particularly in food manufacture, where their incorporation into foods can promote the growth of certain bacteria in the gut (Ito *et al*, 1993), which are purported to have health benefits. In this way, GOS are said to have a *nutraceutical* role.

Batch experiments and a review of the literature also revealed that GOS are formed as intermediates *en route* to complete lactose hydrolysis (Prenosil *et al*, 1987, Yang and Okos,1988, Lopez-Leiva and Guzman, 1995). With this in mind, it was hypothesised that the strategies applied to reaction control in the residence time studies could be used to manipulate reaction to optimise yield of GOS. According to Lopez-Leiva and Guzman (1995) these GOS intermediates eventually break down to their component monosaccharides, glucose and galactose; this was confirmed by the results of experiments in a 1 metre long CBRS. A means of controlling reaction was also needed to arrest break down of GOS; raising temperature was identified as a possible means of achieving this within a CBRS.

This led to the conception of the dithermal chromatographic bioreactor-separator (DTCBS), which had two discrete temperature zones: the first zone kept at a temperature favouring reaction with the second zone being heated to a temperature sufficiently high to inhibit further reaction and breakdown of GOS. Increased temperature, it was thought, should also benefit product separation. Pilot experiments on a 1 metre column length showed that the temperature selected for the separation section, 82°C, was sufficiently high to reduce reaction rate considerably.

The method of operation was designed such that GOS levels were allowed to reach a peak while leaving the maximum possible length of column for purification. The CBRS was operated at a high flow rate over the 1 metre long reaction zone, giving a residence time which enabled GOS concentrations to reach a maximum. A step change in flow-rate, down to the normal CBRS separation flow-rate, was then imposed, accompanied by an increase in temperature, such that further reaction was inhibited and product separation was promoted.

It was estimated that a 3 metre long separation section would give adequate purification of GOS, making an overall length of 4 metres, which was the practical upper limit of column length. Repeated stirred batch experiments were then performed to amass a significant volume of GOS-rich product mixture; pulses of this mixture were subsequently applied to the 3 metre long column to demonstrate

its ability to purify the GOS fraction. Also, sufficient volumes of GOS were generated to permit GPC analysis and confirmation of the purified material as being GOS.

The 4 metre long x 1.5 cm i.d. dithermally operated chromatographic bioreactor-separator (DTCBS) was then designed and built. The DTCBS was shown to produce pure GOS, whereas isothermal operation did not.

## 2.2 Thesis Structure

**Chapter 1** describes the background to the thesis and introduces the initial research objectives.

**Chapter 2** describes how the experimental programme evolved, together with an outline of the thesis structure. Because of extensive coverage by previous Aston researchers a *brief* review of chromatographic bioreactor-separators is presented at the beginning of the thesis report; the reader is also referred to the extensive literature reviews presented by Taddei (1994) and Shieh (1994). Relevant literature is then incorporated into the appropriate chapters, reflecting how the focus of the thesis changed from the original objectives.

**Chapter 3** is concerned with the detailed design of the chromatographic bioreactor-separator system used by the author.

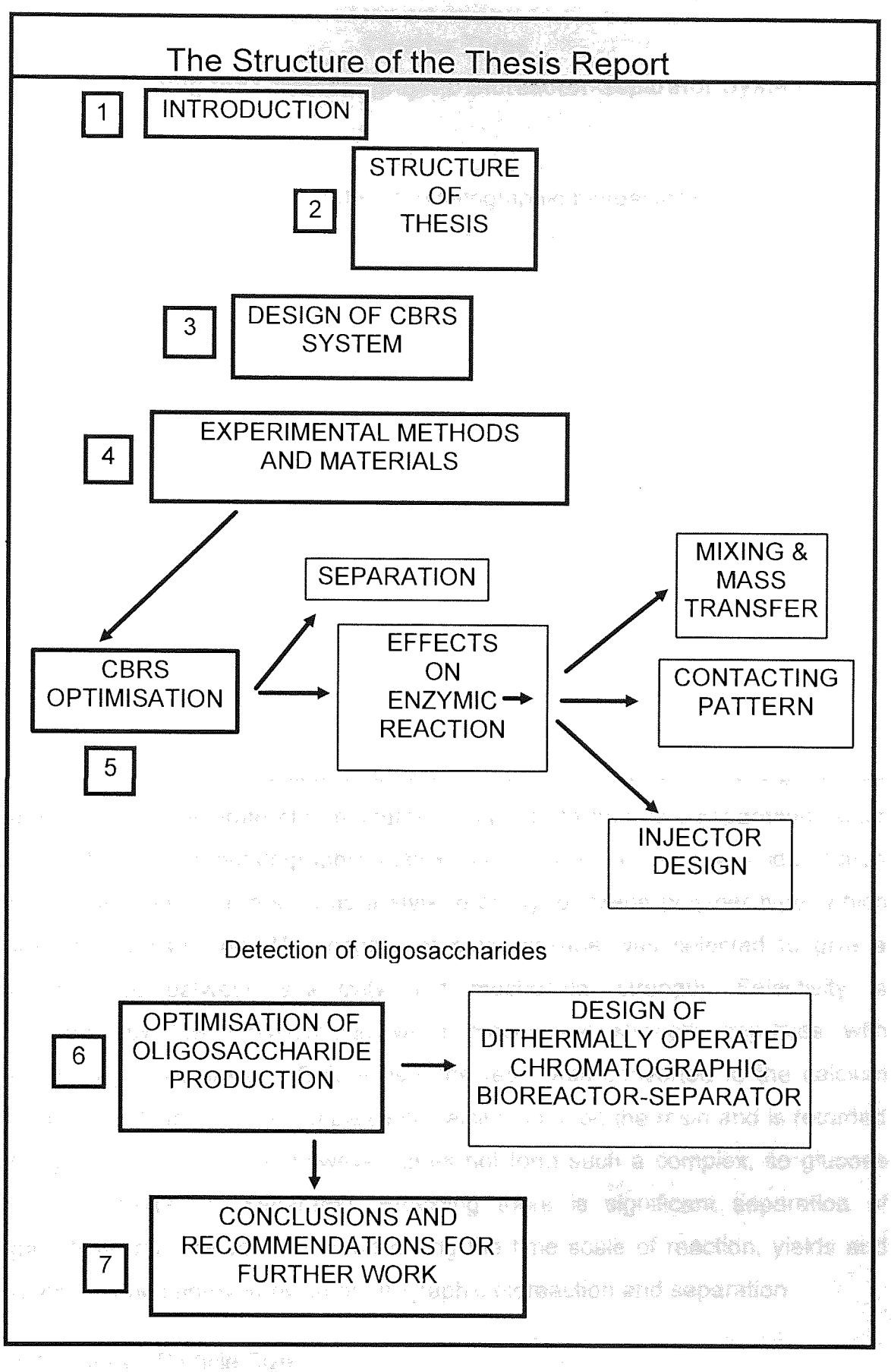
**Chapter 4** describes the core experimental methods and materials used throughout the research.

**Chapter 5** focuses on optimising CBRS operation. The chromatographic bioreaction and separation process was broken down to its component processes, such as substrate pulse injection, bulk mixing between the substrate and the enzyme eluant, reaction, mass transfer and product separation. These processes were considered in isolation, together with their combined effects.

In **Chapter 6**, the focus of the research changes to oligosaccharide production, using the experience gained in the CBRS optimisation studies to design and build a dithermally operated chromatographic bioreactor-separator.

In **Chapter 7**, the conclusions of the research are laid down, followed by recommendations for further work.

The structure of the thesis report is described diagramatically overleaf.


The Structure of the Thesis Report
1
INTRODUCTION
2
STRUCTURE OF THESIS
3
DESIGN OF CBRS SYSTEM
4
EXPERIMENTAL METHODS AND MATERIALS
CBRS OPTIMISATION
5
SEPARATION
EFFECTS ON ENZYMIC REACTION
MIXING & MASS TRANSFER
CONTACTING PATTERN
INJECTOR DESIGN
Detection of oligosaccharides
6
OPTIMISATION OF OLIGOSACCHARIDE PRODUCTION
DESIGN OF DITHERMALLY OPERATED CHROMATOGRAPHIC BIOREACTOR-SEPARATOR
7
CONCLUSIONS AND RECOMMENDATIONS FOR FURTHER WORK

# Chapter Three

# Designing the Chromatographic Bioreactor-Separator System

## Scope of Chapter

In this chapter, the design of the chromatographic bioreactor-separator system is discussed. Reasons are given for the for selection of key components, and routine operational features are described. Since the reaction to be studied in the chromatographic bioreactor-separator is the hydrolysis of lactose in the presence of the enzyme β-galactosidase, the interaction (with the stationary phase) of the enzyme, lactose and the hydrolysis products glucose and galactose is considered.

## 3.1 The Stationary Phase

### 3.1.1 Choice of Matrix

The principal reaction studied was the enzymic hydrolysis of lactose to glucose and galactose in the presence of the enzyme β-galactosidase. Separation of galactose from the reaction zone may be beneficial since galactose has been shown to inhibit this reaction (Friend & Shahani 1982). Glucose and galactose are difficult to separate chromatographically due to their low separation factor (1.2-1.4). The chromatographic matrix chosen was a strongly acidic cation exchange resin. The resin was a styrene-divinyl benzene polymer type, which was 8% cross-linked. This degree of cross linkage was selected to give a compromise between selectivity and mechanical strength. Selectivity is favoured by low cross-linkage while mechanical strength improves with increasing cross-linkage. Before use, the resin was converted to the calcium form. Galactose forms a complex with calcium ions on the resin and is retarded (Angyal 1973). Glucose, however, does not form such a complex, so glucose and galactose are separated. Providing there is significant separation of galactose from the reaction zone during the time scale of reaction, yields and rates may be improved by chromatographic bioreaction and separation.

### 3.1.2 Resin Particle Size

In previous work at Aston, Taddei (1994) and Shieh (1994), used a similar resin which had an average particle size of 150-200 μm. In order to improve the resolution of glucose and galactose, a resin of smaller average particle

diameter was chosen for this work. The particle size of the resin was stated by the supplier to be 38-75 μm when dry and in the hydrogen form. Ion exchange resins tend to swell to varying degrees when wet and resin volume may change with ionic form. The particle size of the resin was therefore determined, using a laser scattering particle size analyser (Malvern instruments). In early experiments, it became apparent that the resin could not be readily suspended in aqueous solution; the particles settled too rapidly during the laser analysis, leading to inconsistent results. Resin samples were therefore prepared for analysis by suspension in 20% w/v calcium nitrate. This minimised the density difference between resin and suspending liquid, so that the Stokes settling velocity was insignificant when compared to the time-scale of the analysis. 90% of the resin particles were found to lie within the size range: 57-99 μm and this was found to be consistent when comparing different batches of resin. The median value, based on this range is 78 μm; since the size distribution curve appeared normal over this range, this value was used for calculation purposes. The size distribution is shown in Figure 3.1

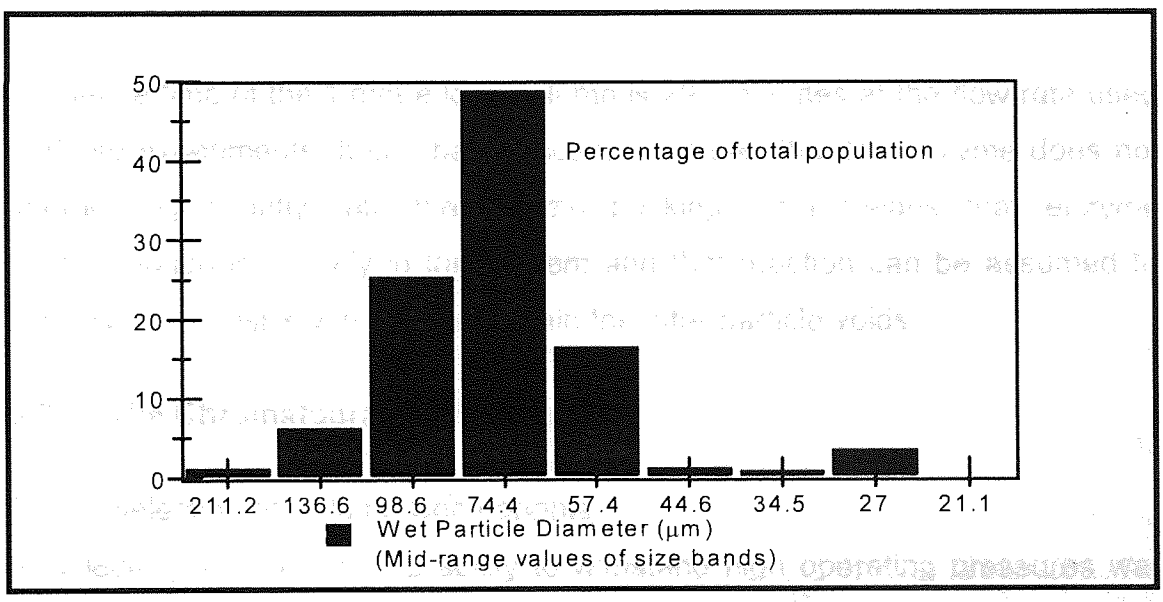



**Figure 3.1 Particle Size Distribution of a Typical Batch of Ion Exchange Resin.** Size was determined by a laser scattering particle size analyser. 90% of the resin particles were found to lie within the size range: 57-99 μm.

### 3.1.3 Interactions With The Column Packing

The molecular weight exclusion limit of the resin was stated by the manufacturers to be 1000 for globular proteins. The molecular weight of lactase from Aspergillus oryzae is 90,000 dalton (Gekas and Lopez-Leva,1985). The enzyme, therefore, will not enter the pores of the resin and is not likely to be retained. However, glucose and galactose, having a molecular weight of 180,

will be able to enter the pores of the resin. Disaccharides and oligosaccharides will be separated from glucose and galactose by size-exclusion dominated mechanisms. Any immobilisation of the enzyme, however transient, would have a bearing on the chromatographic reaction.

The interaction of the enzyme and sugars with the column packing was investigated in a 1 m long x 1.5 cm i.d. column. Pulses of glucose, galactose, lactose, dextran 2000 kd and enzyme solution were added to the column and eluted at 2.1 $cm^3$ $min^{-1}$. The residence times were measured and mean values are presented in Table 3(a).

**Table 3(a) Residence Times of Pulses Added to the 1 m long x 1.5 cm i.d Column.** Pulse sizes were equivalent to 1.25% of the volume of the column when empty. Experiments were performed in triplicate and varied by no more than 0.1 minutes from the mean in each case.

| Enzyme | Dextran 2000 kd | Lactose | Glucose | Galactose |
|---|---|---|---|---|
| 30.6 min | 30.7 min | 34.5 min | 38.7 min | 43.2 min |

Within experimental error, the residence time of the enzyme pulse was identical to that of Dextran 2000 kd, which is not retained due to its size. The calculated residence time of the 1 metre long column is 29.3 minutes at the flow rate used in these experiments. It can be deduced, therefore, that the enzyme does not interact significantly with the column packing. This means that enzyme immobilisation is unlikely in this system and that reaction can be assumed to take place exclusively in solution, within the inter-particle voids.

## 3.2 The Chromatographic Column

### 3.2.1 Selection of Column Components

In selecting the column, the ability to withstand high operating pressures was essential, due to the small diameter packing which was to be used. Also, to enable comparison, geometric similarity to previous systems was sought (Taddei (1994) and Shieh (1994) used a column of 1.96 cm i.d.).

Process visibility was also considered important, due to the possibility of gas break-out and microbial fouling with their adverse effects on separation. Additionally, packing the column is easier if the column can be viewed internally. For these reasons, borosilicate glass was selected as the material of construction. Columns were supplied (Omnifit, Cambridge, UK) in 1 metre

lengths of 1.5 cm internal diameter, with an external thread at each end to allow attachment of end-pieces. Two-metre long columns were formed by fusing two one-metre columns which had been supplied by the manufacturers with a thread at one end only. By using combinations of one and two metre lengths, 1, 2, 3 and 4 metre-long columns could be constructed.

The maximum feasible operating flow rate was 10 $cm^3$ $min^{-1}$, this limit being the maximum output of the pumps. The maximum pressure drop that would be encountered across the 4 metre long column was estimated to be 5.5 Bar (550 KPa) using the Carmen-Kozeny equation (Holland and Bragg 1995):

$$\Delta P = \frac{180 \cdot (1-\varepsilon)^2 \cdot \mu \cdot L \cdot u}{(\varepsilon^3 \cdot dp^2)}$$

Voidage ($\varepsilon$) was estimated at 0.35 (see Appendix A-2 for calculation). The manufacturers stated that the columns could withstand an operating pressure of 20 bar (2 MPa).

PTFE tubing and fittings were used for the necessary connections. Small internal diameter tubing (0.8 mm I.D.) was used for inter-column connections, so that plug flow conditions were encouraged. Connectors were chosen which minimised dead volume, thereby reducing the likelihood of mixing. Experiments involving the introduction of glucose and galactose pulses demonstrated that the 2 metre length of tubing used to connect columns had no effect on the degree of dispersion. This was shown by there being no difference in NTP values when the 2 metre length of tubing was inserted between the column exit and the detector. The residence time in the connecting section was negligible (1.7 seconds) compared to that for the column (29.3 min per metre of column length) at a volumetric flow-rate of 2.1 $cm^3$ $min^{-1}$.

### 3.2.2 Column Sanitation

Occasionally, microbial growth was seen on the column packing, in some of the early experiments, indicated by pink colouration of the resin. A means of sanitation was therefore required. Three eluant treatments were considered:

(i) Sodium azide

(ii) 20% ethanol

(iii) Sodium bisulphite (150 ppm).

Sodium azide is commonly used to store HPLC columns during shut-down periods. However, it was thought unsuitable for these experiments due to its high toxicity, thus creating handling hazards. Also, it is not suitable for use in what could feasibly be a food grade process. Sodium bisulphite is frequently used as a food preservative ingredient and, like ethanol, presents no toxicity problems at these concentrations.

The efficacy of the latter two treatments was evaluated as follows. A sample of infected column packing was taken and stirred to ensure homogeneity of contamination. Three 5 g samples of resin were placed in three sterile, screw-capped bottles. One sample was suspended in 3 ml 20% ethanol, and another in distilled and deionised water containing 150 ppm sodium bisulphite. 3 ml distilled and deionised water were added to the remaining sample. The three samples were then left on the bench for 48 hours, simulating a weekend shut-down period. 5 ml sterile saline were then added to each bottle and mixed by shaking. 0.2 ml saline was then aseptically removed before spreading on aerobic and anaerobic nutrient agar. The plates were next incubated at 37°C for 3 days. One yeast colony was seen on the aerobic ethanol plate while no growth was seen on the plates from the bisulphite treatment. 150 ppm sodium bisulphite was added to all distilled and deionised water passed through the columns in later experiments. No further problems of microbial growth were experienced.

### 3.2.3 Pre-column filtration

Although the enzyme solution had been centrifuged, some particulate material was visible. In order to protect the column packing against fouling, a 10 cm long x 1.5 cm i.d. guard column was fitted in line after the eluant pump.

## 3.3 Hydrodynamic Considerations

### 3.3.1 Flow Rates Employed

The operating flow rates chosen for the experiments were dictated by the pump output range and the ability of the column and associated plumbing to

withstand the accompanying back-pressures. Resin particle size combined with interstitial liquid velocity influenced the flow patterns generated within the column; in particular, the degree of axial dispersion. Some axial dispersion is needed for the substrate pulse to mix with the enzyme solution so that reaction can take place; excessive axial dispersion, however, would result in poor chromatographic separation. Flow conditions must be close enough to plug flow (as defined, for example, by Levenspiel, 1972), to enable chromatographic separation, but with sufficient axial dispersion to allow intimate contact between enzyme and substrate. Hydrodynamics will be considered further in Chapter 5.

### 3.3.2 Pressure Drop Measurement.

Prior to packing the column, the system was pressure tested using deionised water. It was necessary to partially close off the valve at the column exit in order to generate the desired pressure. Pressure was indicated by two Budenberg gauges, one placed after each HPLC pump, indicating the pressure drop across the column. A sudden drop in pressure indicated a leak, while pressure pulsation was symptomatic of pump malfunction.

### 3.3.3 Reynolds Number

For the range of flow rates and temperatures used, Reynolds numbers ranged 1 to 15 (based on interstitial velocity); these values fall within the laminar region. The method of calculation used, together with the significance of these values will be discussed in detail in Chapter 5.

## 3.4 Injector Systems

The chromatographic reaction/separation injector systems used in this work are described in Figure 3.2. The injection assemblies were constructed from PTFE. The sinter injector was used exclusively in chromatographic bioreactor-separator experiments in this work. Liquid entered the column via a 1 mm diameter orifice, passing immediately through the frit. The frit was stated by the manufacturers (Omnifit, Cambridge, UK) to have an average pore size of 70 μm and a voidage of 0.53. The needle injector system was constructed so as to mimic the injector types used by Taddei (1994) and Shieh (1994). This enabled comparison of the flow patterns generated by each injector using Magnetic Resonance Imaging (which is discussed in Chapter 5).

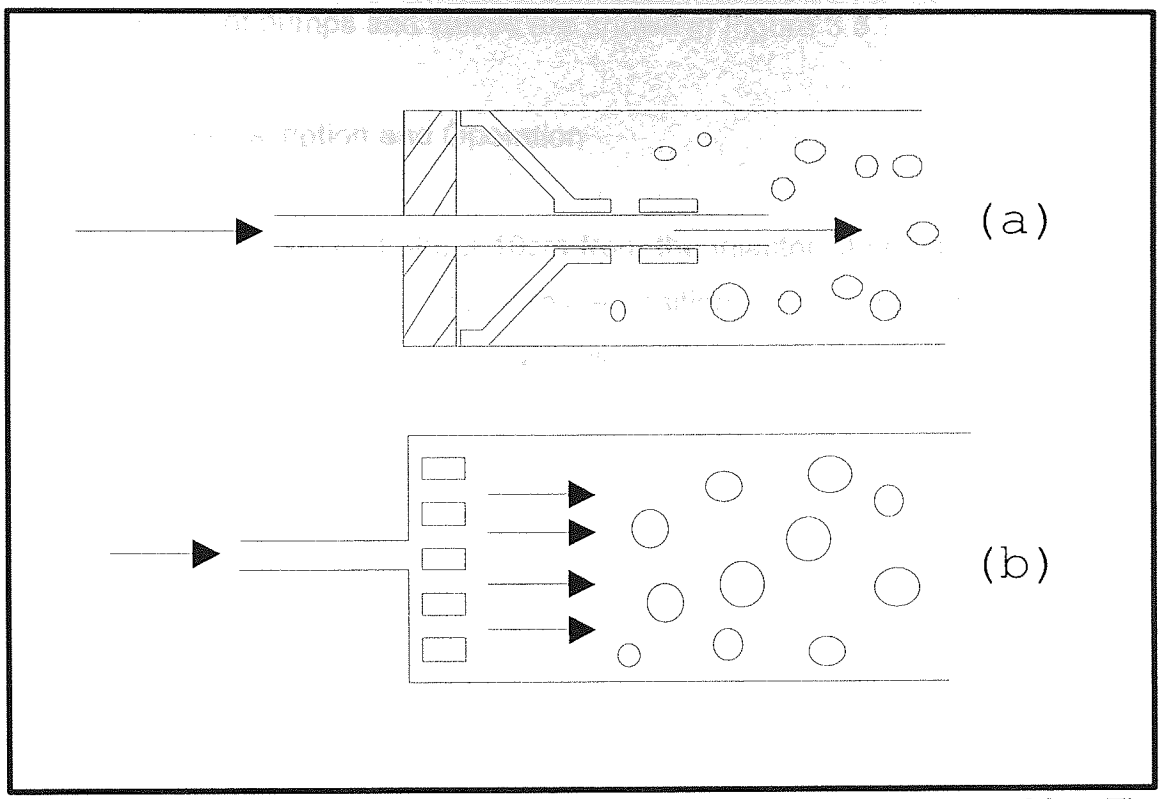



**Figure 3.2 Injection Assemblies (a) Needle Injection Assembly.** The terminus of a 3 m long x 0.8 mm i.d. PTFE tube formed the needle through which both pulses and eluant were injected into the column, about 3 cm along its packed length.**(b) Dispersed Flow Injection Assembly.** The injected liquid was dispersed by a porous frit composed of compressed PTFE fibres, which was placed on top of the column packing.

## 3.5 Description and Operation of Pumps and Valves.

### 3.5.1 Pump Description

The pumps used in this system were as follows:

Pump 1 (P1) HPLC pump (Alltech Model 301, Carnforth, UK)

Pump 2 (P2) HPLC pump (BioRad Model 1330, Watford, UK)

Pump 3 (P3) Peristaltic proportioning pump (Technicon Auto Analyser (London, UK).

Pumps 1 and 2 were standard HPLC pumps, selected for accurate, pulse free delivery against high back pressures. Pump 3 was fitted with two lengths of peristaltic tubing, each taking 0.8 $cm^3$ $min^{-1}$ from the column effluent flow.

The locations of pumps and valves are shown in Figure 3.3.

### 3.5.2 Valve Description and Operation

Valve 1 (V1) was located about 10cm from the injector. It could be switched to direct eluent flow on to the column (elute position), or, alternatively, to its purge position. In the purge position, flow could be recirculated to the eluant tank or, more commonly, to the 50 $cm^3$ burette which was used to check flow rates. V1 was electronically operated, controlled by a switch at the base of the column.

Valve 2 (V2) was positioned on top of the injector. In its pulse position it allowed substrate pulses to flow on to the column. In its purge position it enabled the line to be purged with sugar solution right up to the injector itself. V2 was operated manually and, while in its purge position, prevented accidental introduction of carbohydrate solution on to the column.

Valve 3 (V3) was situated directly (about 10 cm) before V2. In its pulse position it allowed substrate pulses to flow on to the column. In its purge position it enabled the line to be purged with sugar solution right up to V2. V3 was operated electronically and controlled from the same control box as V1, at the base of the column.

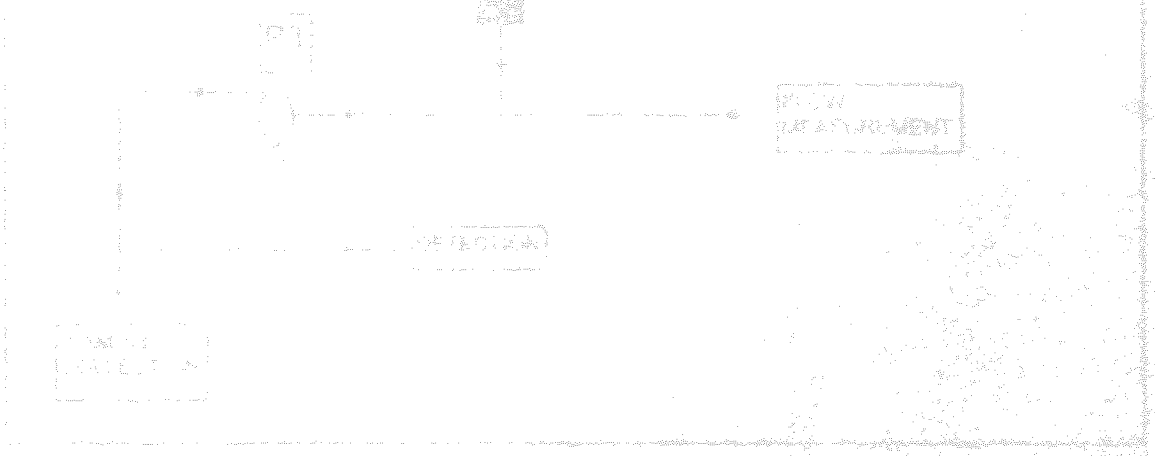


Figure 3.3 Pump and Valve Layout for the Chromatographic Bioreactor-Separator. Pumps are labelled P1 to P3 while valves are labelled V1 to V3. Valve conditions are summarised in Table 3(b) overleaf.

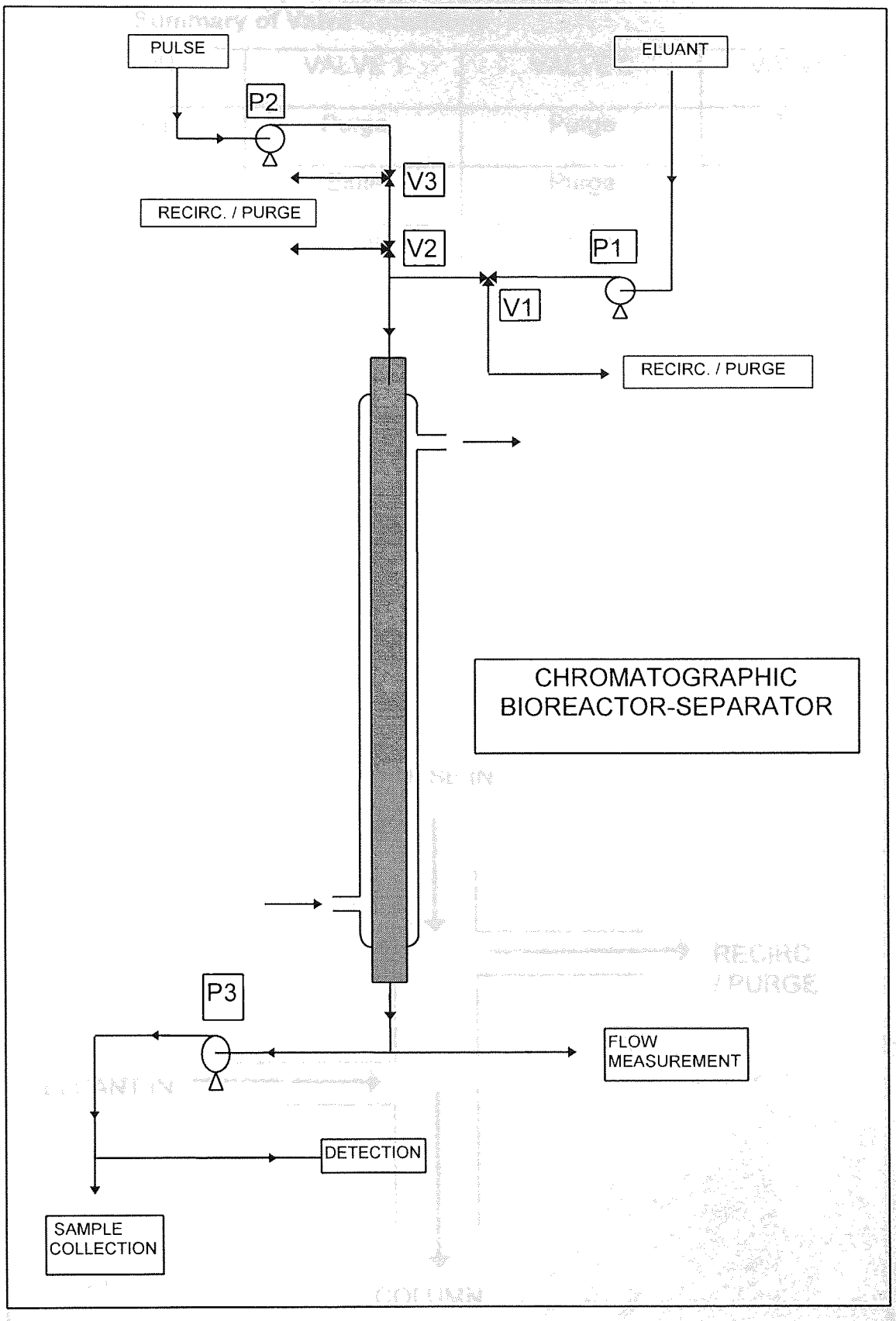



**Figure 3.3 Pump and Valve Layout for the Chromatographic Bioreactor-Separator.** Pumps are labelled P1 to P3 while valves are labelled V1 to V3. Valve conditions are summarised in Table 3(b) overleaf.

**Table 3(b) Summary of Valve Conditions**

| OPERATION | VALVE 1 | VALVE 2 | VALVE 3 |
|---|---|---|---|
| Line Purging | Purge | Purge | Purge |
| Elution | Elute | Purge | Purge |
| Pulse Introduction | Purge | Pulse | Pulse |

Flow paths through the valves are shown in Figures 3.4(a) and 3.4(b).

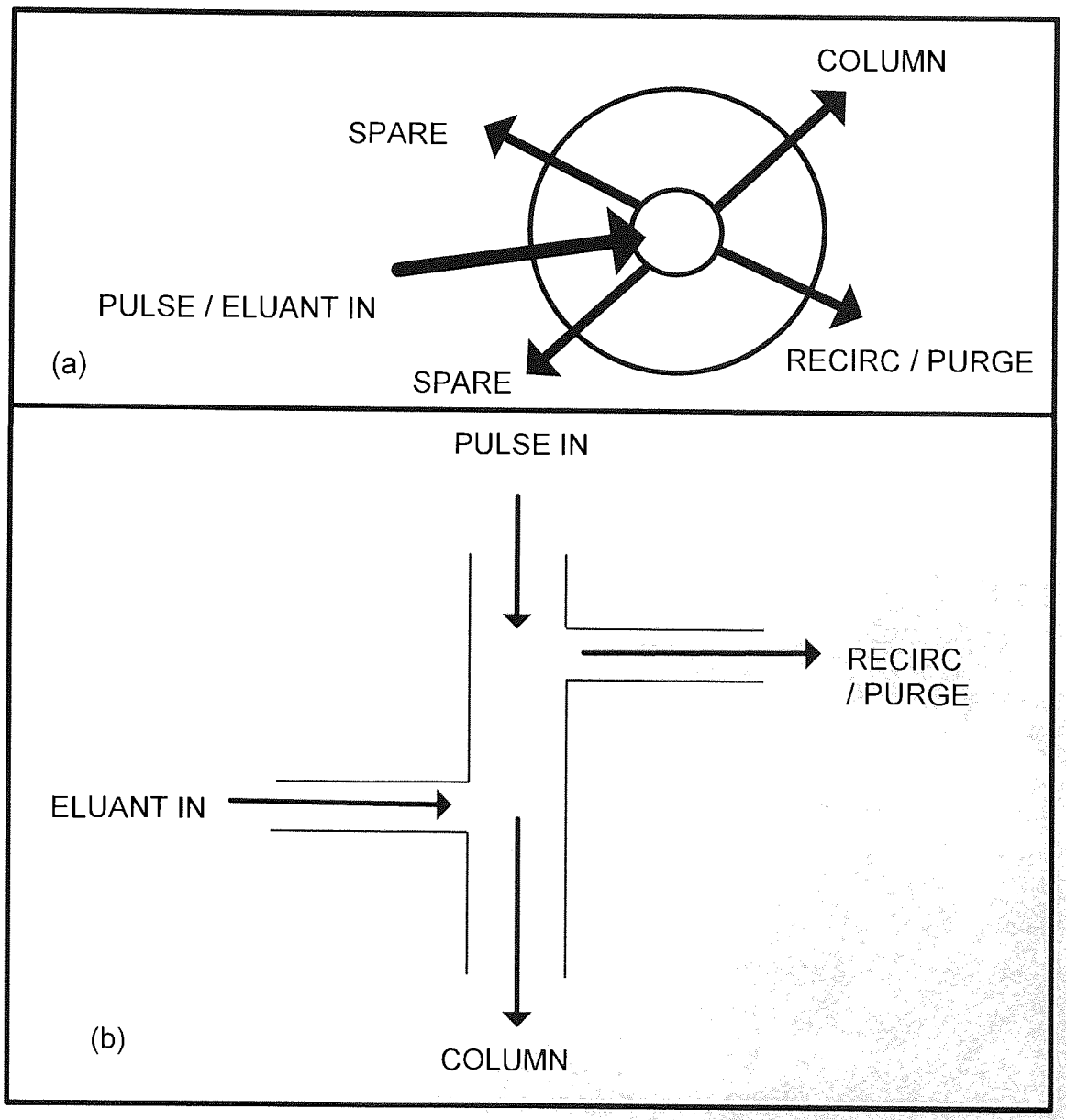



**Figure 3.4 Flow paths through the electronic valves V1 and V3 (a) and the manual valve V2 (b).** In each of the electronic valves, the two spare valve outlets were not blanked-off. This meant that if either valve was accidentally switched to either position, the pump would not be forced to operate against a dead end.

### 3.6 Column Heating and Temperature Measurement

The heating system consisted of an insulated steel tank with a stirrer-type thermostatic heater. Heated water was circulated through the reactor heating jackets via a central-heating type pump (Gundfos Selectric), together with copper piping (1.1 and 2.2 cm i.d.). Temperature was monitored by a series of four thermocouples (R.S. Components), linked to an eight channel selector (Comark 2508), which was, in turn, connected to an L.E.D. indicator (Comark 2501).

# Chapter Four

## Core Experimental Methods and Materials

### Scope of Chapter

Column packing is discussed, along with preparation of carbohydrate and enzyme solutions. There then follows a description of the sequence of operations involved in adding a pulse of sugar solution to the column. The process is almost identical for both characterisation experiments and bioreaction-separations. The only significant difference is that degassed and deionised water is substituted for enzyme solution when characterising the column. The means of monitoring column effluents are described, as are the methods of sample collection, quenching and analysis.

### 4.1 Column Packing

The resin was mixed with distilled and deionised water to form a slurry then washed five to six times to remove fines. The column end-piece was removed and the column filled with resin by pouring the resin slurry from a beaker while stirring the resin constantly. The column end-piece was replaced and deionised water pumped through the bed at a flow-rate of 3.0 $cm^3$ $min^{-1}$. The level of the resin was then marked and flow continued until the resin level was constant.

Supernatant liquid was removed via a syringe with a 0.5 m length of silicone rubber tubing attached until a depth of approximately 5 cm of supernatant remained. The top 5 cm of packed bed was mixed with the supernatant before adding further resin. This mixing reduced layering due to gravity settling. The resin addition process was repeated until the column was filled to around 2 cm below the top of the column.

#### 4.1.1Conversion of Resin to the Calcium Form

The resin was converted from the hydrogen form (as supplied) to the calcium form prior to use. The conversion was performed *in situ* by passing the equivalent of four void volumes of 10% w/v calcium nitrate through the column at a flow rate of 2.1 $cm^3min^{-1}$. The resin was then eluted overnight with degassed deionised water at 55°C and a flow rate of 2.1 $cm^3min^{-1}$. This step served not only to remove excess calcium nitrate, but also to evacuate air bubbles trapped in the voids of the bed. In order to maintain optimal column

performance, the ion exchange resin was regenerated periodically, typically after ten enzymic runs. Regeneration involved repetition of the process by which the resin was converted to the calcium form.

## 4.2 Eluant Preparation

### 4.2.1 Distilled and Deionised Water

When the column was not being used for simultaneous bioreaction and separation, the eluant used was deionised and distilled water. This water was used to make up enzyme and carbohydrate solutions. 150 $mgl^{-1}$ sodium bisulphite was added to prevent microbial growth on the column packing.

### 4.2.2 Preparation of enzyme solutions

Water was degassed by heating to 100°C and placed in a volumetric flask. The flask was then filled to its lip, to allow for liquid contraction on cooling to 20-25°C. The appropriate quantity of enzyme was weighed out and just enough of the degassed water added to form a thin paste. This paste was ground with a plastic stirring rod until no enzyme particles were visible. The remaining degassed water was then added and the mixture placed on a magnetic stirrer. The pH of the enzyme solution was adjusted from, typically, a value of 6.2, to pH 5.2, the optimum pH for enzymic reaction. Sodium acetate buffer was then added to give a final concentration of 0.0001m.

## 4.3 Preparation of Carbohydrate Solutions

Carbohydrate solutions were prepared by weighing out the appropriate amount of carbohydrate and dissolving in degassed deionised water. Solutions were then heated to 100°C while stirring, thereby expelling any air introduced while adding the carbohydrate powder. The carbohydrate solutions used in this work were prepared from powders on a percentage weight / volume (% w/v) basis.

## 4.4 Characterising the Chromatographic Columns.

Characterisation enabled values for HETP, N, Kd and Separation Factor (see Appendix A-1) to be obtained under conditions representative of those of the experiments. Pulse volume was equivalent to 1.25% of the total empty column volume (TECV) and the pulse concentration was 2% w/v sugar. Low sample volumes and concentrations were selected so that characterisation could be carried out under conditions of infinite dilution. The eluant used was distilled and deionised water, which had a pH of around 5.5. The eluant flow-rate

chosen was 2.1 $cm^{-1}$ $min^{-1}$, selected such that it was representative of the flow-rates to be used in the planned CBRS experiments. Characterisation was carried out at 55°C, the normal operating temperature of the chromatographic bioreactor-separator.

## 4.5 Operation of the Chromatographic Bioreactor-Separator.

The layout of the Chromatographic Bioreactor-Separator system is illustrated by Figure 4.1 overleaf.

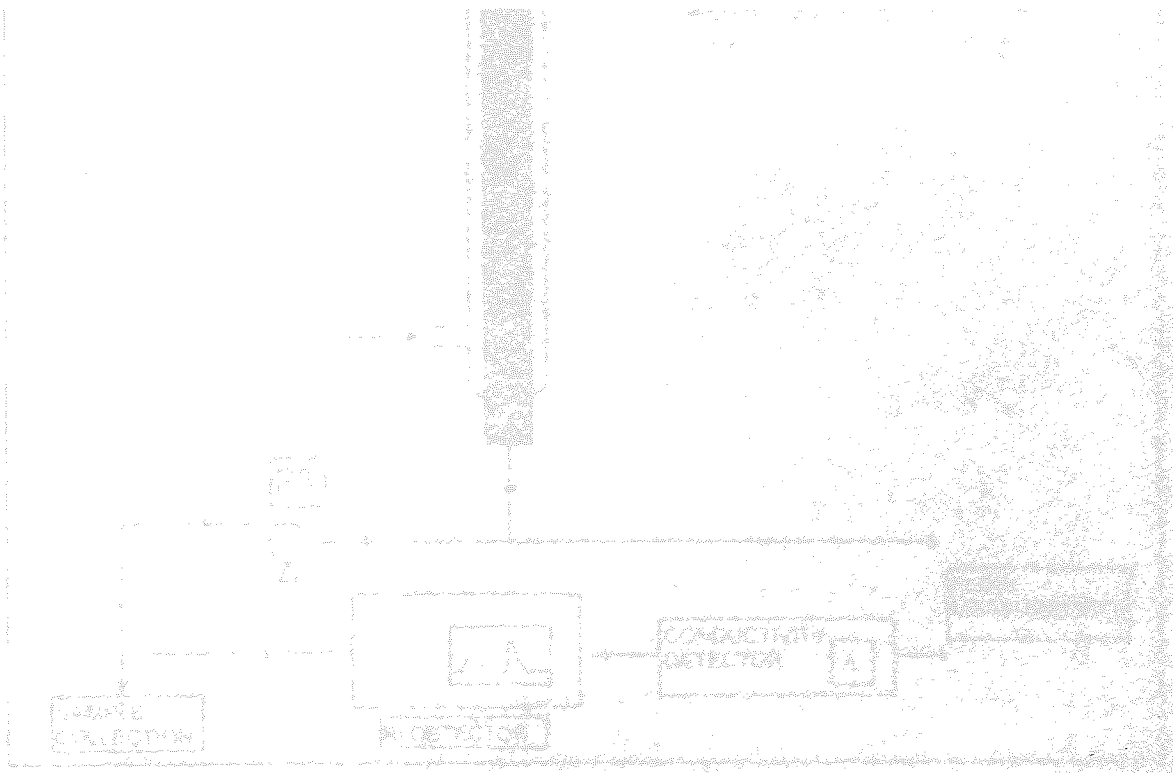


Figure 4.1 Flow Diagram Depicting the Chromatographic Bioreactor-Separator system showing the layout of key components. A detailed description of pumps and valves is given in Chapter 3.

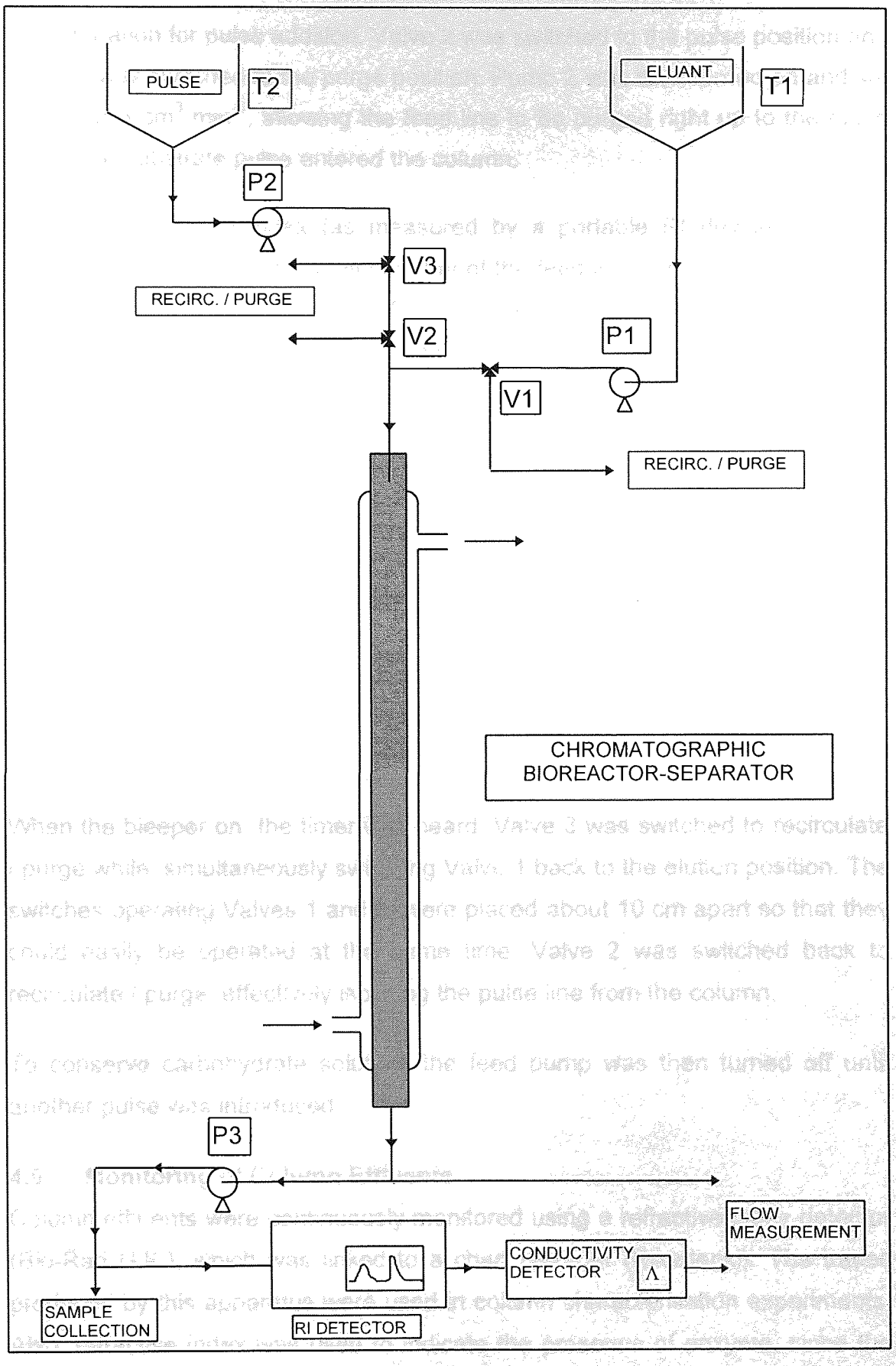



**Figure 4.1 Flow Diagram Depicting the Chromatographic Bioreactor-Separator** system. Showing the layout of key components. A detailed description of pumps and valves is given in Chapter 3.

In preparation for pulse addition, Valve 2 was switched to the pulse position and Valve 3 was switched to the purge position. Pump 2 was then turned on and set to deliver 3 $cm^3$ $min^{-1}$, allowing the feed line to be purged right up to the place where the substrate pulse entered the column.

When the refractive index (as measured by a portable RI device) of liquid exiting the recirculation line matched that of the feed in Tank 2, pulses could be initiated. Pump 2 was switched off while Valve 2 was switched to the pulse position. Once the refractive index monitor at the column outlet produced a stable base-line, Valve 3 was switched to purge/recirculate. Next, Pump 2 was switched and set to deliver the desired flow rate.

To introduce a pulse to the column, Valve 3 was switched to the pulse position while simultaneously switching Valve 1 to recirculate / purge. Immediately after pulse initiation, the timer was started. At the same time, the marker button on the refractive index detector was pressed, marking the introduction of the pulse on the chart recorder trace. The feed was then delivered for a time interval corresponding to the volume of the pulse being injected (at the elution flow - rate).

When the bleeper on the timer was heard, Valve 3 was switched to recirculate / purge while simultaneously switching Valve 1 back to the elution position. The switches operating Valves 1 and 3 were placed about 10 cm apart so that they could easily be operated at the same time. Valve 2 was switched back to recirculate / purge, effectively isolating the pulse line from the column.

To conserve carbohydrate solution, the feed pump was then turned off until another pulse was introduced.

### 4.6 Monitoring of Column Effluents

Column effluents were continuously monitored using a refractive index detector (Bio-Rad U.K.), which was linked to a chart recorder (Rikadenki). The traces produced by this apparatus were used in column characterisation experiments. Also, refractive index was used to indicate the presence of enzyme, since the enzyme solution had a higher refractive index than that of the degassed and deaerated water which formed the eluant in between enzyme runs.

## 4.7 Sample Collection and Quenching

At the column outlet, a T-piece was fitted, splitting the eluant flow between a Pump 3 and a loop of tubing which rose above the top of the column, thereby balancing the hydrostatic head. At Pump 3, a further T-piece divided the flow between two lengths of pump tubing, each taking 0.8 $cm^3$ $min^{-1}$ from the column effluent. Samples were collected into test tubes which contained 1.2 $cm^3$ 0.05m sodium hydroxide. This ensured an end pH of around 12, which had been shown to quench the enzymic reaction as effectively as boiling while being more rapid (see Appendix A-4). Also, this served as a dilution step, providing a sample volume adequate for HPLC analysis. The dilution factor was noted and incorporated into spreadsheet calculations (see Appendix A-6).

## 4.8 Analysis of Saccharides

20 μl of each sample was injected on to the HPLC column (7.8 mm i.d. x 300 mm long) (Model HPX 87C) (BioRad, Watford, UK) via an automatic injection device (Model ASI-3)(Talbot Scientific, Alderley Edge, Cheshire, UK). Samples were then eluted at 85°C with a flow rate of 0.5 $cm^3$ $min^{-1}$. The column was supplied pre-packed with the same ion exchange resin as was used to pack the experimental columns: 8% cross-linked cation exchange resin in the calcium form (BioRad, Watford, UK), except the particle size was smaller (7-10 μm).
The column was protected by a guard column (Hibar-Lichochart Cartridge, BDH Chemicals, Atherstone, Warwickshire, UK).

The detection and integration system comprised a refractive index monitor (Model 1755) (BioRad, Watford, UK) linked to an integrator (Model SP 4270) (Spectra Physics, St. Albans, Herts., UK). The HPLC system was calibrated by the use of standard solutions (1% (w/v)) of glucose, galactose and lactose. A set of three standards was analysed after every fifteen samples.

## 4.9 Materials Used for the Experiments

Enzyme

The enzyme used in this work was a fungal lactase from Aspergillus oryzae. Its pH optimum is pH 4.5-5.5 and its temperature optimum is 55° C. Enzyme activity determination is described in Appendix A-3. The materials used in this project are listed in Table 4(a) overleaf.

**Table 4(a) Materials Used in this Project**

| Product | Specification | Supplier | City/Country |
|---|---|---|---|
| β-galactosidase | Biolactase (Food Grade) | Biocon Biochemicals Ltd. | Cork/Ireland |
| Buffer solutions pH4 and pH7 | General purpose reagent | BDHLaboratory Supplies | Poole (UK) |
| Calcium nitrate | General purpose reagent | Fisons Scientific Equipment | Loughborough(UK) |
| Cation Exchange Resin | Dowex-50WX8 Av. dp 75μm | Sigma-Aldrich Chemical Co. | St. Louis / USA |
| Dextran 2000kd | mw approx 2000kd | Sigma-Aldrich Chemical Co. | St. Louis / USA |
| Galactose | General purpose reagent | Fisons Scientific Equipment | Loughborough(UK) |
| Glucose | General purpose reagent | BDH Laboratory Supplies | Poole (UK) |
| Hydrochloric acid | General purpose reagent | BDH Laboratory Supplies | Poole (UK) |
| Lactose | Analytical grade reagent | BDH Laboratory Supplies | Poole (UK) |
| Sodium acetate | General purpose reagent | BDH Laboratory Supplies | Poole (UK) |
| Sodium hydroxide | General purpose reagent | Fisons Scientific Equipment | Loughborough(UK) |
| Sodium bisulphite | ACS reagent | Sigma-Aldrich Chemical Co. | St. Louis / USA |

# Chapter Five

## Optimisation of the Performance of a Chromatographic Bioreactor-Separator

### Scope of Chapter

The aim of the work reported in this chapter was to optimise the performance of a Chromatographic Bioreactor-Separator (CBRS). The processes involved in chromatographic bioreaction and separation namely: injection, mixing, reaction and separation were examined and the individual and combined effects of these processes considered.

In Section 5.1 the separative power of the CBRS was evaluated. The effect on separation of reducing the particle diameter of the column packing was first gauged. The system was then evaluated in terms of its ability to enable complete conversion of lactose with simultaneous separation of the products galactose and glucose.

In Section 5.2, inlet design was considered in terms of its effect on mixing between enzyme and substrate as pulses of substrate are injected. Hydrodynamic calculations were used, supplemented by magnetic resonance imaging (MRI).

Section 5.3 is concerned with the factors affecting enzymic reaction in a CBRS. Initially, the time scales involved in reaction and separation were compared. The approach taken by the author was to operate the CBRS at different residence times. This resulted in a range of lactose conversion values, so that the progress of reaction could be studied. The effect of reactor loading was then investigated, in terms of both substrate concentration and pulse volume. Mixing between enzyme and substrate was also studied, providing estimates of the enzyme concentrations reached within the reaction zone. Finally, attention was turned to the influence of dispersion and chromatography on chromatographic bioreaction and separation. This involved consideration of the flow characteristics of the bulk liquid, together with the relative time scales involved in the processes of reaction, mass transfer, adsorption and desorption.

Section 5.4 examines the effect of contacting pattern between enzyme and substrate, in particular the difference between introducing components over a period of time as in a CBRS, and almost instantaneous mixing as in a conventional stirred batch reaction.

## 5.1 Assessment of Bioreactor-Separator Performance

Having made changes in Chromatographic Bioreactor-Separator (CBRS) design, with the goal of optimising its separative capability, experiments were performed to establish whether this aim had indeed been achieved.

### 5.1.1 Assessment of Separative Performance

Introduction

The design improvements described in Chapter 3 enabled the CBRS system to be operated with a packing material of smaller diameter. Zafar (1986) and Taddei (1994) were constrained by the column material of construction (PYREX glass); while robust in terms of withstanding temperature fluctuations, the columns could not be operated safely at high pressures. This, as was pointed out by Zafar (1986), restricted the overall length of columns. Taddei (1994), nevertheless, extended column length from 2 metres to 4 metres, increasing the number of theoretical plates (N) for glucose and galactose from around 500 to about 1000.

Shieh (1994), in a collaborative study with Taddei, further increased column length to 7.8 metres, gaining an improvement in number of theoretical plates (N) from 1000 to about 1200 for glucose and galactose. Shieh's column was constructed from stainless steel which allowed higher pressure operation but process visibility was lost. The borosilicate glass column material used by the author enabled a 4 metre long column, of similar internal diameter to that used by Taddei (1994), to be operated with a smaller particle size packing, with the goal of improving separation. This pressure-resistant column material also gave greater flexibility to the system, allowing operation at flow rates up to the maximum pump setting of 9.9 $cm^3min^{-1}$.

As described in Chapter 3, HPLC pumps were adapted for use with the CBRS system. HPLC pumps were not only able to cope with the higher back-pressures developed with a smaller particle size system, but they were also capable of more accurate and consistent delivery than the peristaltic pumps used by previous workers (Taddei 1994, Zafar 1986).

Experimental

The 2 metre long x 1.5 cm i.d. CBRS was characterised by injecting separate 4.4 $cm^3$ pulses (representing 1.25% of the column's volume when empty) of 1% w/v glucose and galactose and operating the CBRS at the normal temperature and flow rate, 55°C and 2.1$cm^3min^{-1}$. From the resulting refractive index traces, values were obtained for N, HETP and Kd, these being calculated as described in Appendix A-1.

The separative performance of the the 2 metre long x 1.5 cm i.d. CBRS was then evaluated by injecting a 4.4 $cm^3$ pulse of a 20% w/v equimolar mixture of glucose and galactose and operating the CBRS at the normal temperature and flow rate. Samples were collected and analysed by HPLC to produce an elution profile from which the resolution of the two sugars could be calculated.

Results and Discussion

Characterisation

At 55°C and a superficial velocity of 1.16 cm $min^{-1}$, values of the equilibrium distribution coefficient (Kd), number of theoretical plates (N) and theoretical plate height (HETP) were found for glucose and galactose at infinite dilution (see Table 5(a)).

**Table 5(a) Results for Characterisation of the 2 m long x 1.5 cm i.d. CBRS.**

| Run No. | N-gluc/2 m | H-gluc (mm) | Kd-gluc | N- gal/2m | H- gal (mm) | Kd-gal |
|---|---|---|---|---|---|---|
| 1 | 2724 | 0.72 | 0.17 | 3056 | 0.64 | 0.21 |
| 2 | 2645 | 0.76 | 0.12 | 2592 | 0.77 | 0.18 |
| 3 | 2655 | 0.75 | 0.15 | 2600 | 0.77 | 0.31 |
| 4 | 2860 | 0.70 | 0.15 | 2812 | 0.71 | 0.22 |
| 5 | 2833 | 0.71 | 0.22 | 2788 | 0.72 | 0.15 |
| Mean | 2743 | 0.73 | 0.16 | 2770 | 0.72 | 0.21 |

The mean value of N was 2743± 43 for glucose and 2770± 93 for galactose (per 2m bed depth). Taddei (1994) and Shieh (1994) obtained values of 532 for N (glucose) and 481 for N (galactose) respectively in a 2 metre long x 1.96 cm i.d.

CBRS packed with a resin of mean particle size 200μm. The CBRS used in this thesis was packed with a resin of mean particle size 75μm. So, by reducing the resin particle size by 62.5%, an approximately 5.5 -fold increase in the number of theoretical plates was achieved with respect to the two monosaccharides.

According to Giddings (1965), theoretical plate height (H) is approximately proportional to the square of particle diameter:

$$H \propto dp^2$$

So, reducing particle diameter from 200 μm to 75 μm should, theoretically, have given the following reduction in H:

$$\left(\frac{75}{200}\right)^2 = 0.375^2 = 0.14 \text{ x reduction in H.}$$

Taddei (1994) quotes theoretical plate height of 4.16 mm for galactose and 3.76 mm for glucose. Applying the above calculation to these figures gives predicted H values of 0.58mm for galactose and 0.53mm for glucose with the 75 μm particle diameter resin used in this thesis. These figures correspond to N values of 3448 and 3774 for galactose and glucose respectively. Given the fairly crude simplifications made of Giddings' equations, the values obtained for the 2 metre long x 1.5 cm i.d. CBRS were acceptable when compared to those which theory predicts.

Separation of Glucose and Galactose

The elution profile for the separation experiment is presented in Figure 5.1. For comparative purposes, an elution profile (Figure 5.2) has been reproduced from the thesis of Shieh (1994). Separation of glucose and galactose was improved significantly by reducing particle diameter from 200 μm to 75 μm. Resolution was increased from 0.58 to 1.82 for approximately the same pulse size, despite sugar concentration being increased from 10% w/v to 20% w/v.

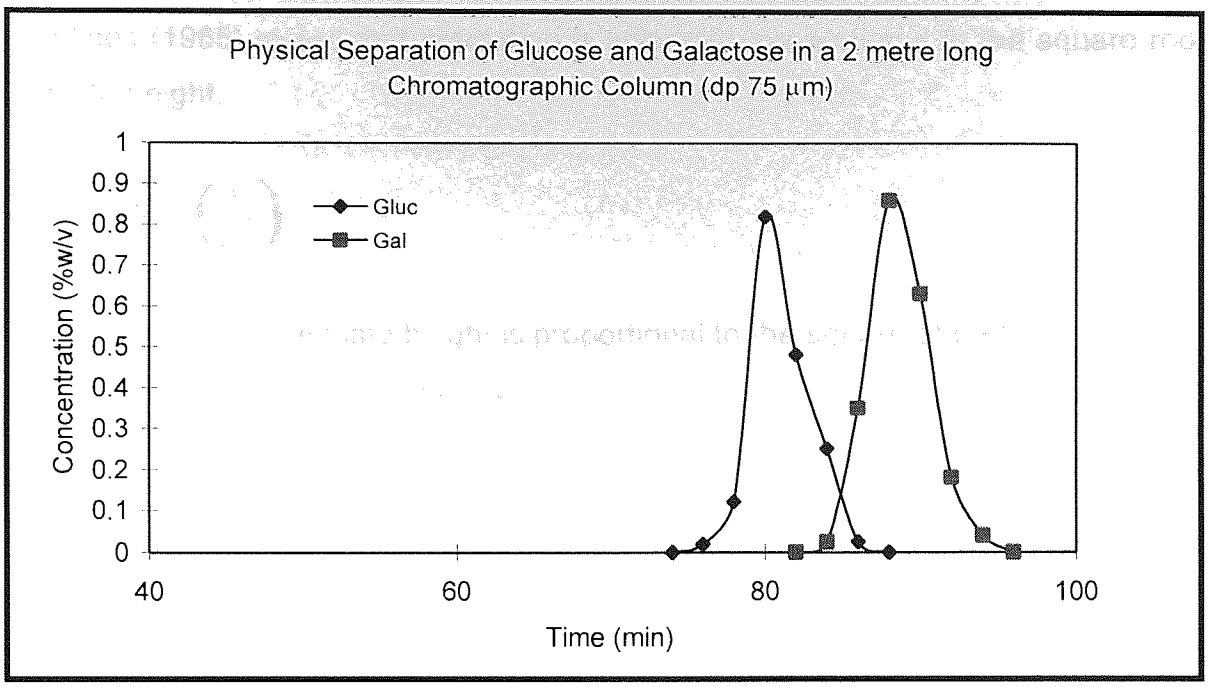



**Figure 5.1 Separation of Glucose and Galactose in a 2 metre long x 1.5 cm i.d. CBRS with a Packing Particle Diameter of 75 μm.** Pulse volume was equivalent to 1.25% of the column volume when empty and pulse concentration was 20% w/v.

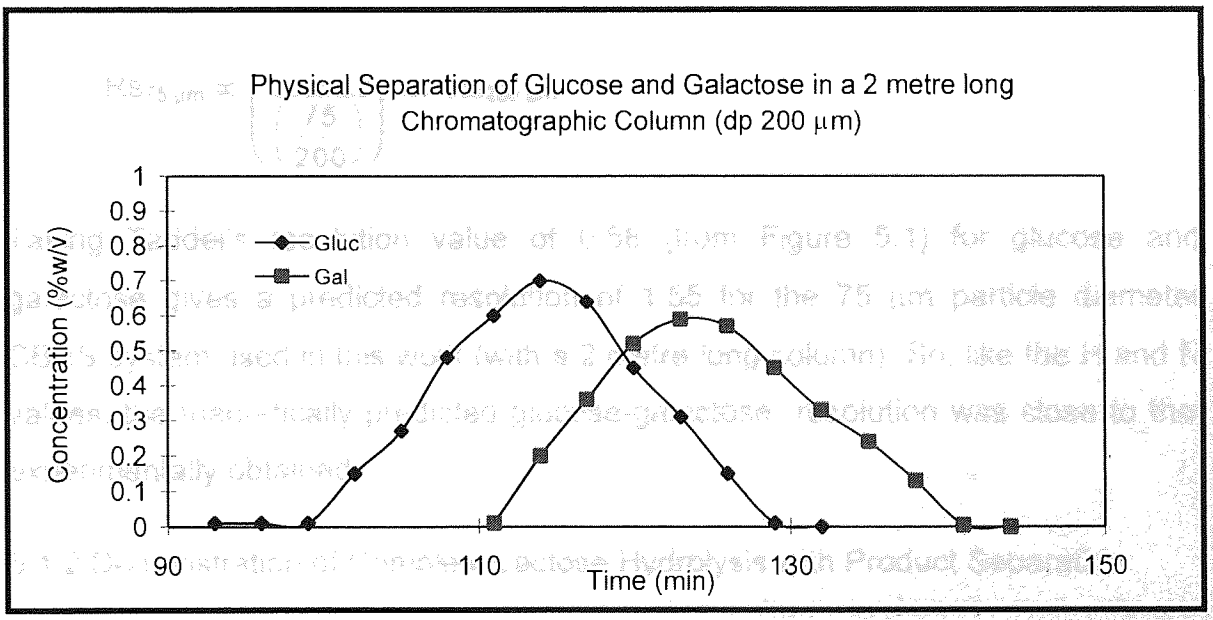



**Figure 5.2 Separation of Glucose and Galactose in a 2 metre long x 1.96 cm i.d. CBRS with a Packing Particle Diameter of 200 μm.** Pulse volume was equivalent to 1.5% of the column volume when empty and pulse concentration was 10% w/v.

Giddings (1965) stated that resolution is inversely proportional to the square root of plate height.

$$Rs \propto \left(\frac{1}{\sqrt{H}}\right)$$

He also reported that plate height is proportional to the square of particle diameter. So, combining these two expressions:

$$Rs \propto \left(\frac{1}{\sqrt{dp^2}}\right)$$

or:

$$Rs \propto \left(\frac{1}{dp}\right)$$

Theoretically, therefore, on reducing particle diameter from 200 μm to 75 μm, resolution would be expected to increase as follows:

$$Rs_{75\ \mu m} \propto \left(\frac{1}{\left(\frac{75}{200}\right)}\right) \times Rs_{200\ \mu m}$$

Taking Taddei's resolution value of 0.58 (from Figure 5.1) for glucose and galactose gives a predicted resolution of 1.55 for the 75 μm particle diameter CBRS system used in this work (with a 2 metre long column). So, like the H and N values, the theoretically predicted glucose-galactose resolution was close to that experimentally obtained.

### 5.1.2 Demonstration of Complete Lactose Hydrolysis with Product Separation

#### Introduction

Having demonstrated that improved separation was achieved by reducing the particle size of the column packing from 200 μm to 75 μm, it was necessary to show that complete conversion of lactose, together with separation of glucose and galactose was achievable in this system. Only a limited number of experiments

were performed, in view of the amount of work undertaken by Taddei (1994) using a CBRS system which, apart from the size of ion exchange resin used to pack the column, was similar to that used in this thesis.

Experimental

A 1 metre long x 1.5 cm i.d. chromatographic bioreactor separator was assembled and attemperated to 55°C. The CBRS was then operated at a flow rate of 2.1 $cm^3min^{-1}$, under the following conditions:

| | |
|---|---|
| Pulse Volume | 4.4 $cm^3$ |
| Pulse Concentration | 2% w/v |
| Enzyme Activity | 30 $Ucm^{-3}$ |

Results and Discussion

As can be seen from Figure 5.3, complete hydrolysis of lactose was achieved, no lactose being detected at the column outlet.

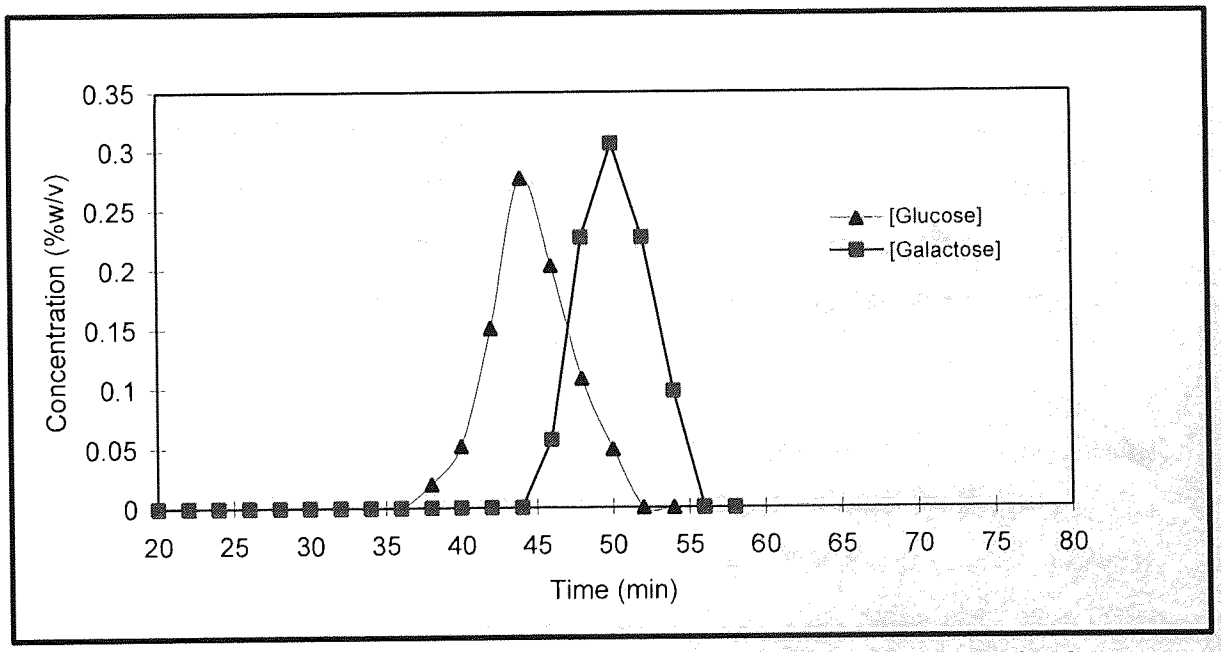



**Figure 5.3 Elution Profile Showing Complete Lactose Conversion in the 1 m long x 1.5 cm i.d. CBRS with Product Separation.** Complete conversion of lactose was achieved, together with reasonable separation of glucose and galactose, a resolution of 0.82 being measured.

This improvement becomes yet more significant when it is considered that Taddei's figure of 0.6 was measured while operating the column in purely

separative mode, that is to say, injecting a pulse of glucose and galactose and eluting with distilled, deionised water.

So, it would appear that the CBRS system could be used to completely hydrolyse lactose and separate the products. This was demonstrated by the fact that a slightly improved separation was achieved in a column of half the length of that used by Taddei (1994). This result was not surprising when considered in the light of the increase in the number of theoretical plates demonstrated in the previous section.

## 5.2 The Effect of Injector Type on Chromatographic Bioreactor-Separator Performance

Introduction

In this thesis, it was thought that the means by which pulses are injected on to the column may have implications with respect to overall CBRS performance. On a visit to Unilever Research, a demonstration of magnetic resonance imaging (MRI) techniques raised the question: "Could MRI be used to study the CBRS injection process?". As a consequence, an experimental programme was proposed and completed at Unilever Research, Port Sunlight.

Magnetic resonance imaging (MRI) is an established diagnostic tool in medicine, where its non-invasiveness enables images of internal organs to be produced, pinpointing abnormalities, such as tumours. It has been realised that MRI has applications in chemical engineering, in particular in the non intrusive study of the behaviour and distribution of liquids. Current and potential applications of MRI in chemical engineering have been extensively reviewed by Gladden (1994). Two main types of injector have been used in chromatographic bioreactor-separator systems, a needle type, as used by Taddei (1994), where substrate is injected jet-like *into* the column packing and the sinter type (as used in this thesis) where the flow of substrate is dispersed, giving a fairly uniform velocity profile across the column diameter. Sinter type injectors were also used by Shieh (1994).

It was thought that the type of injector used may influence CBRS performance. In order to compare the two injection systems, MRI was used in an attempt to visualise the flow patterns generated in each case. The dynamics of injector systems have been examined previously, in the field of analytical chromatography by Knox et al (1976), who used polarographic detection to study radial and axial dispersion in a chromatographic column. Due to the intrusiveness of this technique, however, dispersion behaviour could only be inferred from measurements made *after* the column outlet, rather than monitoring events taking place *within* the chromatographic column. MRI has also been used to follow the progress of a chromatographic band by Bayer *et al* (1989), who produced images at 37, 63 and 110 minutes after injection.

Experimental

The experimental methods used are described in detail in a paper presented at the 5th world Congress of Chemical Engineering, San Diego U.S.A. (see Appendix A-7). Briefly, a miniaturised version of the chromatographic bioreactor-separator was placed within the probe of an MRI apparatus. Pulses of lactose were then injected while imaging the injection area. The two injector configurations are illustrated in Figure 5.4.

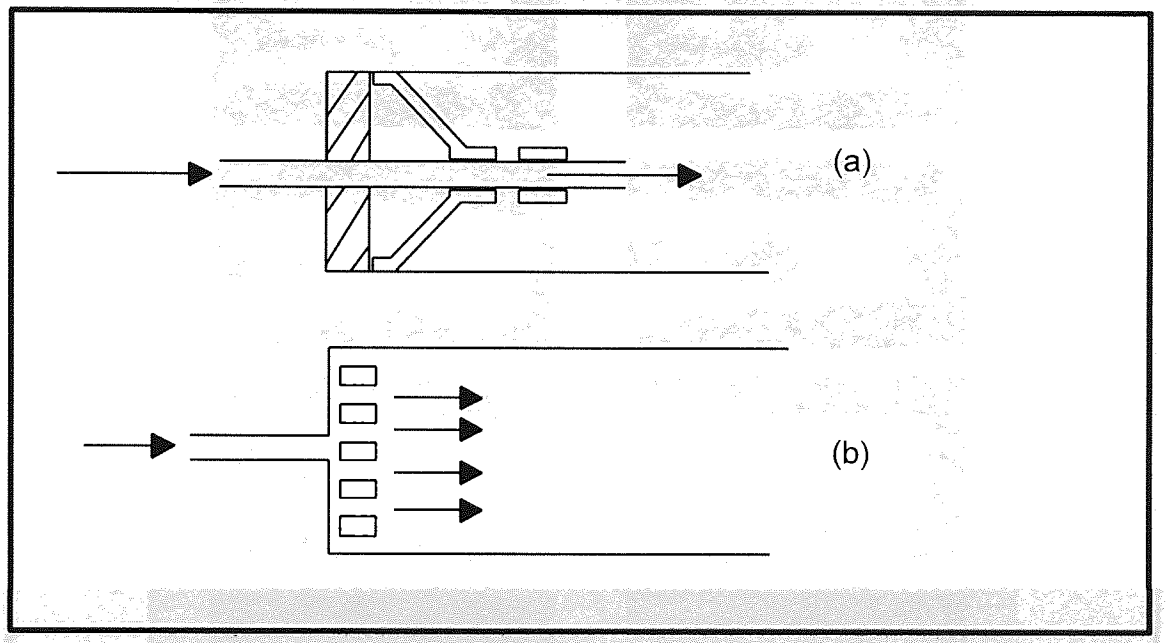



**Figure 5.4 Injection Assembly Types (a) Needle and (b) Sinter.** The two types of injector are likely to create different flow patterns, which will, in turn, affect the concentration profile.

The sinter injector used consisted of a composite PTFE frit, about 2 mm deep, placed about 1 mm after the inlet orifice, which has a diameter of 1.2 mm. Flow of liquid from the orifice was likely to be dispersed by the tortuous pores of the sinter, giving a fairly flat velocity profile across the internal diameter of the column. The needle injector consisted of a PTFE tube of 0.8 mm i.d., which was inserted 2 cm into the top of the column. The PTFE tube itself formed the needle. The needle was likely to produce a jet-like injection, resulting in a velocity profile where the liquid velocity at the centre is significantly greater than at the column walls. In both cases, the injector also acted as the column inlet, so both eluant and pulses entered through the same system.

Results and Discussion

As can be seen from Figure 5.5, the needle injection process was successfully imaged, showing the time course of events from the first ingress of lactose to the stage where fully developed flow is seen.

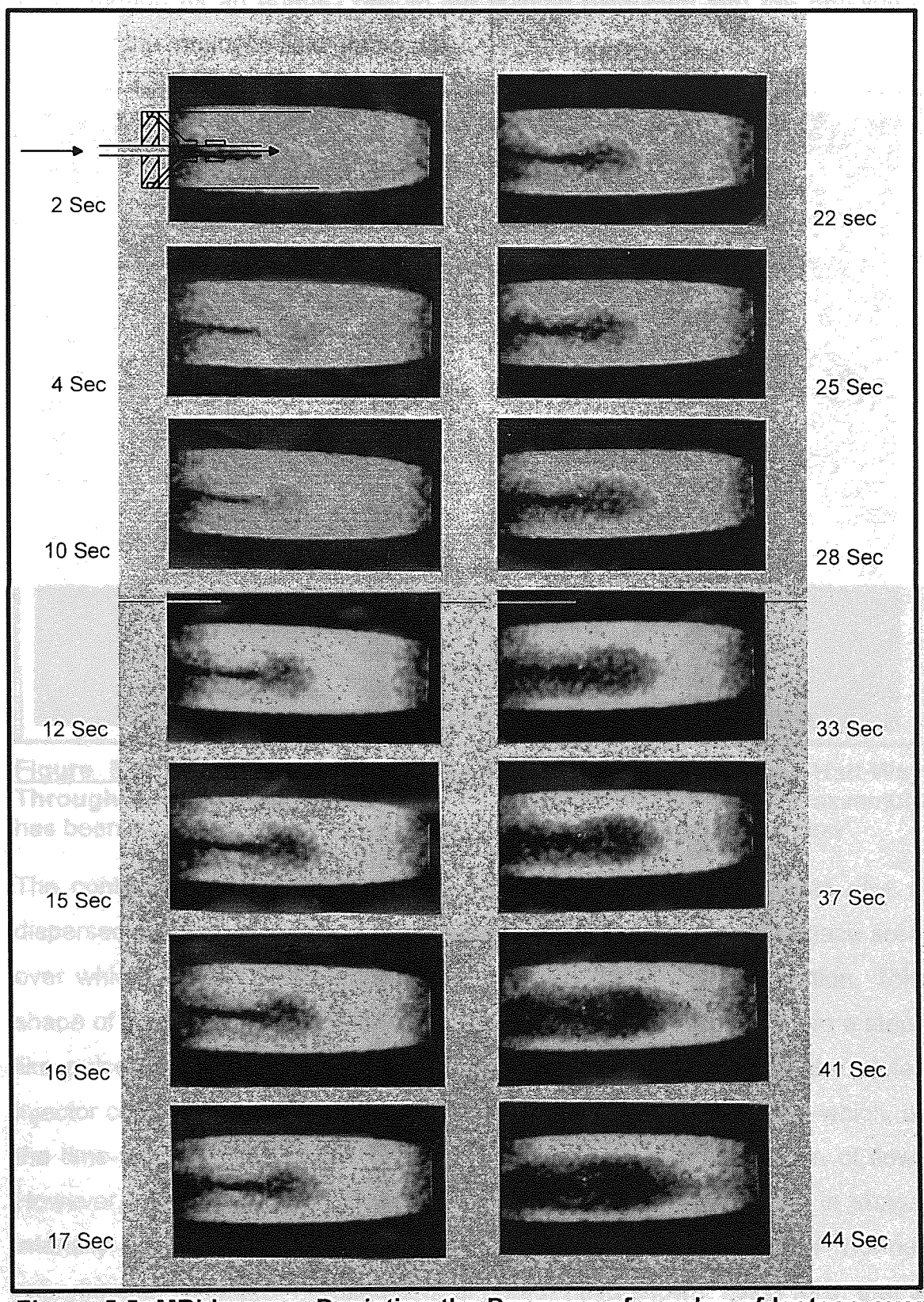



**Figure 5.5 MRI images Depicting the Progress of a pulse of lactose over a 44 second time period.**

The position of the imaging window with respect to the needle is presented in Figure 5.6, which shows the extent of flow pattern development around half-way through the pulse injection period. The apparent curvature of the tube walls was also observed by Fernandez et al (1996), who explained this phenomenon as an artefact due to the column extending into the non-linear region of the magnetic field gradients.

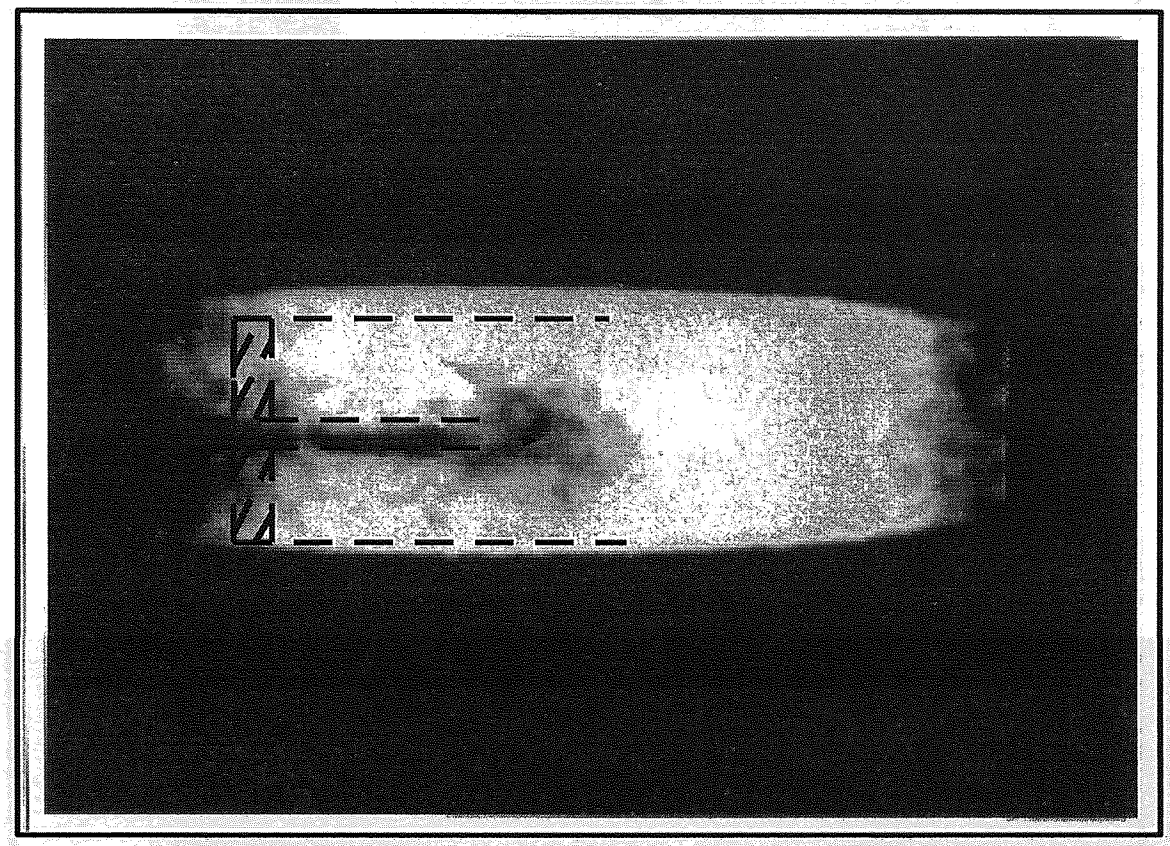


**Figure 5.6 MRI Image Taken at 20 Seconds, Approximately Half-Way Through the Injection Period.** A drawing of the needle injection assembly has been superimposed to show the position of the MRI `viewing window'.

The contacting pattern resulting from needle injection was shown to give a dispersed pulse. This shape of pulse will have a greater interfacial surface area over which diffusion can take place and may benefit enzymic reaction. This shape of pulse (dispersed) is less desirable chromatographically, where a step-like pulse is preferable with minimal axial dispersion. Imaging of the sinter injector configuration was inconclusive. A low intensity image resulted which, at the time was thought to have been due to highly effective dispersion of flow. However, further study of images revealed that the small increase in image intensity was not significant when considered in the light of normal experimental variation. Fernandez *et al* (1996) have shown, using MRI, that a flat velocity profile results from injection via a sinter-like distributor, giving a

step-like input. This was confirmed by Colin (1993), who demonstrated the benefit of this type of injector in terms of resolution. The expected pulse behaviour for the two injector configurations is summarised schematically in Figure 5.7

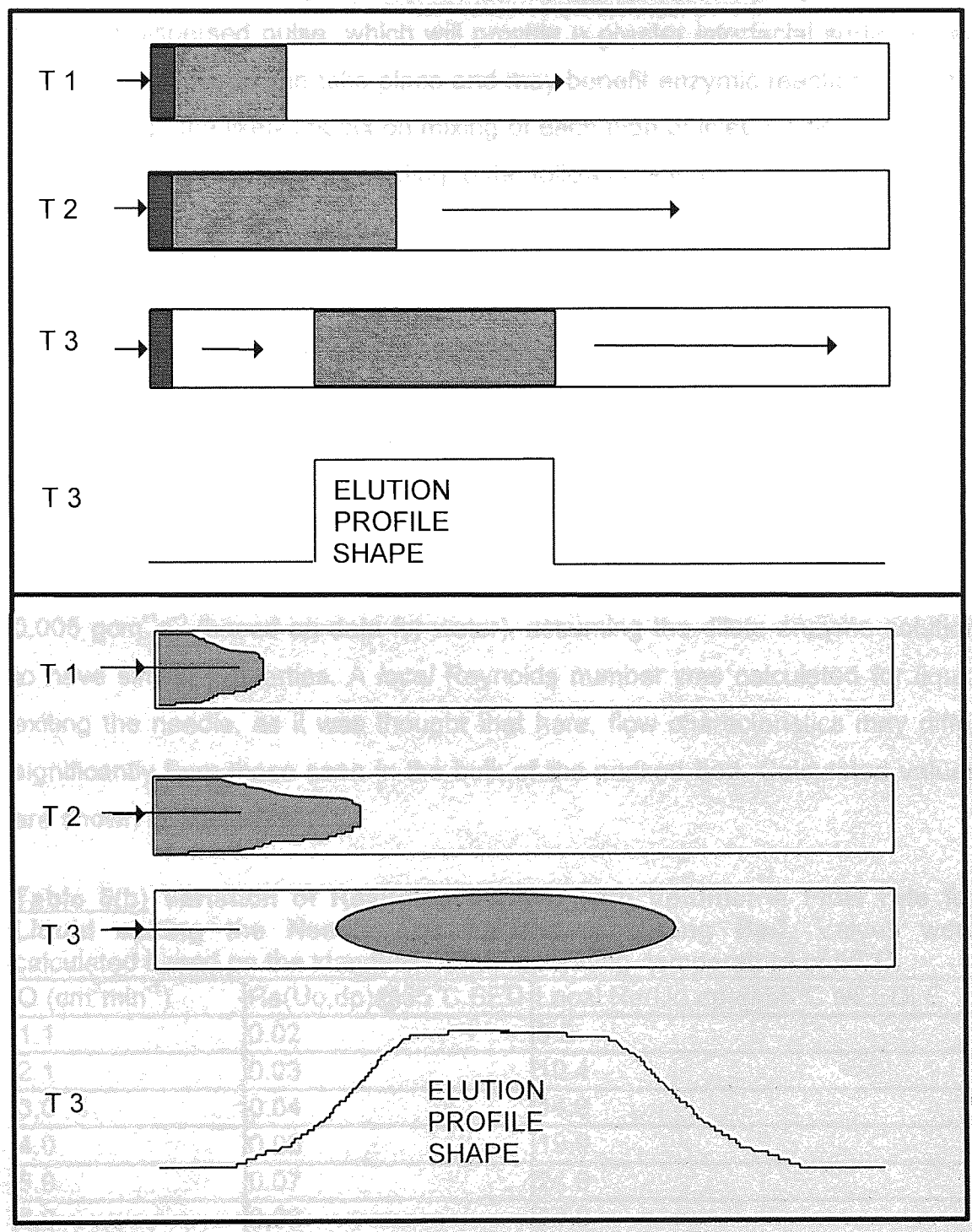



**Figure 5.7 Predicted Pulse Behaviour from (a) Sinter Injection and (b) Needle Injection at T1 (start of injection), T2 (end of injection), and T3, when flow has been switched back to enzyme eluant flow.** In each case, the likely shape of the elution profile is shown.

The step-like input from the sinter injector is more desirable in terms of chromatographic separation while a broad pulse results in loss of chromatographic reactor efficiency (Schweich and Villermaux (1982). However, the jet-like injection from the needle injector may have advantages in terms of enzyme-substrate mixing. As was shown in Figure 5.7, needle injection is likely to give a dispersed pulse, which will provide a greater interfacial surface area over which diffusion can take place and may benefit enzymic reaction. In order to help judge the likely impact on mixing of each type of inlet, Particle Reynolds Numbers were calculated according to the following equation:

$$Re = \frac{U_0 dp \rho}{\mu}$$

where: $U_0$ = Superficial velocity ($cms^{-1}$)

dp = particle diameter of column packing (cm)

$\rho$ = liquid density ($gcm^{-3}$)

$\mu$ = liquid viscosity ($gcm^{-1}s^{-1}$).

Particle diameter was taken as 75$\mu$m, liquid density as 1 $gcm^{-3}$ and viscosity as 0.005 $gcm^{-1}s^{-1}$ (based on data for water), assuming the dilute enzyme solution to have similar properties. A *local* Reynolds number was calculated for liquid exiting the needle, as it was thought that here, flow characteristics may differ significantly from those seen in the bulk of the packed bed. Calculated values are shown in Table 5(b).

**Table 5(b) Variation of Reynolds number with Volumetric Flow rate for Liquid exiting the Needle and for the remaining Bed.** Values were calculated based on the standard CBRS operating temperature of 55°C.

| Q ($cm^3min^{-1}$) | Re(Uo,dp)@55°C BED | Local Re(Uo.dp)@55°C NEEDLE |
|---|---|---|
| 1.1 | 0.02 | 5.5 |
| 2.1 | 0.03 | 10.4 |
| 3.0 | 0.04 | 14.9 |
| 4.0 | 0.06 | 19.9 |
| 5.0 | 0.07 | 24.9 |
| 6.0 | 0.08 | 29.9 |
| 7.0 | 0.10 | 34.8 |
| 8.0 | 0.11 | 39.8 |
| 9.0 | 0.13 | 44.8 |
| 9.9 | 0.14 | 49.3 |

Over the this range of Reynolds Numbers, according to data presented by Gunn (1968), Peclet numbers vary little and are low (around 0.5). Peclet Numbers give an indication of whether mixing is likely to be dominated by convective effects due to liquid flow, or by diffusion effects and are calculated thus:

$$Pe_p = \frac{Udp}{D_L}$$

where: U = Interstitial velocity ($cms^{-1}$)

dp = particle diameter of column packing (cm)

$D_L$ = Longitudinal diffusion coefficient.

Westerterp *et al* (1983) define the Particle Peclet number ($Pe_p$) as the ratio between the transport rate by convection and the transport rate by dispersion. As Peclet Numbers approach zero, liquid-liquid mixing is dominated by dispersion (diffusional) effects, while as Peclet Numbers approach infinity, convective effects become more dominant. Referring back to Table 5(b), for the needle injection zone, Reynolds numbers are about 300 times higher than in the bed, as a consequence of the high exit velocity of liquid leaving the needle. Peclet numbers for liquids do not show significant change until Re>100 (Gunn 1968) and this means $D_L$ is proportional to U, the interstitial velocity. Clearly there will be some effect of convection at the higher flow rates. Reynolds numbers were re-calculated with a particle diameter of 200μm, as used by Taddei (1994) and are presented in Table 5(c).

**Table 5(c) Comparison of Particle Reynolds numbers Obtained for Two Packing Particle Diameters, 75 μm and 200 μm.** Calculations were based on the Superficial velocity of Liquid exiting the Needle.

| Q ($cm^3min^{-1}$) | Re(Uo,dp)55°C (dp=75 μm) | Re(Uo,dp)55°C (dp=200 μm) |
|---|---|---|
| 1.1 | 5.5 | 14.6 |
| 2.1 | 10.4 | 27.9 |
| 3.0 | 14.9 | 39.8 |
| 4.0 | 19.9 | 53.1 |
| 5.0 | 24.9 | 66.3 |
| 6.0 | 29.9 | 79.6 |
| 7.0 | 34.8 | 92.9 |
| 8.0 | 39.8 | 106.2 |
| 9.0 | 44.8 | 119.4 |
| 9.9 | 49.3 | 131.4 |

Reynolds numbers ranged from 15 to 131, with a value of 28 being calculated for the normal operating flow-rate of the CBRS, 2.1 $cm^3min^{-1}$. This Reynolds number falls well within the laminar flow region. It is not likely therefore, that use of a needle type injector has a significant effect on mixing between enzyme and substrate, even when used in conjunction with a larger particle size column packing. The sinter type of injector is likely to produce a uniform velocity profile, resulting in a more step-like input, which is desirable in terms of chromatographic separation. Consequently of the two injector types examined, the sinter type of inlet was judged to give the best all-round performance.

## 5.3 Factors Affecting Enzymic Reaction in a Chromatographic Bioreactor-Separator.

Having designed and built a chromatographic bioreactor-separator with improved ability to separate the products of lactose hydrolysis, attention was turned to the reaction process, with the aim of identifying the factors which affect reaction. This was expected to provide design information which, together with knowledge of how to improve separation, could be used to optimise the overall chromatographic bioreaction-separation process.

### 5.3.1 Following the Progress of Chromatographic Reaction

#### Introduction

Although other workers had looked at factors affecting the reaction, little attention has been given to the relative time scales of the processes of reaction and separation. The aim of this section was to elucidate the progress of hydrolysis under conditions which were representative of chromatographic bioreaction and separation.

#### Experimental

The approach used was to operate a (CBRS) of fixed length (1 metre) at progressively higher volumetric flow rates, resulting in shorter residence times. This was intended to give a picture of the progress of reaction along the column. In the first set of experiments, lactose pulses of concentration 5% w/v and volume 17.66 $cm^3$ were injected on to the column. The CBRS was then operated at each of the following flow rates: 2.1, 3.0, 5.0, 7.0 and 9.0 $cm^3min^{-1}$. From the resultant concentration profiles, the degree of conversion was determined. The experiments were then repeated with a pulse volume of 35.3 $cm^3$. The pulse volumes injected, 17.66 $cm^3$ and 35.3 $cm^3$, were selected because they are convenient fractions (5% and 10%) of the total empty column volume (TECV) of the full scale 2 metre long x 1.5 cm i.d. CBRS (353 $cm^3$). The experiments were then repeated with the same pulse volumes, but with a lactose concentration of 10% w/v. By this time, the practical operating limits of the CBRS system had been expanded to encompass a greater range of flow-rates, giving a wider range of residence times. The flow rates

used in the experiments are shown in Table 5(d), along with the corresponding residence times.

**Table 5(d) Flow Rates Used in Reaction Progress Experiments.** The 1 metre long x 1.5 cm i.d. CBRS was operated at the range flow rates: 1.1 to 9.9 $cm^3min^{-1}$ which corresponded to the practical operating limits of the system.

| Q ($cm^3min^{-1}$) | 1.1 | 1.7 | 2.1 | 3.0 | 5.0 | 7.0 | 9.0 | 9.9 |
|---|---|---|---|---|---|---|---|---|
| $\tau$ (min) | 55.9 | 36.2 | 29.3 | 20.5 | 12.3 | 8.8 | 6.8 | 6.2 |

Enzyme activity was 26 $Ucm^{-3}$ (8g/litre). This is around half the activity used by Taddei (1994). The enzyme activity was set at a lower level as it was thought that a range of lactose conversions would be observed, giving more information about CBRS behaviour than if, as in previous work at Aston, complete lactose conversion was sought.

Results and Discussion

From Figures 5.8 and 5.9, it is interesting to note that for both column loadings, the degree of conversion nears its final level after around 10 minutes.

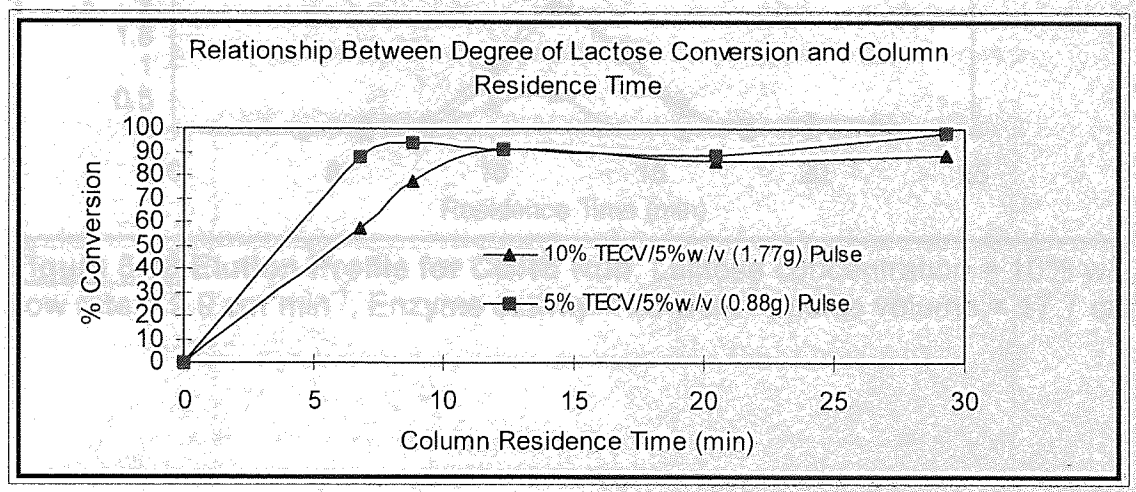



**Figure 5.8 Progress of Reaction for a Lactose Concentration of 5% w/v.**

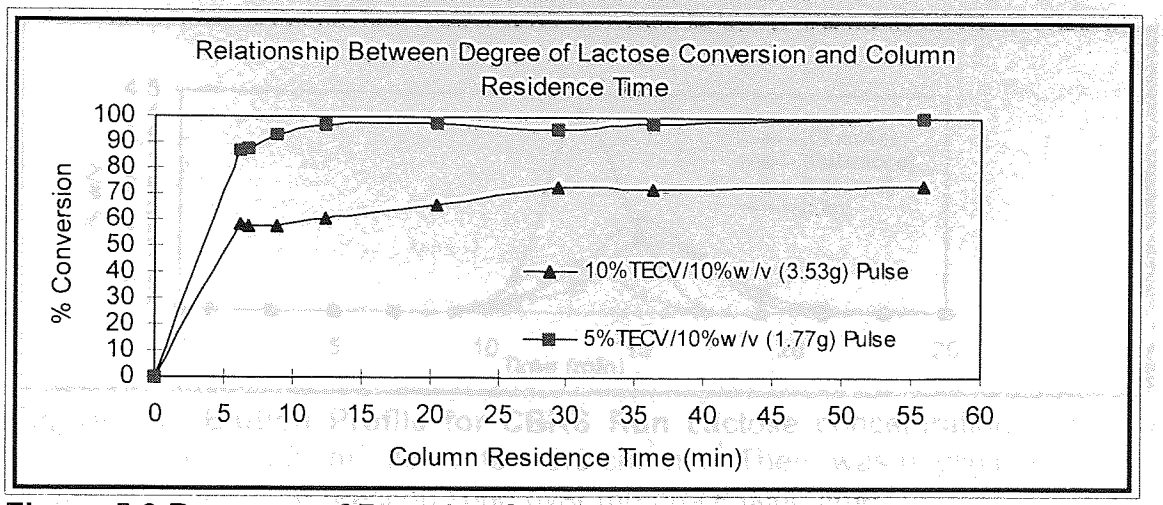



**Figure 5.9 Progress of Reaction for a Lactose Concentration of 10% w/v.** From the 10% w/v, 5% TECV curve, it can be seen that hydrolysis is complete by around 15 minutes.

During this time (as can be seen from Figure 5.10) there is negligible product separation.

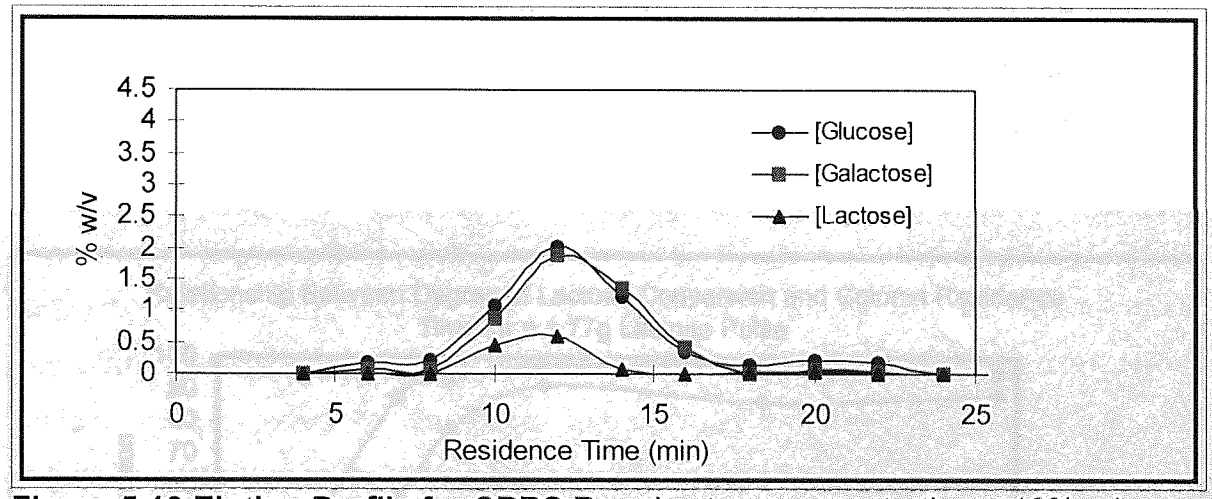



**Figure 5.10 Elution Profile for CBRS Run**. Lactose concentration = 10% w/v, flow rate = 9.9 $cm^3min^{-1}$, Enzyme activity = 26 $Ucm^{-3}$ , pulse volume = 17.7 $cm^3$.

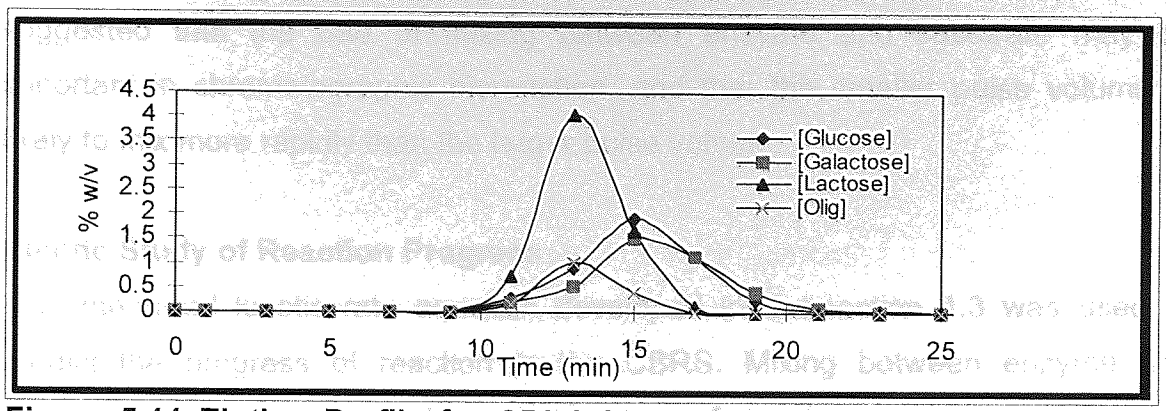



**Figure 5.11 Elution Profile for CBRS Run** Lactose concentration = 10% w/v, pulse volume = 35.3 $cm^3$. flow rate = 9.9 $cm^3min^{-1}$ There was negligible separation of galactose from the reaction zone over the processing time.

The slightly longer retention times seen in the larger pulse volume experiments (Figure 5.11) were likely to be due to the longer pulse delivery time. When analysing the data from which Figures 5.8 and 5.9 originated, it was realised that the same mass of lactose (1.77g) was injected in each of the two series of experiments, but in a different pulse volume. Also, it was noticed that the rate and degree of lactose conversion were different according to the pulse volume injected. To make comparison easier, the two reaction progress profiles were plotted on the same graph, shown in Figure 5.12.

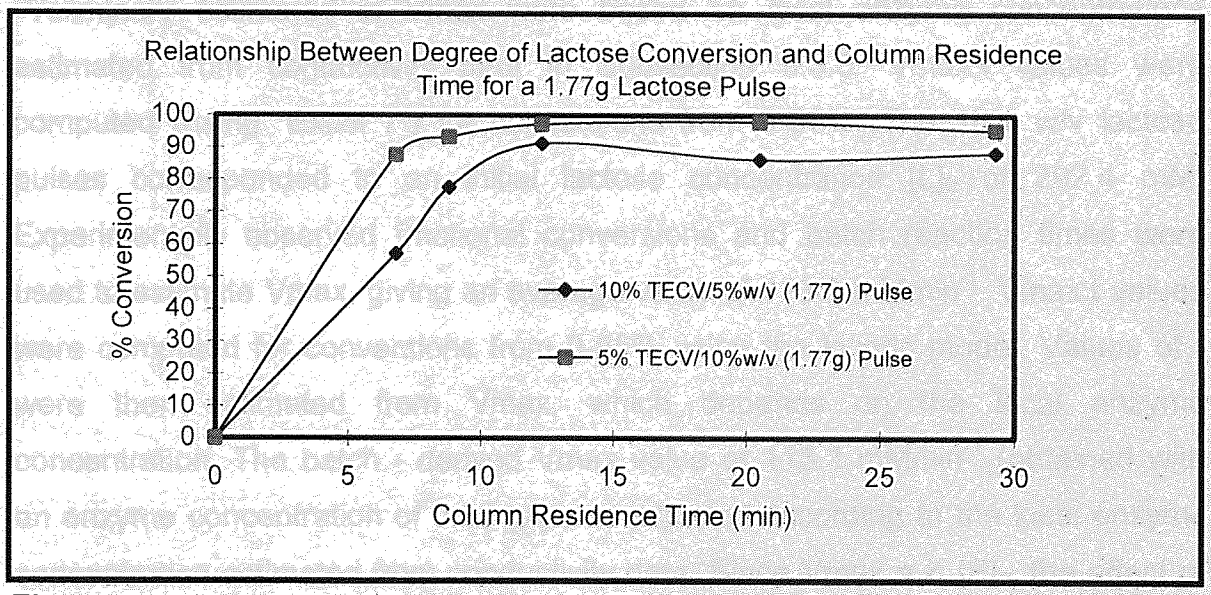



**Figure 5.12 Progress of Reaction for 1.77g Lactose Pulses Resulting from Two Different Concentration and Volume Combinations**

Referring to Figure 5.12, it is noticeable that the rate of lactose conversion was higher for the low pulse volume / high pulse concentration combination. This

suggested that the rate of mixing between enzyme and substrate may be important in chromatographic bioreaction, and that the smaller pulse volume is likely to mix more rapidly than the larger pulse volume.

**Kinetic Study of Reaction Progress**

The integrated kinetic rate equation developed in Subsection 1.3 was used to predict the progress of reaction in the CBRS. Mixing between enzyme and substrate was considered by making use of local enzyme concentrations estimated from conductivity data.

$$Vmax \,.\, t = \left(1 - \frac{Km}{Ki}\right).\left([L]_0 - [L]\right) + \left(\frac{Km}{Ki}.[L]_0 + Km\right).\ln\frac{[L]_0}{[L]}$$

The Km and Ki values used were taken from Tack's analysis (1997) and are comparable with other literature values:

Km = 154 mM

Ki = 4.3 mM.

Preliminary estimates of Vmax were based on local enzyme concentrations estimated from conductivity data in Subsection 5.3.3. Vmax.t values were computed (using "Excel 7") for conversions from 0-30%. The 10% w/v lactose pulses corresponded to an initial lactose concentration $[L]_0$ of 292.4 mM. Experimentally observed fractional conversions and batch reaction times were used to estimate Vmax, giving an average value of 113.3 $mMmin^{-1}$. Vmax.t values were computed for conversions from 0-95% using the kinetic model. Values of t were then estimated from Vmax, which depends on the local enzyme concentration. The batch - derived Vmax value of 113.3 $mMmin^{-1}$ (obtained with an enzyme concentration of 0.53 g/l) was adjusted according to the local enzyme concentration estimated from conductivity data. Since $Vmax = K\,[E]_0$, the effect of a change in $[E]_0$ on Vmax could be readily calculated. Vmax was estimated using both the average enzyme concentration and that expected in the centre of the lactose pulse. For example, for the 17.7 $cm^3$ lactose pulse an average enzyme concentration of 5.7 g/l was estimated from conductivity experiments.

$$V\,max = 113.3 \times \left(\frac{5.7}{0.53}\right) = 1218.5\ mMmin^{-1}$$

Similarly, a Vmax value of 684 $mMmin^{-1}$ was estimated using the enzyme concentration expected in the centre of the pulse. The process was repeated for the 35.3 $cm^3$ pulse volume, giving Vmax values of 897.8 $mMmin^{-1}$ and 85.5 $mMmin^{-1}$, based on the average and centre-based enzyme concentrations respectively.

Predicted and experimental data are presented in Figures 5.13 and 5.14.

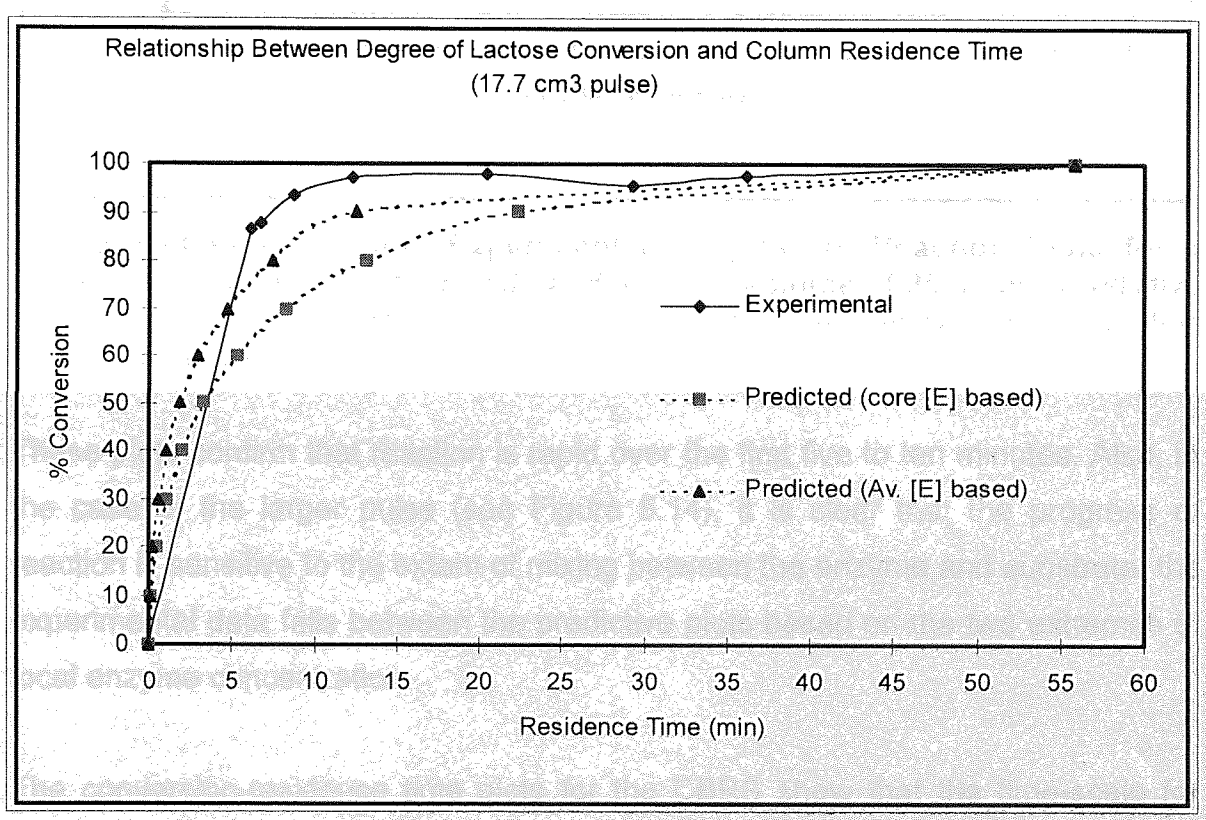



**Figure 5.13 Predicted and Experimental Progress of Reaction Plots for a Lactose Concentration of 10% w/v and a Pulse Volume of 17.7 $cm^3$.** Predictive data was produced considering both the expected average enzyme concentration and that anticipated at the core of the pulse.

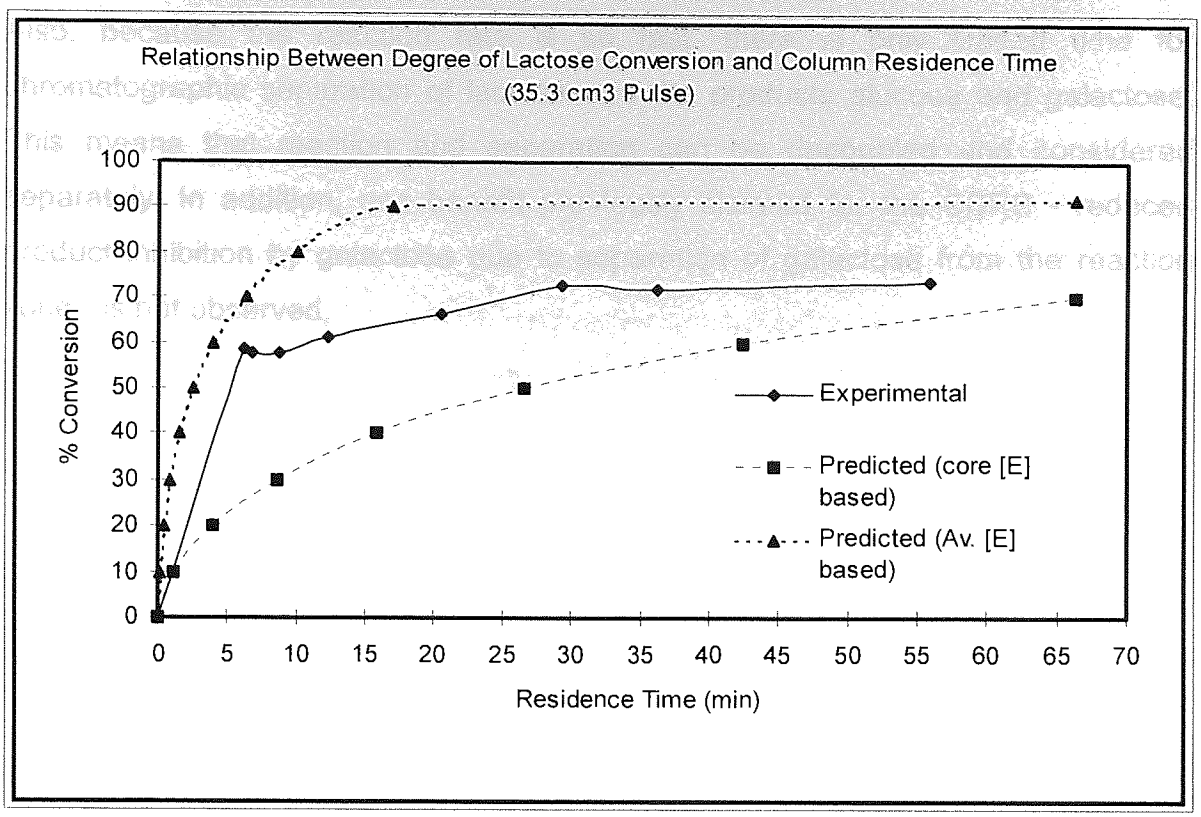



**Figure 5.14 Predicted and Experimental Progress of Reaction Plots for a Lactose Concentration of 10% w/v and a Pulse Volume of 35.3 $cm^3$.** Predictive data was produced considering both the expected average enzyme concentration and that anticipated at the core of the pulse.

These plots confirm that reaction is rapid over the first five to ten minutes. Also, in the case of the larger pulse (see Figure 5.14), it is clear that the progress of reaction is sensitive to the extent of mixing between the enzyme and substrate: the experimental data falls between the predictive plots based on the two extremes of local enzyme concentration.

The conversion-residence time plots for the CBRS show that the time-scale for complete reaction is of the order of 10 minutes for a pulse size of 17.7 $cm^3$ and considerably longer (~100 min.) for a pulse size of 35.3 $cm^3$. The time scale of minutes compares with that of seconds for the rate processes involved in chromatography, as discussed in later sections. Consequently, the assumption of instantaneous equilibrium between components in the mobile and stationary phases holds when reaction is taking place; in other words, the rate of reaction will not be affected locally by the adsorption-desorption process.

Also, because the reaction rate is so fast, there is only limited time for chromatographic separation of lactose and the products glucose and galactose. This means that reaction and separation can be decoupled and considered separately. In addition, one benefit previously claimed for the CBRS - reduced product inhibition by galactose due to separation of galactose from the reaction zone - is not observed.

### 5.3.2 Effect of Reactor Loading Upon Degree of Lactose Conversion

Introduction

Results presented in the previous section (5.3.1) suggested that a time-related factor such as mixing becomes more significant as the system limits are explored. To investigate this further, experiments were designed which examined the combined effects of pulse volume and pulse concentration in terms of degree of lactose conversion attained over the notional 8 minute residence time of the 1 metre long x 1.5 cm i.d. CBRS when operated at a flow rate of 9.0 $cm^3min^{-1}$.

Experimental

The 1 metre long x 1.5 cm i.d. CBRS was operated at a temperature of 55°C and a flow rate of 9.0 $cm^3min^{-1}$. This flow rate was chosen as it corresponds to a calculated mean residence time of 6.8 minutes for a pulse of lactose in the column. Since the post column piping had a measured residence time of 1.2 minutes, a notional residence time of around 8 minutes was assumed. In the previous section, it was demonstrated that reaction is almost complete after this time. Lactose concentration was fixed while the pulse volume was varied, covering a range of pulse sizes which were equivalent to 5 to 90% of the column's void volume (see Table 5(e)).

**Table 5(e) Pulse Sizes Injected Expressed as Actual Volumes and as a Percentage of the Void Volume of the 1 metre long x 1.5 cm i.d. CBRS used in the Reactor Loading Experiments.**

| %TECV (2 metre Column) | Pulse Volume ($cm^3$) | Percentage of Void Volume of 1 metre Column |
|---|---|---|
| 0.625 | 2.2 | 3.6 |
| 1.25 | 4.4 | 7.2 |
| 5 | 17.7 | 29 |
| 10 | 35.3 | 57.9 |
| 15 | 53 | 86.9 |

The experiments were designed by a factorial method, as used by Bridges (1990). However, in this thesis, full statistical analysis was not applied to this set of results due to the small number of factors varied. It was thought that in these experiments, due to the small number of factors varied, any effects or trends

should be highlighted by plotting simple graphs. Only two factors were varied, pulse concentration (expressed as % w/v lactose), and pulse volume, expressed as a percentage of the volume of the 2 metre long x 1.5 cm i.d. CBRS in its unpacked form (%TECV = Percentage of Total Empty Column Volume). At the time of these experiments, there were no plans to extend the CBRS beyond its then two metre length, so pulse volumes were related to the 2 metre column volume.

Factorial Experimental Design

The factors chosen were denoted by a capital letter:

A = Pulse Volume (% TECV) and B = Pulse Concentration (%w/v)

The levels were represented by a numbered subscript:

$A_1 = 0.625$, $A_2 = 1.25$, $A_3 = 5.0$, $A_4 = 10.0$, $A_5 = 15.0$

$B_1 = 2.0$, $B_2 = 5.0$, $B_3 = 15.0$

The following combinations were used, giving a total of 15 experiments:

| | | | | |
|---|---|---|---|---|
| $A_1 B_1$ | $A_2 B_1$ | $A_3 B_1$ | $A_4 B_1$ | $A_5 B_1$ |
| $A_1 B_2$ | $A_2 B_2$ | $A_3 B_2$ | $A_4 B_2$ | $A_5 B_2$ |
| $A_1 B_3$ | $A_2 B_2$ | $A_3 B_3$ | $A_4 B_3$ | $A_5 B_3$ |

Results and Discussion

On examining Figures 5.15, 5.16 and 5.17 (overleaf), it appears that mass transfer limitations have a more pronounced effect as concentration increases. For each of the initial lactose concentrations studied, 2, 5 and 10% w/v, the effect of increasing pulse volume was to reduce percentage conversion achieved during a residence time of 8 minutes. This effect became more pronounced with increasing initial lactose concentration, and it is demonstrated by the increased divergence of the "Mass Injected" and "Mass Converted" profiles as lactose pulse concentration was increased. The difference is greatest with the highest (10% w/v) lactose concentration. This could be due to the theoretical maximum convertable mass of lactose being approached.

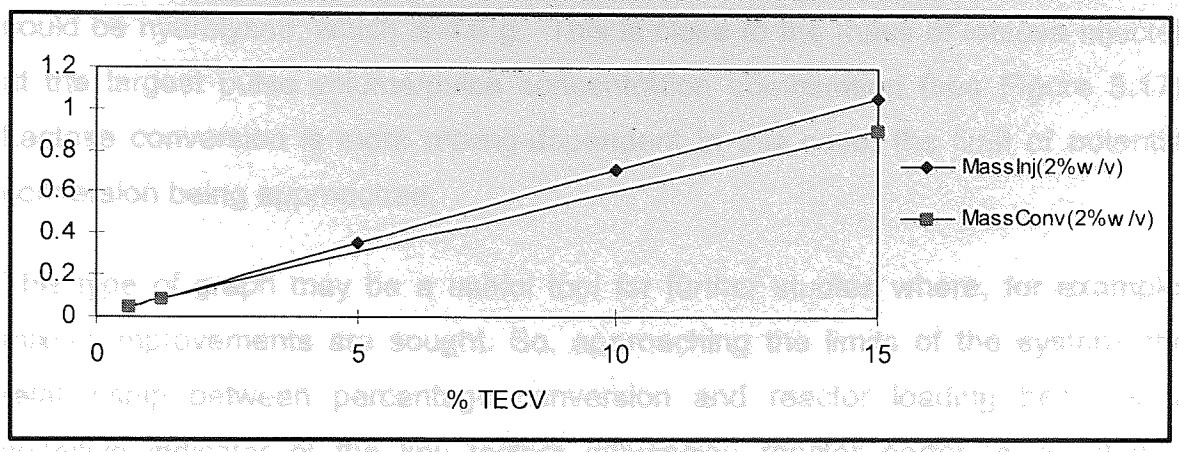



**Figure 5.15 Relationship Between Mass (g) Injected and Mass Converted for a Lactose Concentration of 2% w/v.**

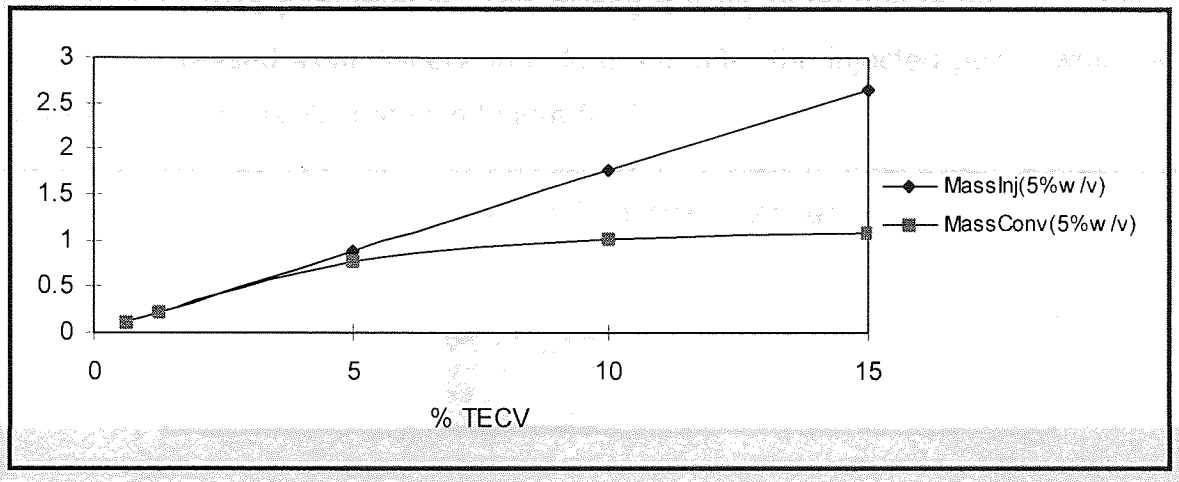



**Figure 5.16 Relationship Between Mass (g) Injected and Mass Converted for a Lactose Concentration of 5% w/v**

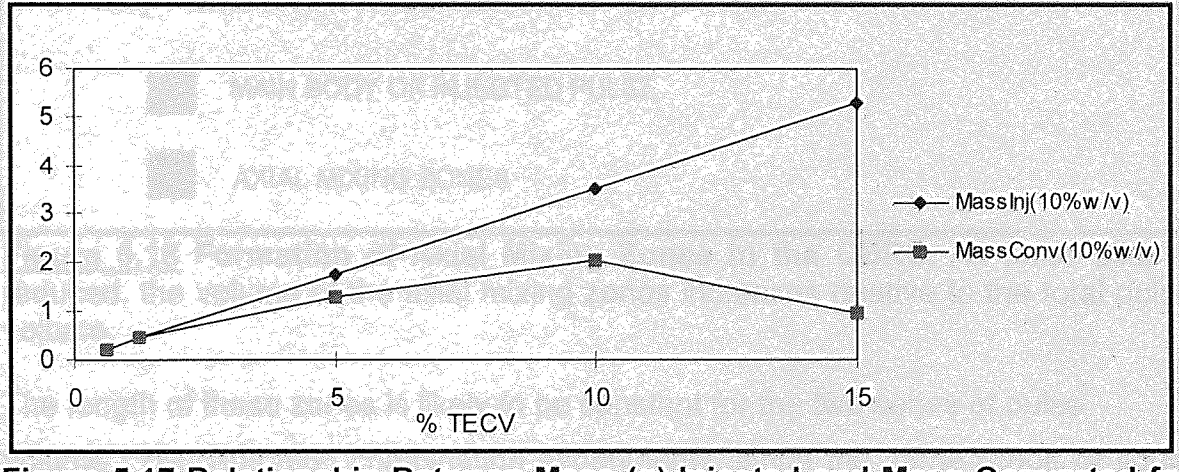



**Figure 5.17 Relationship Between Mass (g) Injected and Mass Converted for a Lactose Concentration of 10% w/v**

The activity of the enzyme was 26 $Ucm^{-3}$, which means 26 μmoles of lactose could be hydrolysed per minute. So, in 8 minutes, 61 x 26 x 8 x ($10^{-6}$) moles of lactose

could be hydrolysed, which is 4.6 g. This is close to the mass of lactose injected at the largest pulse volume/pulse concentration combination (see Figure 5.17). Lactose conversion is more mixing-dependent in this case, the limit of potential conversion being approached.

This type of graph may be a useful tool for further studies where, for example, mixing improvements are sought. So, approaching the limits of the system, the relationship between percentage conversion and reactor loading becomes a sensitive indicator of the key factors influencing reactor performance. It was concluded that mixing between enzyme and substrate is a significant factor when considering CBRS performance. This mixing is likely to be due to the existence of zones of increased axial dispersion before and after the injected pulse, which act as mixing zones, as illustrated in Figure 5.18.

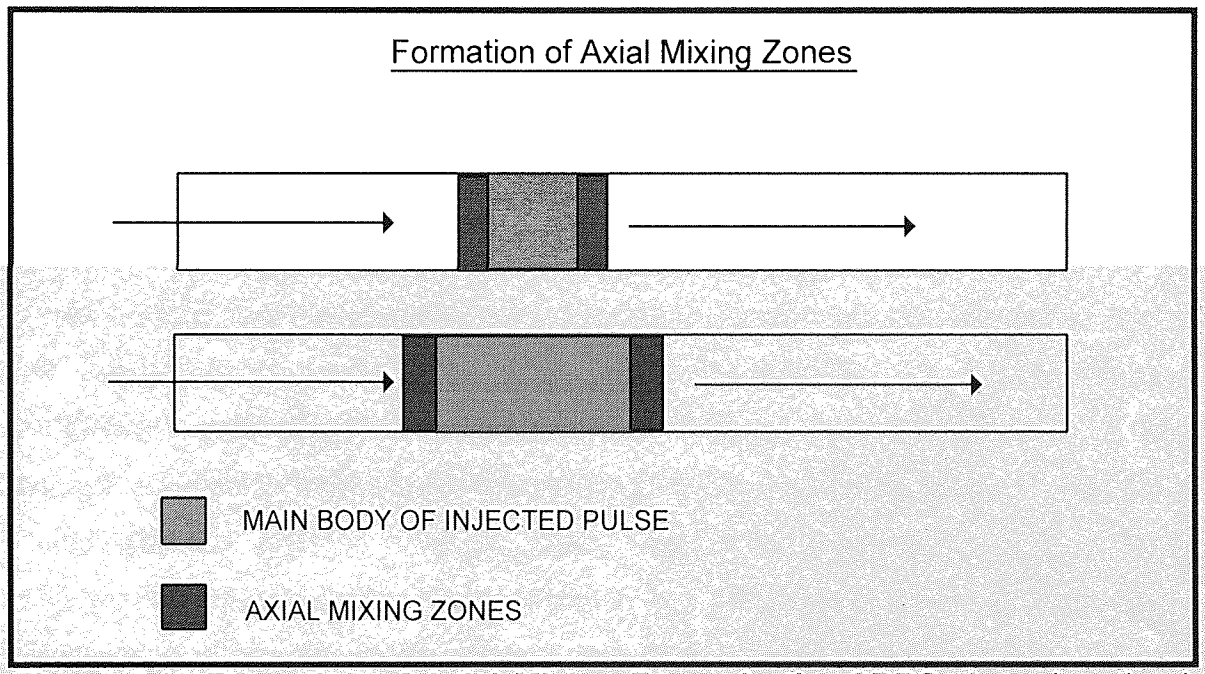



**Figure 5.18 Formation of Axial Mixing Zones in the CBRS.** As pulse size is reduced, the volume of the axial mixing zones increases relative to the total pulse volume.

The length of these zones is likely to be constant for the two series of pulse volume : concentration combination experiments, since the same range of liquid velocities was used, resulting in similar Peclet numbers. As the volume of pulse is reduced, the size of the mixing zones will increase relative to the volume of the pulse "slug", resulting in more intimate contact between enzyme and substrate.

The converse is likely as pulse size is increased, having the effect of reducing the rate and extent of enzyme : substrate mixing, with an accompanying reduction in conversion over a given residence time.

### 5.3.3 Mixing between Enzyme and Substrate

Introduction

The experiments in Section 5.3.2, which investigated the effect of CBRS loading upon lactose conversion, suggested that mixing between enzyme and substrate is important in chromatographic bioreaction and separation. In this section, the nature and degree of enzyme : substrate mixing is studied by monitoring changes in the conductivity of the column effluent.

Experimental

The conductivity of the column effluent was monitored during CBRS operation, using an in-line conductivity detector (`Econo-Gradient' Model, Bio-Rad (UK). The conductivity cell had a dead Volume of 8 μl, so it was unlikely to have any effect on the flow pattern due to back-mixing. The enzyme solution had a conductivity of about 40 μS and it was noticed that the conductivity of enzyme solution decreased as the reaction products emerged from the column outlet. Conductivity was used, therefore, as a sensitive indicator of the arrival of carbohydrates at the column outlet.

It was noticed that the shape of the conductivity profile was very similar to that of the concentration profile of sugars exiting the CBRS. Since the minimum conductivity was recorded at the retention time of the carbohydrates, it was thought that conductivity may be decreasing as sugar solutions (conductivity 5 μS) mixed with the enzyme solution. Serial dilution experiments showed a linear relationship between enzyme concentration in a glucose solution and conductivity. Experiments were performed which, it was hoped, would indicate the nature and degree of mixing between enzyme and substrate. Effluent conductivity was monitored for a series of experiments on the 1 metre long x 1.5 cm i.d. CBRS as it was operated at 55°C. Pulses of 10% w/v lactose were injected in each case, at the flow rate of the eluant. The experiments were performed with pulse sizes of 17.7 $cm^3$ and 35.3 $cm^{-3}$ and were planned using a factorial method.

Factorial Experimental Design

The following factors and levels were chosen:

A = Pulse Volume ($cm^3$) B = Flow rate ($cm^3 min^{-1}$)

$A_1$ = 17.7 $cm^3$, $A_2$ = 35.3 $cm^3$

$B_1$ = 1.1, $B_2$ = 1.7, $B_3$ = 2.1, $B_4$ = 3.0, $B_5$ = 5.0, $B_6$ = 7.0, $B_7$ = 9.0, $B_8$ = 9.9

The following combinations were used, giving a total of 16 experiments:

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| $A_1 B_1$ | $A_1 B_2$ | $A_1 B_3$ | $A_1 B_4$ | $A_1 B_5$ | $A_1 B_6$ | $A_1 B_7$ | $A_1 B_8$ |
| $A_2 B_1$ | $A_2 B_2$ | $A_2 B_3$ | $A_2 B_4$ | $A_2 B_5$ | $A_2 B_6$ | $A_2 B_7$ | $A_2 B_8$ |

Results and Discussion

A typical enzyme conductivity / sugar concentration plot is shown in Figure 5.17, from the combination $A_2 B_8$.

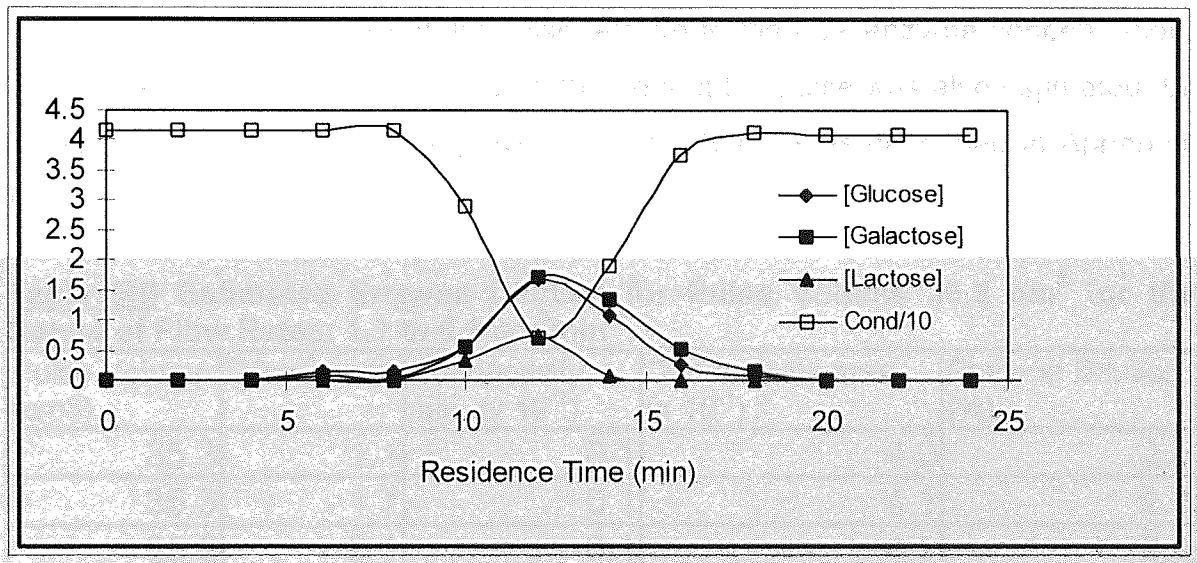



**Figure 5.19 Typical Enzyme Conductivity/Elution Profile.** CBRS effluent conductivity was measured in micro Siemens (μS). Conductivity values were divided by 10 to enable plotting against the same Y axis scale as carbohydrate concentration (measured by HPLC). Flow rate was 9.9 $cm^3 min^{-1}$, pulse volume was 35.3 $cm^3$.

Referring to Figure 5.19 it is apparent that at either side of the minimum, conductivity increased with decreasing sugar concentration. This suggested that at the centre of the pulse/slug, the enzyme was poorly mixed with the sugar solution, whereas, moving outwards to the periphery of the pulse, the enzyme became more intimately mixed with the sugar solution, shown by a gradual return to the

conductivity of the pure enzyme solution. This supports the theory proposed in section 5.3.2 that zones of axial mixing exist at the extremities of the pulse/slug.

Estimation of Degree of Enzyme Mixing

For each pulse size, the degree of dilution of enzyme due to mixing with the carbohydrate pulse was estimated thus, subtracting the baseline conductivity of the degassed and deionised water used to make up sugar and enzyme solutions:

$$\left(\frac{\text{Conductivity Minimum} - \text{Baseline Conductivity}}{\text{Enzyme Conductivity} - \text{Baseline Conductivity}}\right) \times 100 = \%\ \text{Dilution of Enzyme}$$

The resulting enzyme dilution values are presented, together with conductivity data in Tables 5(f) and 5(g). It should be noted that this is really a measure of enzyme concentration at the centre of the pulse, representing the reduction in enzyme concentration at the centre of the pulse relative to the bulk enzyme concentration. The average concentration of enzyme throughout the pulse was also estimated, by measuring the area of the (negative) conductivity peak as described in Appendix A-5.

**Table 5(f) Estimated Enzyme Dilution for Pulse Volume 35.3 $cm^3$ for the Range of Flow Rates: 1.1 to 9.9 $cm^3min^{-1}$.**

| Pulse Volume (cm3) | Q($cm^3min^{-1}$) | Conductivity min. (x $10^{-1}$) | Conductivity max. (x $10^{-1}$) | Enzyme Dilution (%) |
|---|---|---|---|---|
| 35.3 | 1.1 | 0.1 | 4.0 | 2.5 |
| 35.3 | 1.7 | 0.1 | 4.1 | 2.4 |
| 35.3 | 2.1 | 0.1 | 4.1 | 2.4 |
| 35.3 | 3 | 0.2 | 4.2 | 4.8 |
| 35.3 | 5 | 0.2 | 4.3 | 4.7 |
| 35.3 | 7 | 0.2 | 3.9 | 5.1 |
| 35.3 | 9 | 0.4 | 3.9 | 10.3 |
| 35.3 | 9.9 | 0.2 | 3.6 | 5.6 |

For the 35.3 $cm^3$ pulse experiments, the mean minimum effluent conductivity was $2.0 \pm 0.4\mu S$, giving a mean enzyme dilution of $4.7 \pm 1\%$. Since concentration in the eluant was 8 $gl^{-1}$ enzyme this suggested that enzyme concentration was around

(0.05)x 8 = **0.4 $gl^{-1}$** at the centre of the carbohydrate pulse. The average enzyme concentration throughout the pulse was estimated as being (0.52)x 8 = **4.2 $gl^{-1}$**.

**Table 5(g) Estimated Enzyme Dilution for Pulse Volume: 17.7 $cm^3$ for the Range of Flow Rates 1.1 to 9.9 $cm^3min^{-1}$.**

| Pulse Volume (cm3) | Q($cm^3min^{-1}$) | Conductivity min. (x $10^{-1}$) | Conductivity max. (x $10^{-1}$) | Enzyme Dilution (%) |
|---|---|---|---|---|
| 17.7 | 1.1 | 1.1 | 3.9 | 28.2 |
| 17.7 | 1.7 | 0.9 | 3.9 | 23.1 |
| 17.7 | 2.1 | 1 | 4 | 25.0 |
| 17.7 | 3 | 2.1 | 4.1 | 51.2 |
| 17.7 | 5 | 1.8 | 4 | 45.0 |
| 17.7 | 7 | 2.1 | 4.1 | 51.2 |
| 17.7 | 9 | 2.2 | 4.4 | 50.0 |
| 17.7 | 9.9 | 2.1 | 4.3 | 48.8 |

For the 17.7 $cm^3$ pulse experiments, the mean minimum effluent conductivity was 1.7 $\pm$ 2$\mu$S, giving a mean enzyme dilution of 40.3$\pm$3.5%. Since concentration in the eluant was 8 $gl^{-1}$ enzyme this suggested that enzyme concentration was around (0.40)x 8 = **3.2 $gl^{-1}$** at the *centre* of the carbohydrate pulse. The *average* enzyme concentration throughout the pulse was estimated as being (0.71)x 8 = **5.7 $gl^{-1}$**.

So, it appeared that, compared to the 35.3 $cm^3$ pulses, the 17.7 $cm^3$ pulses became more intimately mixed with the enzyme solution. This lends further support to the theory proposed in section 5.3.2, that zones of axial mixing exist at the extremities of the pulse/slug. This would also result in differences in the rate of reaction over the length of the pulse, these zones becoming larger in relation to the total pulse volume as pulse size is decreased. This, in turn, explained the greater degree of mixing of enzyme with the smaller carbohydrate pulse shown in the conductivity tracer experiments.

### 5.3.4 The Effects of Dispersion and Chromatography on Mixing Between Enzyme and Substrate

Introduction

From the experimental programme so far, it was clear that mixing between the enzyme solution and slugs (pulses) of substrate was important in both CBRS design and operation. This mixing arises in part from the hydrodynamic mixing processes associated with flow through the packed bed and in part from the chromatographic processes taking place in the column. In this section a quantitative assessment is made of the relative importance of these two mixing mechanisms.

Mixing Due to Dispersion

The hydrodynamic mixing processes can be characterised in terms of a Peclet Number and the particle Reynolds Number. For the experimental conditions used in this chapter, the particle Reynolds Numbers ($Re_p$) range from 0.016 to 0.14. The particle Peclet Number is calculated as follows,

$$Pe_p = \frac{Udp}{D_L}$$

where U is interstitial velocity in $cms^{-1}$ and $D_L$ is the longitudinal dispersion coefficient. $Pe_p$ has a constant value of about 0.5 for liquid systems in this region (Westerterp 1983), which means that for a constant particle diameter, dp of 0.0075 cm, $D_L$ can be calculated thus:

$$D_L = \frac{0.0075}{0.5} \times U \ cm^2s^{-1}.$$

As shown in Table 5 (h) overleaf, the calculated dispersion coefficients range from $7 \times 10^{-4}$ $cm^2s^{-1}$ to $6 \times 10^{-3}$ $cm^2s^{-1}$; these values are orders of magnitude greater than the value of the molecular diffusivity for lactose of $5 \times 10^{-6}$ $cm^2s^{-1}$. Consequently, it can be concluded that the main mechanism for mixing is statistical dispersion arising from flow of liquid through the packing.

**Table 5(h) Characterisation of Mixing in the CBRS** Values were calculated for the range of operating flow rates1.1 to 9.9 $cm^3min^{-1}$.

| Volumetric Flow Rate Q ($cm^3min^{-1}$) | 1.1 | 9.9 |
|---|---|---|
| $Re_p$ (based on superficial liquid velocity, $U_0$.) | 0.016 | 0.14 |
| Dispersion Coefficient $D_L$ ($cm^2s^{-1}$) | $7.1 \times 10^{-4}$ | $6.2 \times 10^{-3}$ |
| External Mass Transfer Coefficient $k_e$ ($cms^{-1}$) | 0.0052 | 0.0108 |
| External Mass Transfer "Rate Constant" $k_ea$ ($s^{-1}$) | 2.75 | 5.70 |
| Intraparticle Mass Transfer Coefficient $k_i$ ($cms^{-1}$) | $3.8 \times 10^{-4}$ | $3.8 \times 10^{-4}$ |

The dispersion effect can also be interpreted in terms of the number of theoretical plates (N) using the relationship:

$$N = \frac{UL}{2D_L}$$

where $\frac{UL}{D_L}$ is the axial Peclet Number.

For a column of 1 metre length, N is about 3300; which means that the height of a theoretical plate is 0.03 cm, equivalent to four particle diameters. More importantly, these figures confirm that flow was plug-like under all conditions used in the experimental programme.

A rough estimate of the penetration depth either side of the eluant solution / substrate solution boundary can be made by treating dispersion as a classic diffusion process. According to Beek and Muttzall (1975),

$$\text{Penetration Depth} = 2\sqrt{\pi\left(\frac{D_L}{UL}\right)L^2}.$$

Since $\frac{UL}{D_L}$ is constant, there will be no difference in penetration depth over the range of Particle Reynolds Numbers used in the experiments. For L = 100 cm, the penetration depth is about 4 cm either side of the pulse of substrate solution. The length of packed column occupied by the standard pulses (17.7 $cm^3$ and 35.3 $cm^3$)

is 30 cm and 60 cm respectively. As a result, there are likely to be factors other than statistical dispersion involved in the mixing of enzyme and substrate.

Mixing and Chromatographic effects

First, attention will be given to the relative rates of external mass transfer and intraparticle diffusion processes. The numerical value of the external mass transfer coefficient between the mobile and stationary phases can be estimated from the correlation:

$$Sh = \frac{1.09}{\varepsilon} Re^{0.33} Sc^{0.33}$$

for the range of Reynolds Numbers used in the experimental work. Using the following physical properties for lactose at 55°C:

$\rho = 1.0\ gcm^{-3}$

$\mu = 0.005\ gcm^{-3}$

$D_m = 0.48 \times 10^{-5}\ cm^2s^{-1}$

it can be shown that $k_e$, the external mass transfer coefficient ranges from 0.0052 to 0.0108 $cms^{-1}$ (see Table 5h). Knowing the bed voidage (0.34) and average particle size (0.0075 cm), the interfacial area per unit bed volume is found to be 528 $cm^2cm^{-3}$. As a consequence of this high value, the $k_La$ values are such that the time scale for mass transfer is of the order of seconds.

$k_i$, the intraparticle mass transfer coefficient, depends on molecular diffusivity ($D_{eff}$) within the gel-like ion-exchange resin particles. Estimates of $D_{eff}$ can be made from the relationship:

$$D_{eff} \approx \frac{\varepsilon_p}{\tau} . D_m$$

where $\varepsilon_p$ is the particle voidage and $\tau$ is the tortuosity factor.

A conservative estimate of $D_{eff}$, based on $\varepsilon_p$ and $\tau$ of 0.5 and 5 respectively, suggests

$$D_{eff} \approx \frac{D_m}{10}.$$

This leads to a value for $k_i$ of $3.8 \times 10^{-4} cms^{-1}$, using the formula suggested by Storti *et al* (1993):

$$k_i = \frac{6 D_{eff}}{dp}.$$

Once more, this value indicates that the intraparticle diffusion step, like external mass transfer, will be relatively rapid and so it is reasonable to conclude that mass-transfer steps will not be rate-controlling in the overall chromatographic process. This is in agreement with the findings of Wilhelm and Riba (1989), who studied the chromatographic separation of D-xylose and D-mannose on an ion exchange resin which was very similar to that used in this thesis. They found that internal diffusion was very fast and could be neglected.

Having established that adsorption-desorption phenomena control the chromatographic process, it is possible to examine their effect on mixing between the enzyme solution and the substrate, lactose, in the CBRS. If it is assumed that equilibrium is rapidly achieved between substrate in the mobile and stationary phases (and that there is a linear adsorption isotherm), experimental data can be interpreted in terms of the classic $Kd_i$ approach. This enables estimates to be made of the relative residence time difference between an adsorbed component and a non-adsorbed species:

$$tr_i = tr_0\left(1 + \frac{1-\varepsilon}{\varepsilon} Kd_i\right).$$

In the case of lactose relative to the enzyme, lactase,

$$tr_{lactose} \sim 1.1x\ tr_{enzyme}.$$

It is then possible to estimate the relative velocity between these key components and hence the relative penetration or mixing. This means that the size of the axial mixing zones, as described in subsection 5.3.2, can be estimated. For example, at the minimum operating flow rate of 1.1 $cm^3min^{-1}$, the trailing edge of the lactose slug will travel at a velocity of 1.67 $cms^{-1}$. The leading edge of the following enzyme eluant, however, will travel at a velocity of 1.8 $cms^{-1}$. This means that, as the leading edge of the following enzyme eluant travels 100 cm along the CBRS, the trailing edge of the lactose slug will only travel 91.9 cm. There will therefore be 100 - 91.9 = 8.1 cm of overlap which will enhance the hydrodynamically-induced mixing due to statistical dispersion.

As pointed out by Villermaux *et al* (1993), extracolumn factors can also affect peak broadening; these include non-ideal pulse form, flow in connecting pipes and the detector characteristics. However, these factors were considered with great care when designing and operating the system so as to minimise their effect.

Concluding Remarks

Because of the complexity of the mixing processes involving the substrate pulse and the enzyme-based eluant, it is the experimental results in Section 5.3.3 that give the best picture of the enzyme profile in the pulse. What has been established is that statistical dispersion and chromatographic effects are key factors in the overall mixing process.

## 5.4 Effect of Enzyme-Substrate Contacting Pattern on Enzymic Reaction

Introduction

In earlier experiments, the performance of a plug flow chromatographic reactor was compared with that of a well-mixed stirred tank reactor. The basis for comparison was the degree of lactose conversion attained over a nominal residence time of eight minutes. Given the attendant mixing limitations of a plug-flow reactor, it was surprising to see an improvement in lactose conversion performance when compared to that in a well-mixed batch reactor.

It was hypothesised that this improvement in lactose conversion rate could be due to the contacting pattern between lactose and enzyme solution. In a stirred batch reactor, the enzyme is almost immediately contacted with the entire charge of lactose, while in the CBRS, the lactose charge is introduced as a pulse which is added over a period of typically 4 minutes. If performance is shown to depend on the type of contacting pattern used, then differences in the initial enzyme to substrate concentration ratio could mean that substrate inhibition is the limiting factor. Therefore, a high initial lactose concentration (20% w/v) was chosen for the experiments, so that this effect, if present, would be magnified to the point where any performance differences were measurable. Since, in the earlier experiments, conditions were not comparable in terms of reactor volume and contacting pattern, a set of experiments was designed based on the same pulse volume : void volume ratio as in a CBRS.

Standard stirred batch reactions were performed in parallel with fed batch reactions and reactions in the CBRS. As far as possible, conditions were made comparable, with the aim of establishing whether the contacting pattern between enzyme solution and substrate had any effect on performance in terms of lactose conversion rate. If this were so, there would be implications for the study of CBRS performance in general, since benefits over stirred batch systems are attributed to the separation of reaction components from the reaction zone. However, performance differences could be due to the fact that, in the CBRS, the enzyme is exposed to the substrate gradually rather than encountering the maximum concentration at the start of reaction. In introducing the charge gradually, its

concentration reduces during the injection period, so that the enzyme does not encounter the substrate concentration it would meet at the start of a stirred batch reaction.

Experimental

Since the chromatographic reactor was operated in non-separative mode, it could be regarded as a conventional plug-flow reactor (PFR). The 1 metre long x 1.5 cm i.d. CBRS was operated at 40°C in the manner described in Chapter 4; a flow rate of 9.0 $cm^3min^{-1}$ was used and a 35.3 $cm^3$ pulse of 20% w/v lactose was added over a period of 3 minutes 55 seconds.

In the absence of knowledge of the degree of mixing between enzyme and substrate, it was difficult to mimic the concentration of lactose and β-galactosidase when designing the stirred batch experiments. The void volume of the column is 61 $cm^3$, so it was assumed that the lactose pulse is diluted in this volume of enzyme solution over the length of the column. The initial lactose concentration was calculated such that mixing resulted in a concentration of 20% w/v. So, the concentration of the lactose charge prior to mixing was:

$$20 \times \frac{61}{35.3} = 34.6\% \text{ w/v.}$$

In the stirred batch reactions, 35.3 $cm^3$ of 34.6% w/v lactose was added to

$$61 - 35.3 = 25.7 \text{ cm}^3$$

of enzyme solution while stirring, so that mixing could be assumed to be instantaneous. In the fed batch experiments, the same volume of lactose was added by placing the tip of the pump outlet tube in the neck of the flask and running the pump at the flow rate used for the PFR experiments, that is 9.0 $cm^3min^{-1}$ for 3 minutes 55 seconds; the enzyme concentration was 1.25 g/l. The local enzyme and lactose concentration reached in the CBRS reaction zone was difficult to mimic in stirred batch experiments, due to lack of information on mixing rate and the simultaneous lactose concentration reduction due to reaction. Due to reaction during the pulse introduction period coupled with mixing-related dilution,

the lactose concentration encountered by the enzyme at any time was likely to be lower than that experienced in the stirred batch reactor where the full lactose charge was added instantaneously at the start of the run.

Results and Discussion

Progress of reaction profiles from the batch versus fed batch experiments are presented in Figure 5.20. The data is also presented in tabular form in Table 5(i).

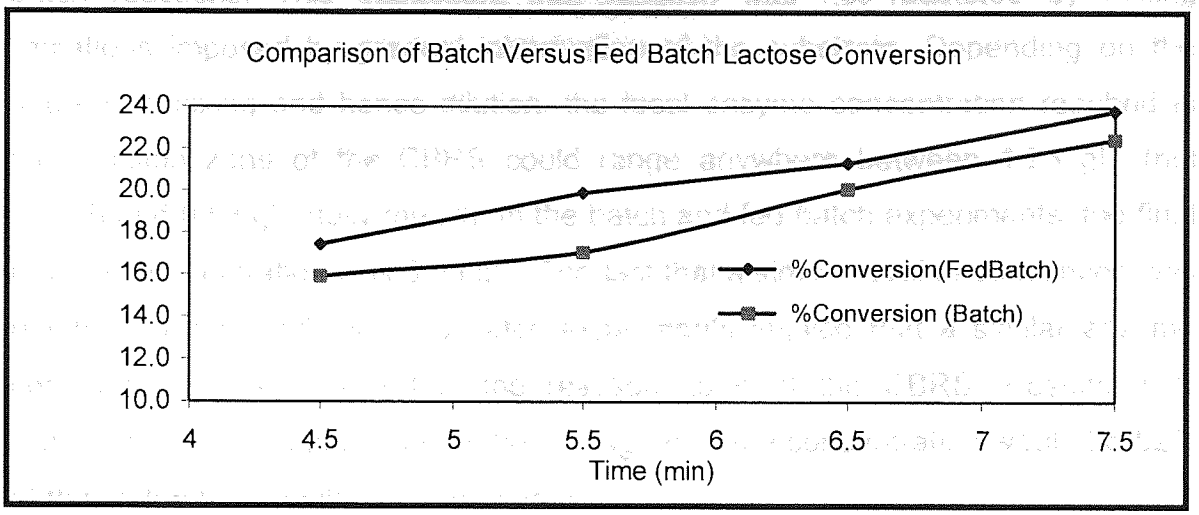



**Figure 5.20 Comparison of Batch and Fed Batch Lactose Conversion Over a 7.5 Minute Reaction Period at 40°C.** This residence time approximates to that of the 1 metre long x 1.5 cm i.d. CBRS.

**Table 5(i) Data from Batch Vs Fed Batch Experiments Presented in Figure 5.20.**

| Time (min) | %Conversion(FedBatch) | %Conversion(Batch) | %Conversion(PFR) |
|---|---|---|---|
| 4.5 | 17.5 | 15.9 | |
| 5.5 | 19.9 | 17.0 | |
| 6.5 | 21.3 | 20.1 | |
| 7.5 | 23.8 | 22.5 | 24.9 |

Although slightly higher conversions were seen in the fed batch reaction than in the batch reaction, the difference in the final conversion level (at 7.5 minutes) was not significant when considered in the light of typical experimental variance: (± 1.25%). Overall, a variance of ± 0.8 % was seen when comparing lactose conversions after 7.5 minutes for the batch, fed batch and PFR reactions. The CBRS experiments resulted in a mean lactose conversion of 24.9 ± 1.25%.

So, the contacting pattern had no significant effect on reactor performance. It appeared that the enzymic reaction was rapid enough to compensate for the fact that in the fed batch and CBRS experiments, the complete lactose charge had not been introduced until about 4 minutes after the start of reaction; by contrast, in the stirred batch experiment, it had been introduced four minutes earlier.

In the CBRS, similar conversion levels were reached to those seen in the stirred batch reactions. This suggested that reaction was not restricted by mixing limitations imposed by gradual introduction of the substrate. Depending on the degree of mixing and hence dilution, the local enzyme concentration reached in the reaction zone of the CBRS could range anywhere between 1.25 $gl^{-1}$ (not mixed) and 0.53 $gl^{-1}$ (fully mixed). In the batch and fed batch experiments, the final enzyme concentration was 0.53 $gl^{-1}$. The fact that a similar level of conversion was reached to the batch and fed batch experiments implied that a similar enzyme concentration was reached in the reaction zone of the CBRS. Results from Subsection 5.3.3 suggested that the average enzyme concentration would be 52% of that in the bulk solution, about 0.65 $gl^{-1}$.

When the batch and fed batch experiments were designed, it was thought that substrate inhibition was possible in the lactose-lactase reaction. Later reference to experimental results produced by Taddei (1994) revealed that the initial rate of lactose conversion was still increasing linearly with a 16% w/v initial lactose concentration. So, in retrospect, based on initial rate data, lactose conversion rate was unlikely to be suppressed by substrate inhibition even at the highest initial lactose concentration of 20% w/v encountered by the enzyme in the stirred batch experiments.

## 5.5 Conclusions

Experiments which showed the likely progress of reaction along the length of a CBRS revealed that there was negligible chromatographic separation over the column length occupied by enzymic reaction, so that reaction and separation could be de-coupled and considered independently. Progress of reaction plots showed reasonable agreement with those predicted by an integrated rate equation, reflecting the effect of pulse volume on mixing between enzyme and substrate.

The effect of inlet design was studied, and it was concluded that use of a needle type injector was unlikely to improve mixing between the enzyme solution and the substrate pulse at normal CBRS operating flow-rates. Also, use of the needle type injector was thought likely to have detrimental effects on chromatographic separation. The sinter type injector was judged to give the best overall CBRS performance.

Tracer experiments, using the inherent conductivity of the enzyme solution, gave an insight into the degree of mixing between enzyme and substrate. It was proposed that zones of axial mixing exist at the extremities of an injected pulse of substrate; these zones providing adequate contact between enzyme and substrate. The influence of these mixing zones was predicted to diminish with increasing pulse volume. Similar conversion levels to those seen in stirred batch reactions were reached in the CBRS, suggesting that reaction was not restricted by mixing limitations imposed by gradual introduction of the substrate. Superimposed on this effect appeared to be one of chromatographic mixing, brought about by retention of the substrate relative to the enzyme. This would increase the amount of enzyme which comes into contact with lactose molecules.

In CBRS operation, there is an apparent contradiction. Plug flow conditions may be assumed in such a column when packed with small diameter spherical particles. Although ideal for the chromatographic progress, these conditions would seem to be unsuitable for the enzymic reaction where a degree of axial dispersion would be required. It appeared that with smaller pulse volumes, there is sufficient axial dispersion to provide adequate contact between enzyme and substrate.

However, as pulse size is increased, this degree of axial mixing becomes a limiting factor on enzymic reaction.

Attention was turned to the relative influence of mass transfer and chromatography on chromatographic bioreaction and separation. This involved consideration of the flow characteristics of the bulk liquid, together with the relative time scales involved in the processes of reaction, mass transfer, adsorption and desorption. The main mixing mechanism, it was decided, was that of statistical dispersion of lactose due to flow of liquid through the column packing. The relative retention of enzyme and lactose molecules appeared to significantly increase the degree of mixing between enzyme and substrate. Mass transfer processes appeared to be very rapid and not rate-controlling.

Finally, it was shown that the contacting pattern between enzyme and substrate in a CBRS, where the pulse of substrate is introduced gradually, does not confer any advantage over that of a conventional stirred batch reactor, where the substrate is introduced at the start of reaction.

## Chapter Six

# Design and Construction of a Chromatographic Bioreactor-Separator for The Production of Oligosaccharides.

### Scope of Chapter

During the experiments described in Section 5.3.1, in which the progress of reaction was studied in a 1 metre-long CBRS, small amounts of higher oligosaccharides were detected. On surveying the literature, it seemed likely that these oligosaccharides were galactooligosaccharides (GOS). These sugars are galactose-containing oligomers with a chain length of 2 or more monomer units. GOS were detected and identified as early as the 1950's by, for example, Aronson (1952), Roberts and McFarren (1953), and Pazur *et al* (1958). Until recently, GOS have been regarded as nuisance by-products of the enzymic hydrolysis of whey lactose (Betschart and Prenosil 1984). However, over the past decade GOS have attracted much commercial interest, particularly in Japan; where in 1995, annual production of GOS was around 15,000 tonnes (Crittendon and Playne, 1996).

The reason for this high demand for GOS lies in their popularity as food ingredients which are purported to have health benefits. GOS are recognised as being probiotics, which are defined by Gibson and Roberfroid (1995) as: "microbial food supplements that beneficially affect the host by improving its intestinal microbial balance". Their action involves an increase in number of bacterial groups such as Bifidobacterium and Lactobacillus in the gut (Ito *et al* 1993). Such microorganisms are commonly incorporated into "live" yoghurts. Other applications of oligosaccharides are currently under investigation, including the treatment of bacterial infections by interfering with the adhesion of human pathogens (Zopf and Roth 1996).

Because of the growing importance of oligosaccharides, it was decided to change the original research objectives and focus on oligosaccharide production rather than on lactose hydrolysis. This chapter describes studies undertaken to optimise the yield of oligosaccharides, culminating in the design, construction and evaluation of a dithermal chromatographic bioreactor-separator.

The chapter is arranged as follows:

Section 6.1 covers work with a 1 metre long x 1.5 cm i.d. chromatographic bioreactor-separator (CBRS) which, supplemented by stirred batch studies, aided the choice of operating conditions in the reaction zone of the dithermal chromatographic system.

Oligosaccharides are intermediates which are formed en route to complete lactose hydrolysis, in the presence of the enzyme β–galactosidase.

LACTOSE $\Rightarrow$ [OLIGOSACCH] $\Rightarrow$ GLUC + GAL

So, to maximise the yield of oligosaccharide, it was necessary to control enzyme activity. This was achieved by changing the operating temperature along the length of the bioreactor-separator. The results of this work are presented in Section 6.2.

Attention was then turned to the separation of the components of the reaction mixture. Since it was first necessary to produce a relatively large amount of this mixture, the opportunity was also taken to generate pure oligosaccharide fractions for detailed analysis. Information about both chromatographic separation and analysis of the oligosaccharides is recorded in Section 6.3.

Finally, Section 6.4 describes how use was made of the earlier work to design and construct a so-called "Dithermal Chromatographic Bioreactor-Separator" for the production and separation of oligosaccharides. Preliminary results confirm the feasibility of this processing approach.

## 6.1 Development of the Reaction Section of the 4 metre long x 1.5cm i.d. Chromatographic Bioreactor-Separator.

Since the chromatographic columns used in this work were conveniently divisible into 1 metre lengths in terms of temperature control jacketing, it was proposed that the first metre of column would act as the reaction section, having an independent heating circuit to the separation section. A series of experiments could then be used to optimise the performance of the reaction section.

### 6.1.1 Time-Course of Oligosaccharide Formation

Introduction

Stirred batch experiments performed in collaboration with P. Tack of this laboratory showed that oligosaccharide concentration reached a peak at around 7 minutes, after which oligosaccharides were broken down to their component monomers. Experiments were performed to study the time course of oligosaccharide formation in a 1 metre long x 1.5 cm Chromatographic Bioreactor Separator (CBRS). This would enable selection of the optimal flow-rate for the reaction section.

Experimental

The approach used was to operate a CBRS of fixed length (1 metre) at progressively higher volumetric flow rates, resulting in shorter residence times. This was expected to indicate the progress of reaction along the column. 10% w/v lactose pulses of volume 17.7 $cm^3$ and 35.3 $cm^3$ were injected on to the column. An enzyme concentration of 8 $gl^{-1}$ was selected, giving an activity of 26 $Ucm^{-3}$. The CBRS was operated (at 55°C) at each of the following flow rates: 1.1, 2.1, 3.0, 5.0, 7.0, 9.0 and 9.9 $cm^3min^{-1}$. This gave column residence times ranging from 6.2 to 56 minutes. The outlet concentration of oligosaccharide was then measured at each residence time.

Results and Discussion

Oligosaccharide concentration reached a peak at around 7 minutes, after which it declined significantly. This is in accordance with the results of stirred batch experiments performed by Tack (1995). A flow-rate of 9 $cm^3min^{-1}$ was therefore selected for the reaction stage giving a residence time of 6.8 minutes

for the reaction section. The likely time-course of oligosaccharide formation in a CBRS is shown in Figure 6.1.

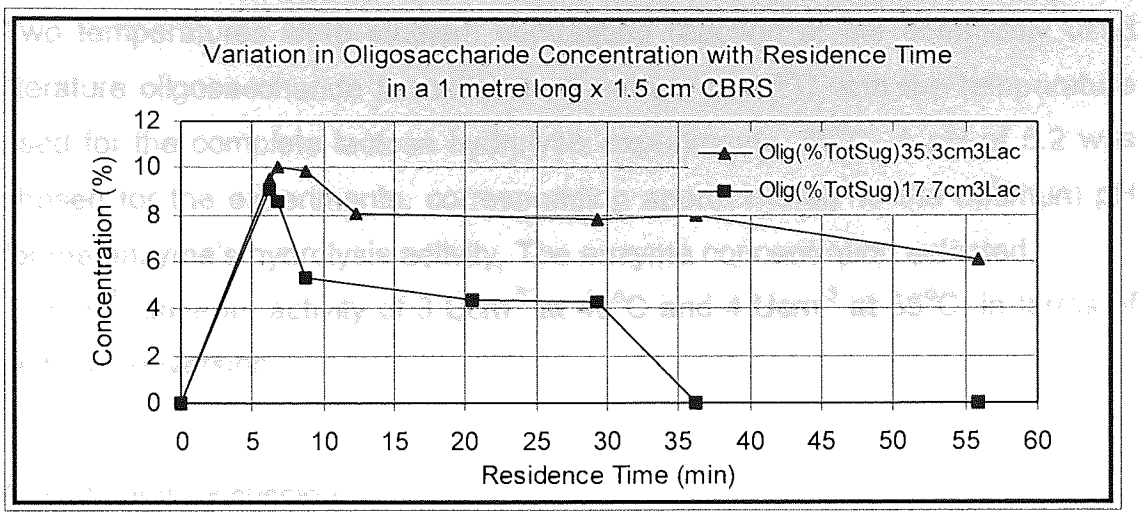



**Figure 6.1 Progress of Oligosaccharide Formation with Residence Time in a 1 metre long x 1.5 cm i.d. CBRS.** Concentration is expressed as a percentage of total sugars in the product mixture.

Studies performed by Lopez Leiva and Guzman (1995) suggest that GOS are formed almost instantaneously. In this thesis however, it was not possible to monitor GOS formation earlier than 6.2 minutes since this was the minimum residence time of the 1 metre long x 1.5 cm i.d. CBRS.

### 6.1.2 Effect of Initial Lactose Concentration and Temperature

#### Introduction

Having selected the operating flow rate for the reaction section, the effects of temperature and initial lactose concentration were studied. This would assist in selecting conditions for operating the CBRS.

#### Experimental

The 1 metre long x 1.5 cm i.d. CBRS was operated in the standard way, as detailed in Chapter 3, at a flow-rate of 9.0 $cm^3min^{-1}$. Although in the literature, initial lactose concentrations of up to 60% (w/v) were used, a range was selected (5, 10, 15 and 20% (w/v)), bearing in mind the known limitations of the separative system in terms of concentration loading. The enzyme concentration selected was 1.25 g/l, giving an activity of 4 $Ucm^{-3}$. This would enable comparisons to be made with the stirred batch studies of Tack (1995) and Iwasaki, Nakajima and Nakao (1996), who each used the same enzyme

as used in this thesis, β-galactosidase from Aspergillus oryzae at a concentration which gave about the same enzyme activity.

Two temperatures were studied, contrasting reaction at the commonly used literature oligosaccharide formation temperature of 40°C with the temperature used for the complete lactose hydrolysis experiments, 55°C. A pH of 5.2 was chosen for the experiments, corresponding approximately to the optimum pH for the enzyme's hydrolysis activity. The enzyme concentration selected, 1.25 $gl^{-1}$, gave an activity of 3 $Ucm^{-3}$ at 40°C and 4 $Ucm^{-3}$ at 55°C, in terms of lactose conversion.

Results and Discussion

The results of the experiments in which lactose concentration was varied are shown in Table 6(a).

**Table 6(a) Variation of Oligosaccharide Production with Initial Lactose Concentration.** The oligosaccharide concentrations shown represent mean values of duplicate experiments.

| $[Lac]_o$ %w/v | Oligosaccharide as % of Total Sugars (40°C) | Oligosaccharide as %Total Sugars (55 °C) |
|---|---|---|
| 5 | 5 | 15 |
| 10 | 12.3 | 8.8 |
| 15 | 10.4 | 9.9 |
| 20 | 11.5 | 12.3 |

These concentrations are, on average, 40% lower than those attained by Tack (1995) in stirred batch reactions performed under similar conditions. This discrepency could be attributed to mass transfer limitations in the plug flow reactor (see also Chapter 5).

At both temperatures studied, an inverse relationship existed between percentage conversion of lactose and initial lactose concentration (see Figure 6.2) overleaf. This is in agreement with the findings of Iwasaki *et al* (1996).

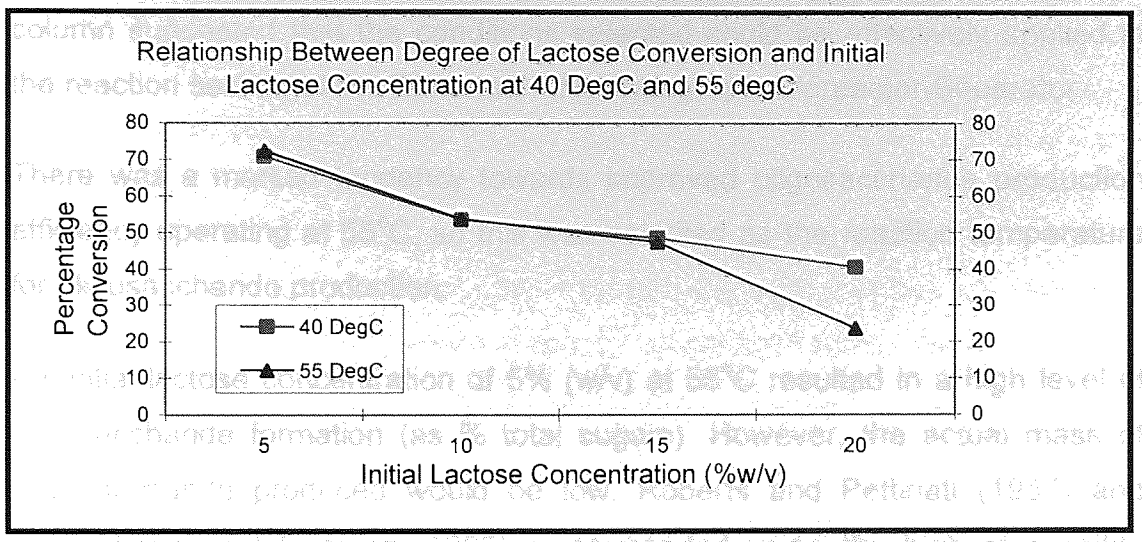



**Figure 6.2 Variation of Degree of Lactose Conversion with Initial Lactose Concentration at 40°C and 55°C.** The experiments were performed in duplicate.

As can be seen from Figure 6.3, at both 40°C and 55°C the proportion of lactose converted to oligosaccharide increased with initial lactose concentration. This trend was also highlighted by Yang and Tang (1988).

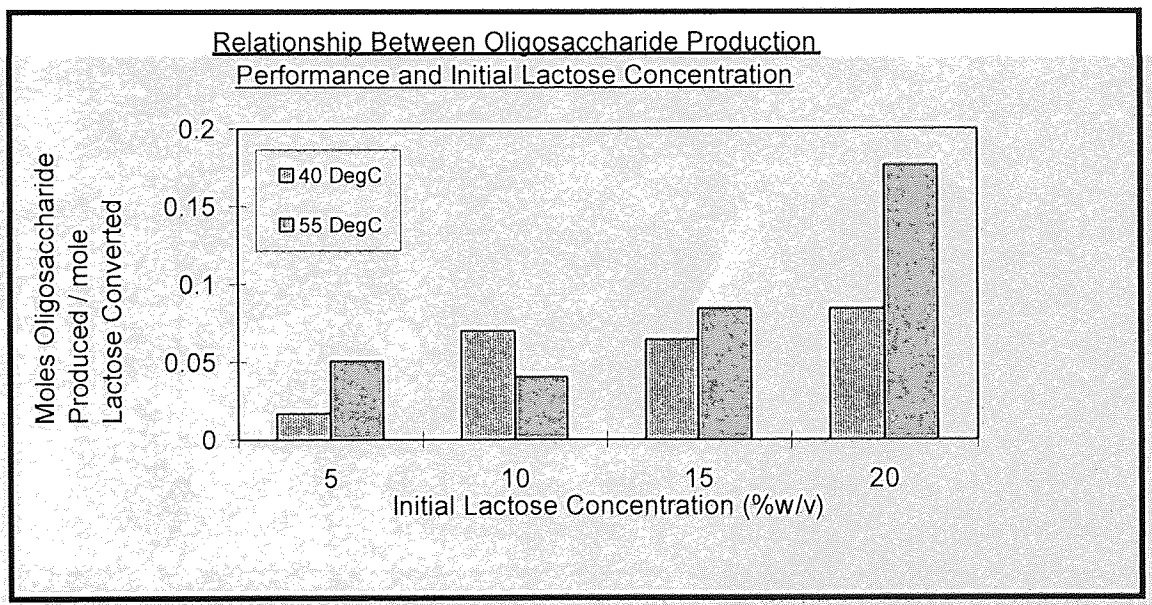



**Figure 6.3 Relationship Between Oligosaccharide Production Efficiency and Initial Lactose Concentration at 40°C and 55°C.** There was a marked tendency towards improved oligosaccharide production efficiency operating at 55°C.

Overall, the 1 metre long x 1.5 cm i.d. column behaved in a similar way to stirred batch systems described in the literature. This meant that useful design information could be extracted from results of the stirred batch studies which have been used almost exclusively by other workers. The performance of the

column suggested that the conditions selected could be effectively applied in the reaction section of the Dithermal Chromatograpic Bioreactor-Separator.

There was a marked tendency towards improved oligosaccharide production efficiency operating at 55°C, so this was selected as the reaction temperature for oligosaccharide production.

An initial lactose concentration of 5% (w/v) at 55°C resulted in a high level of oligosaccharide formation (as % total sugars). However, the actual mass of oligosaccharide produced would be low. Roberts and Pettinati (1957) and Lopez Leiva and Guzman (1995) recommended using the highest possible lactose concentration to maximise GOS production. It was decided to operate the CBRS with a lactose pulse concentration of 15% (w/v), since, from experience, it was known that above this concentration the upper limit of CBRS loading was approached, with detrimental effects on chromatographic separation.

## 6.2 Evaluation of the Use of Raised Temperature to Control the Production of Oligosaccharides in a 1 metre long x 1.5 cm i.d. Chromatographic Bioreactor-Separator.

Introduction

According to Lopez Leiva and Guzman (1995), Oligosaccharides are formed as intermediates which are broken down to monosaccharides. In stirred batch experiments, Tack (1995) observed significant break-down of oligosaccharide over 90 minutes. Since this corresponded to the residence time of the proposed 3 metre long separation section, a means of reaction control was sought. Also, if the reaction zone is relatively short, products are not being formed along the whole column length. This is beneficial in terms of chromatographic separation. On examining Figure 6.4, Shieh's data (Shieh 1994) shows that lactase activity is optimal at 55-60°C, after which it falls sharply to 60% of this activity at 65°C. It was hypothesised, therefore, that temperature could offer a means of reaction control.

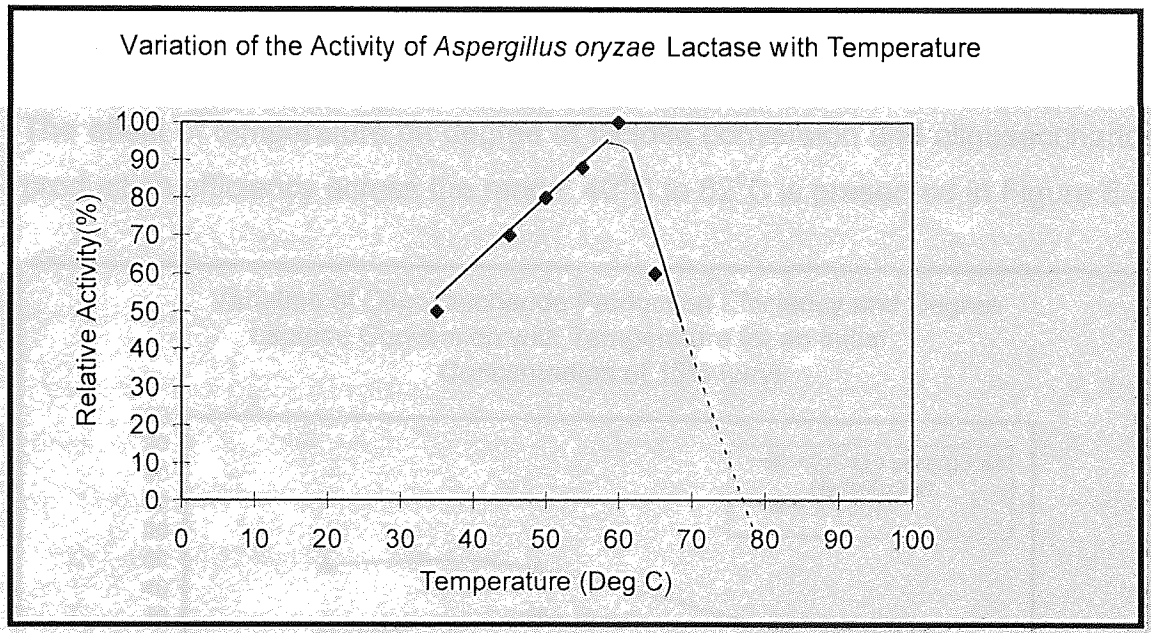



**Figure 6.4 Variation of the Activity of *Aspergillus oryzae* Lactase with Temperature (after Shieh 1994).** Extrapolation of Shieh's data (solid line) suggested that lactase activity could be reduced to negligible levels by raising the temperature to around 80°C.

Extrapolation of Shieh's data (dashed line), suggested that activity should be negligible at approximately 80°C and that a temperature in excess of 80°C would be suitable for limiting further reaction as the reaction mixture entered the separation section.

Experimental

Stirred flask experiments demonstrated that the enzyme precipitated at 85°C, shown by turbidity of the enzyme solution. This would be undesirable in the chromatographic reactor-separator due to the likelihood of column blockage. 82°C was therefore selected as the temperature for the separation section. The performance of the 1 metre long x 1.5cm i.d CBRS at 82°C was then compared with that seen at 40°C and 55°C. The aim was to test the effectiveness of the proposed separation section in terms of controlling the formation of oligosaccharides. The CBRS was operated at three temperatures:40°C, 55°C and 82°C, with a lactose pulse concentration of 15% (w/v) and a pulse volume of 35.3 $cm^3$. Although under the proposed separation conditions, the separation section was to be operated with a flow rate of 2.1 $cm^3$ $min^{-1}$, the actual experiments were carried out at a flow rate of 9.0 $cm^3$ $min^{-1}$, giving a contact time of 6.8 minutes at 82°C. This represented a `worst case' situation where the enzyme had only limited contact time with the high temperature section (in the actual separation section contact time was to be 88 minutes).

Results and Discussion

The effect of temperature on degree of lactose conversion and oligosaccharide production efficiency across the range: 40°C to 82°C is presented in Figure 6.5.

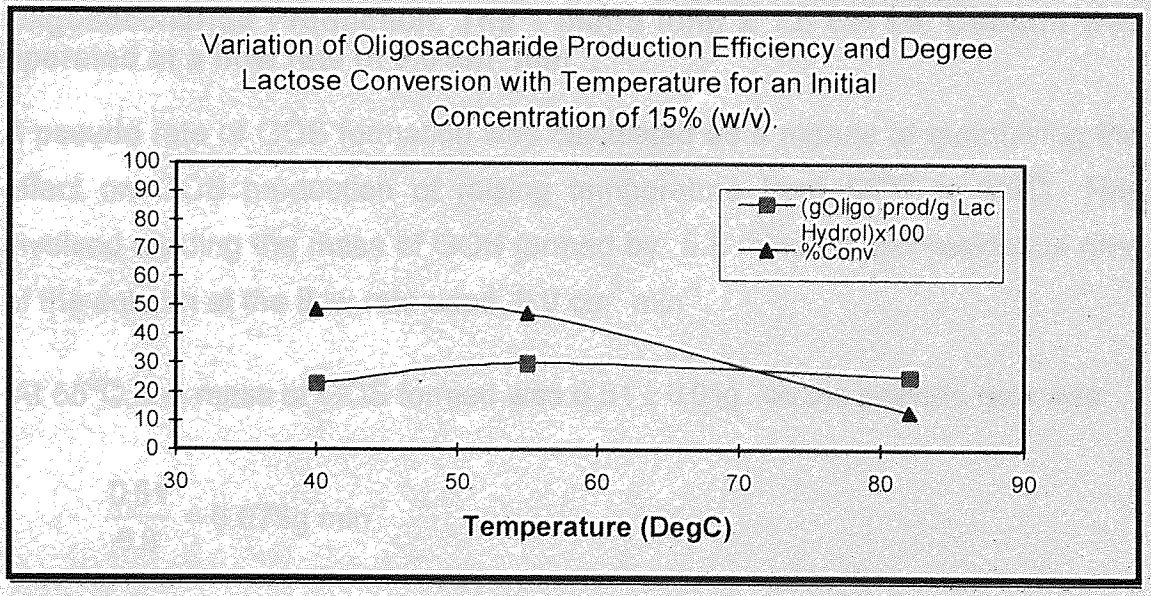



**Figure 6.5 Variation of Oligosaccharide Production Efficiency and Degree of Lactose Conversion with Temperature.** Initial lactose concentration was 15%(w/v).

Oligosaccharide production efficiency increased across the temperature range studied while degree of lactose conversion decreased. These trends mirror those seen by Iwasaki *et al* (1996). Yang and Tang (1988) reported that up to an initial lactose concentration of 15% (w/v), little difference in the extent of oligosaccharide formation was seen over the range 8°C to 50°C.

At 40°C and 55°C, oligosaccharide could be produced in concentrations comparable to those predicted by stirred batch experiments, that is, around 10% of total sugars (see Figure 6.6).

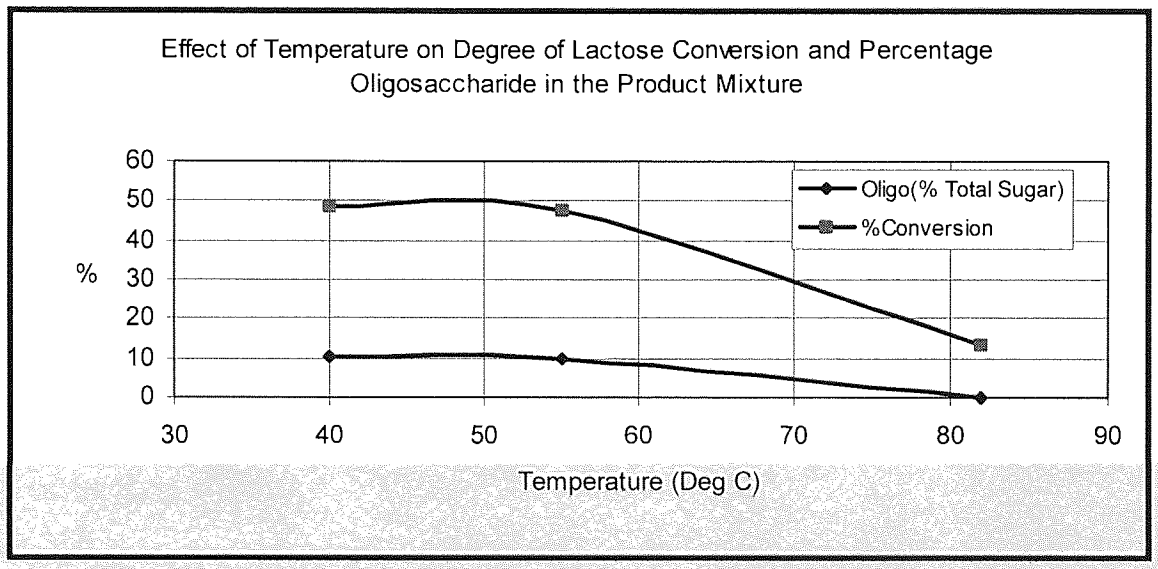



**Figure 6.6 Effect of Temperature on Lactose Conversion and Oligosaccharide Production. The 1 metre long x 1.5 cm i.d. Column was operated at a flow rate of 9.0 $cm^3$ $min^{-1}$.**

A pseudo rate of GOS formation was calculated as a means of quantifying the effect on GOS production of raising temperature from 55°C to 82°C. This involved dividing the mass of GOS formed by 6.8 minutes, the residence time of the column at the flow rate used, 9.0 $cm^3$ $min^{-1}$.

At 55°C, the mass of GOS formed was $0.51 \pm 0.01$g, so the pseudo rate was:

$$\frac{0.51}{6.8} = 0.075\text{g min}^{-1}$$

At 82°C the mass of GOS formed was $0.15 \pm 0.05$g, so the pseudo rate was:

$$\frac{0.15}{6.8} = 0.022\text{g min}^{-1}$$

So, by raising the temperature to 82°C the pseudo rate was reduced to 29.3% of the rate at 55°C. The amount of GOS produced, expressed as a percentage of the total sugar concentration, was reduced from $9.87 \pm 0.79\%$ to $2.56 \pm 0.45\%$. It was concluded, therefore, that the 82°C temperature of the separation section should be sufficient to slow down the reaction enough to enhance separation and hence purity of the GOS product.

## 6.3 Stirred Batch Preparation of Oligosaccharides Followed by Purification in a 3 m long x 1.5 cm i.d. Preparative Chromatographic Column

Introduction

Having designed the reaction section of the Dithermal Chromatographic Bioreactor-Separator (DTCBS), attention was turned to the separation section. This was to be a 3 metre-long x 1.5 cm i.d. section of chromatographic column, of the type described throughout the thesis so far. The main aim of the following experiments was to ensure that it would indeed separate a typical pulse of reaction mixture as it emerged from the reaction section. Also, there was a need to generate pure samples of oligosaccharide, so that Gel Permeation Chromatography could be used to elucidate the chain length of the oligomers. The approach taken was similar to that of Yakult Honsha (1987): stirred batch GOS production followed by chromatographic separation. A 1 litre batch of reaction mixture was produced from multiple stirred batch reactions. This was diluted 50:50 to give a concentration suitable for separation in the 3 metre column length used here, mimicking the separation section of the 4 metre long DTCBS.

Once the separative ability of the column had been confirmed, HPLC analysis was used to identify a cut which would give a high purity oligosaccharide fraction. Repeated pulses were then injected on to the column until around 100 $cm^3$ of oligosaccharide fraction had been generated, sufficient for GPC analysis and acid hydrolysis. It was hoped that these analyses, in combination with mass balance-derived information, would confirm the oligosaccharides to be galactooligosaccharides. Also information would be yielded on the structure of the oligosaccharides and the stoichiometry of the reactions producing them.

### 6.3.1 Preparation of Oligosaccharides

Experimental

Stirred batch reactions were performed as described in Chapter 5. The reaction conditions and analytical results are shown overleaf in Table 6(b). The products of 10 reactions were bulked to give 500 $cm^3$ of heat-quenched reaction mixture. Precipitated enzyme was removed by centrifugation for 10 minutes at 2800

rpm, followed by filtration through a 5 μm membrane. The aim was to give a 'clean', stable solution for the preparative chromatographic purification runs which were to follow.

**Table 6(b) Typical reaction conditions for a Preparative Stirred Batch Reaction.** The concentration of Oligosaccharide attained was comparable to values reported by Tack (1995).

| | |
|---|---|
| Initial Lactose Concentration (%w/v) | 20.00 |
| Enzyme Concentration ($gl^{-1}$) | 1.25 Initial (0.53 on mixing) |
| Temp ($^{o}C$) | 55.0 |
| Reaction Time (minutes) | 8.00 |
| Percentage Conversion (of lactose) | 25.24 |
| Oligosaccharide Formation(as % of total sugars) | 16.15 |
| Final Oligosaccharide Concentration (% w/v) | 0.36 |

### 6.3.2 Preparative Chromatographic Purification of Oligosaccharides

Introduction

A complete elution profile was generated from the 3m long x 1.5cm i.d. Chromatographic Column, demonstrating the production of a pure oligosaccharide fraction. From this profile, a time period was selected over which to sample further preparative runs so that samples were taken up until just before the point where the appearance of lactose was first detected. The samples were then bulked, giving sufficient sample volume for Gel Permeation Chromatography.

Experimental

The oligosaccharide-containing product mixture generated in Section 6.3.2 was diluted 50:50 to give a sugar concentration of about 10% w/v. 17.7 $cm^3$ pulses of product mixture were injected on to the column, this volume representing 5% of the empty column volume of the planned 4 metre long x 1.5 cm i.d. Dithermal Chromatographic Bioreactor-Separator (DTCBS). Approximately ten runs were performed, samples being collected at 1 minute intervals over the period: 83 to 88 minutes inclusive. The resulting HPLC profiles were then examined, confirming the compositional consistency of the preparative cuts. The samples were then bulked, giving around 10 $cm^3$ of sample for each of the 1 minute sampling intervals.

Results and Discussion

A typical elution profile from a preparative run is shown in Figure 6.7.

f

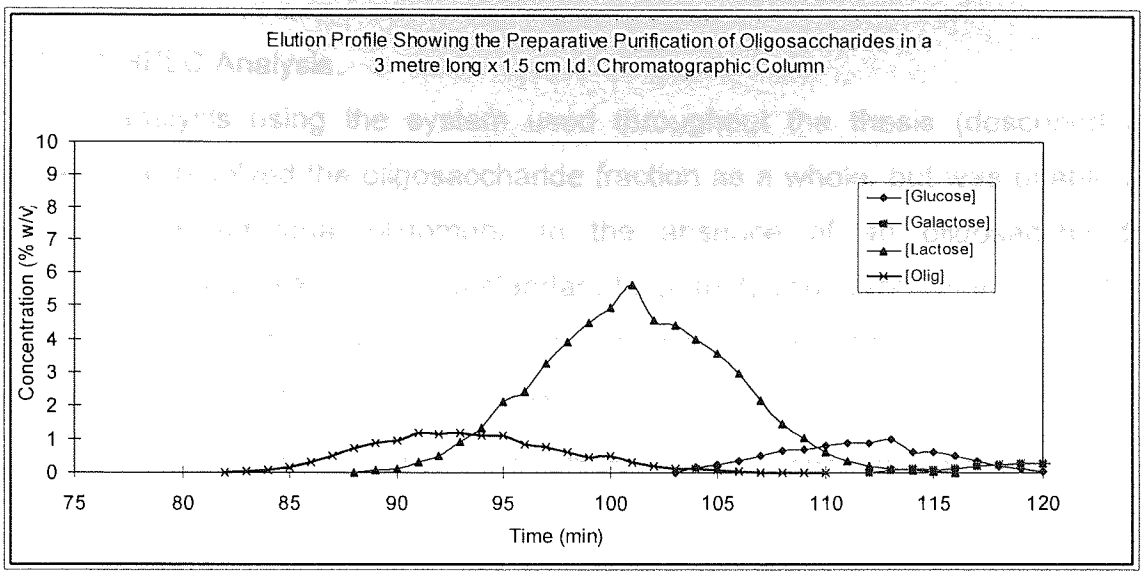



**Figure 6.7 Elution Profile from a Typical Preparative Purification of Oligosaccharides in a 3 metre long x 1.5 cm i.d. Chromatographic Column.** Purification of oligosaccharide is visible from around 84 to 88 minutes.

Resolution values from two typical preparative runs are shown in Table 6(c), from which it can be seen that variability between runs was low. The good run-to-run reproducibility can be attributed to accurate flow delivery and use of electronic valves.

**Table 6(c) Resolution Values from Two Typical Preparative Runs on the 3 metre long x 1.5 cm i.d. Chromatographic Column.** Run-run reproducibility enabled multiple cuts to be bulked for later analysis.

| Pulse Vol. ($cm^3$) | lac Concn. (%w/v) | rsGa-Lac | rsGluc-Gal | rsGluc-Lac | rsGal-OS | rsGluc-OS | rsLac-OS |
|---|---|---|---|---|---|---|---|
| 17.7 | 10 | 0.98 | 0.46 | 0.56 | 1.51 | 1.12 | 0.55 |
| 17.7 | 10 | 0.95 | 0.45 | 0.58 | 1.47 | 1.14 | 0.51 |

So, resolution was good and purification of oligosaccharides was successful, 13.4% of total oligosaccharide being recovered. It was concluded therefore, that the 3 metre long separation section of the DTCBS should give adequate purification of oligosaccharide. It was decided to apply 17.7 $cm^3$ pulses to the DTCBS while increasing pulse concentration to 15% (w/v). This would impose a greater loading upon the reaction and separation system, which would make it more sensitive to the effects of dithermal operation.

6.3.3 Analysis of Oligosaccharides

The preparative oligosaccharide cuts were analysed using a combination of HPLC and Gel permeation Chromatography.

6.3.3.1 HPLC Analysis.

HPLC analysis using the system used throughout the thesis (described in Chapter 4) resolved the oligosaccharide fraction as a whole, but was unable to resolve the individual oligomers. In the absence of an oligosaccharide standard, lactose was used as a standard to quantify oligosaccharides detected in the product mixture, which is common practice in the literature (e.g Jeon and Mantha (1985)). A typical HPLC profile is shown in Figure 6.8.

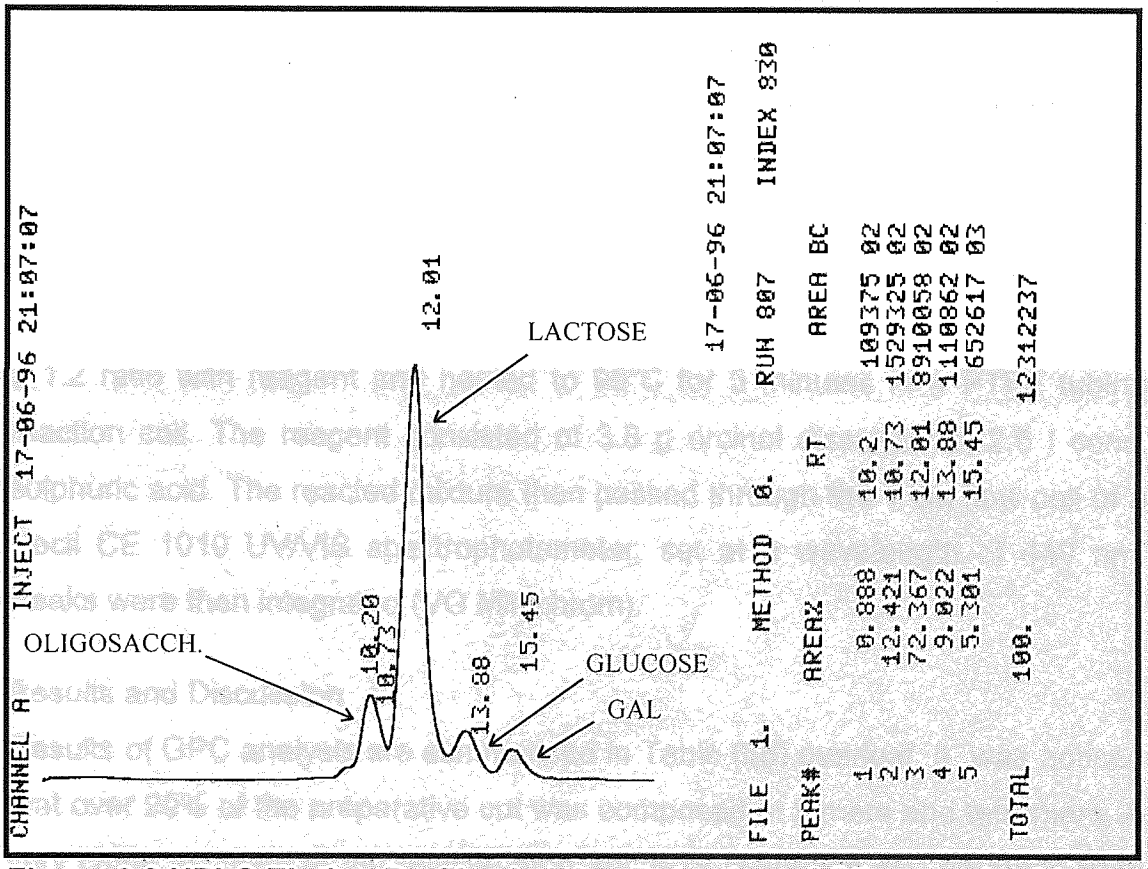



**Figure 6.8 HPLC Elution Profile showing Detection of Oligosaccharides.** The oligosaccharide peak eluted, typically 1-2 minutes earlier than the lactose peak.

Bonn (1985) achieved resolution of oligosaccharides with chain lengths spanning the range 1-12 by linking three HPLC columns. Since only a single-column HPLC system was available during the completion of this thesis, an alternative method was used, Gel Permeation Chromatography.

6.3.3.2 Gel Permeation Chromatography.

Introduction

Oligosaccharide fractions collected from the 3 m long x 1.5 cm i.d. Chromatographic Column were analysed by Alta Biosciences, based at The University of Birmingham. From mass balance analysis an estimate was made of the ratio of glucose to galactose in the oligosaccharide fraction. This estimation was made with the assumption that glucose and galactose not detected as free monomers must be incorporated as oligosaccharides.

Experimental

3 columns were connected in series: 30 cm long x 0.8 cm i.d., packed with Biogel P2 (400 mesh size). The columns were housed in a thermostatically controlled oven which allowed them to be maintained at 70°C during operation. The eluant consisted of HPLC grade distilled water, degassed by an in-line boiler. Eluant was pumped at a flow rate of 0.24 $cm^3$ $min^{-1}$ via a Constametric III Pump. Samples (200 ml) were injected via a Waters WISP 710B injector, without pre-treatment. On emerging from the column, the effluent was mixed at a 1:2 ratio with reagent and heated to 90°C for 5 minutes in a PTFE tubing reaction coil. The reagent consisted of 3.8 g orcinol dissolved in 2.5 l conc. sulphuric acid. The reacted mixture then passed through the 1 cm flow cell of a Cecil CE 1010 UV/VIS spectrophotometer, set at a wavelength of 440 nm. Peaks were then integrated (VG Minichrom).

Results and Discussion

Results of GPC analysis are summarised in Table 6(d) overleaf. It was noticed that over 90% of the preparative cut was composed of trimers and tetramers. A very crude estimate of the relative proportions of oligomers can be made from the percentage composition values in Table 6(d) overleaf, but this applies only to the cut itself and does not represent the overall reaction stoichiometry. This would require GPC analysis over the whole elution profile and could not account for dimers unless some means of differentiating lactose from other dimers was available.

**Table 6(d) Results of GPC Analysis.** The masses of each oligomer detected are listed. The sample times shown represent the times at which collection of 1 minute samples was started.

| SampleTime (Min) | Monomer | Dimer | Trimer | Tetramer | Pentamer | Hexamer |
|---|---|---|---|---|---|---|
| 84 | 0.4 | 2.3 | 45.0 | 40.0 | 6.8 | 1.1 |
| 85 | 0.1 | 2.2 | 52.0 | 36.0 | 5.5 | 0.7 |
| 86 | 0.4 | 4.1 | 59.0 | 30.0 | 4.0 | 0.5 |
| 87 | 0.2 | 6.5 | 65.0 | 24.0 | 2.7 | 0.2 |
| Sum of Masses | 1.1 | 15.1 | 221.0 | 130.0 | 19.0 | 2.5 |
| %Composition | 0.3 | 3.9 | 56.9 | 33.4 | 4.9 | 0.6 |

As can be seen from Table 6(d) and Figure 6.9, a fraction comprising trimers and tetramers was obtained which was of about 90% purity. There is evidence of some resolution of the oligosaccharides on the 3 metre long column. The concentration of tetramer decreased across the sample period of the cut, implying that its maximum occured before that of the trimer, whose concentration *increased* across the same time period. Likewise, the concentrations of pentamers and hexamers *decrease* across the preparative cut. Overall there is evidence of separation by size, as would be expected with the material used to pack the 3 metre long column.

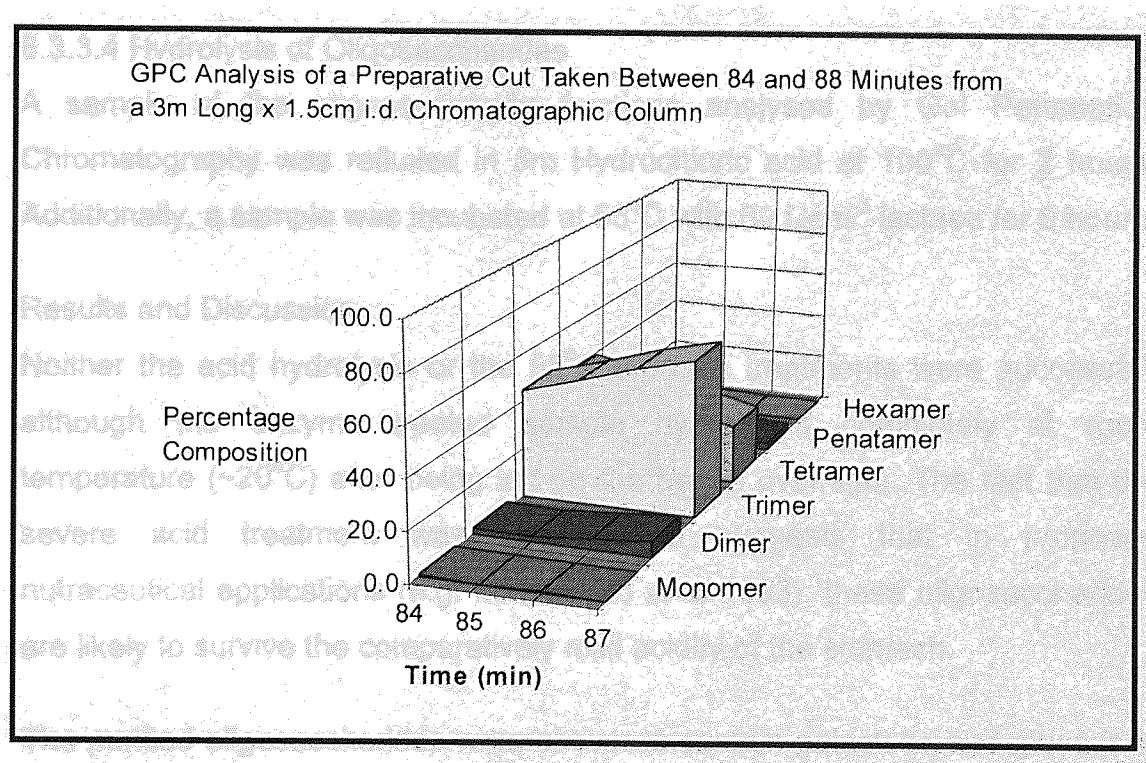



**Figure 6.9 3-Dimensional representation of Preparative Oligosaccharide Cut.** Over 90% of the saccharides present were either trimers or tetramers. The dimers could be lactose, lactulose, galactobiose or cellobiose.

### 6.3.3.3 Estimation of Reaction Stoichiometry for Oligosaccharide Production

Introduction

An estimate was made, from mass balance data, of the likely stoichiometry of the reaction for oligosaccharide production. Results for 15 reactions performed in the 1 metre long x 1.5 cm i.d. CBRS were analysed and the concentrations of unconverted lactose, glucose, galactose and oligosaccharide were normalised by dividing by the mass of lactose injected in each case.

Despite the initial lactose concentrations varying between 5% (w/v) and 20% (w/v), the relative proportions of the above saccharides remained fairly consistent throughout. For each saccharide therefore, the normalised concentrations were averaged to give a summarised reaction mixture analysis. The following average mass balance was estimated from the mass balance data, suggesting that more galactose than glucose was incorporated as oligosaccharides:

$$1\ \text{Lac} \rightarrow 0.46\ \text{Gluc} + 0.28\ \text{Gal} + 0.24\ \text{Oligosacch.}$$

### 6.3.3.4 Hydrolysis of Oligosaccharides

A sample of the oligosaccharide fractions analysed by Gel Permeation Chromatography was refluxed in 5m Hydrochloric acid at 100°C for 2 hours. Additionally, a sample was incubated at 55°C with 60 $Ucm^{-3}$ lactase for 6 hours.

Results and Discussion

Neither the acid hydrolysis or the 55°C enzymic treatments were successful, although the enzyme treated sample hydrolysed completely at room temperature (~20°C) after being left on the bench overnight. The fact that the severe acid treatment was unsuccessful suggests that in proposed nutraceutical applications (e.g. Matsumoto *et al* 1993), these oligosaccharides are likely to survive the comparatively mild acidity of the stomach.

The purified oligosaccharides were shown to contain galactose and glucose in the ratio 1.7:1, so that, in terms of the dominant component, they could be classed as galactooligosaccharides. There is no obvious explanation for the discrepancy between this analytically derived ratio and that estimated from mass balances.

Lack of detailed knowledge of the reaction stoichiometry has, so far, prevented elucidation of the kinetics of GOS formation. Prenosil *et al* (1987) have studied this reaction, highlighting in so doing the complicated nature of the problem. β-galactosidase catalyses both hydrolysis and transfer reactions (Matsumoto *et al* 1993) which, in itself, makes modelling difficult. Complexity increases yet further when mutarotation is considered. Flaschel *et al* (1982) pointed out that b-galactose can undergo mutarotation to the α-form, which has 12 times the competitive inhibitive effect of the β-anomer. Consequently, mathematical modelling of this reaction system has not been attempted in this thesis.

## 6.4 Construction and Evaluation of the 4 metre long x 1.5 cm i.d Dithermal Chromatographic Bioreactor Separator

Introduction

Reference to the work of Tack (1995), combined with the results of pilot experiments in the 1 metre long CBRS presented in Section 6.1, suggested that there would be significant degradation of GOS over the residence time of the 1 metre long reaction section (if it was operated at the normal CBRS separation flow rate of 2.1 $cm^3min^{-1}$). It was decided, therefore that in addition to a step change in temperature over the column length, there would also be a step change in flow rate, so that the residence time of the reaction section corresponded to the time at which GOS concentrations approached peak levels.

From the results of Section 6.3, it was decided to apply 35.3 $cm^3$ pulses to the DTCBS while increasing pulse concentration to 15% (w/v). This would impose a greater loading upon the reaction and separation system, which would make it more sensitive to the effects of dithermal operation. The performance of the DTCBS could then be compared to an isothermal system operated under otherwise identical conditions.

Experimental

A 4 metre long column was assembled such that the heating circuit of the first metre was operated independently to that of the remaining 3 metres. As a control experiment, the column was first operated isothermally at 55°C throughout. The column was then operated dithermally with the first metre of column operated at 55°C and the remaining 3 metres operated at 82°C. The resultant 4 metre long x 1.5 cm i.d. dithermally operated chromatographic bioreactor-separator is shown in Appendix A-8.

A 35.3 $cm^3$ pulse of 15% (w/v) lactose was injected on to the column in each case. The column was operated at a flow rate of 9.0 $cm^3min^{-1}$ for 6 minutes 30 seconds before stepping the flow rate down to the separation flow rate of 2.1 $cm^3min^{-1}$. The stepping down process took 30 seconds, giving a residence time of about 7 minutes in the reaction section. The reacted pulse then entered the 3 metre long separation section.

Typical elution profiles from isothermal and dithermal operation are shown in Figures 6.10 and 6.11 respectively.

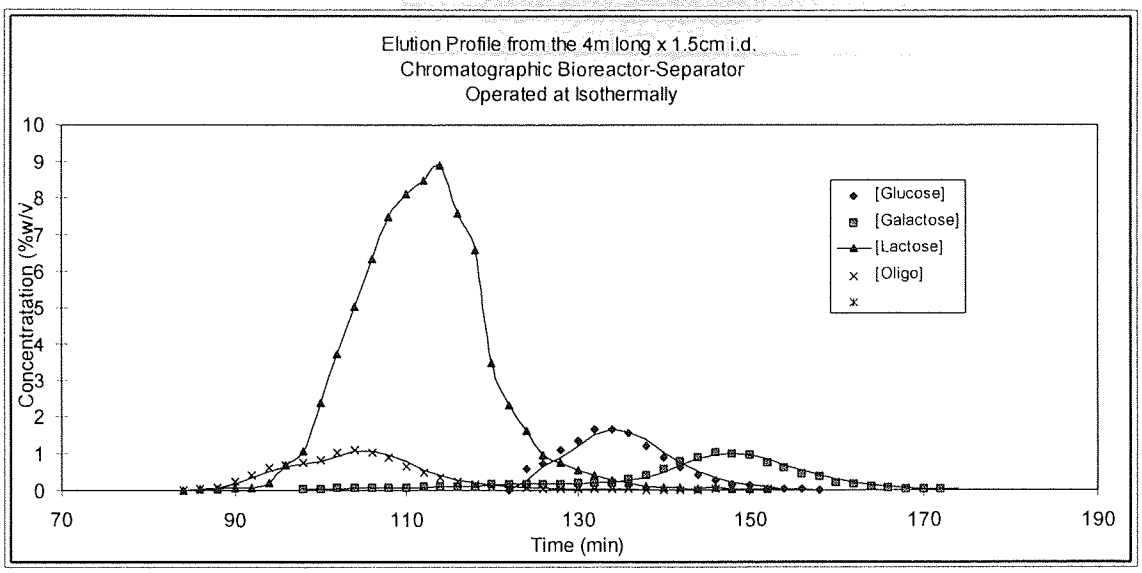



**Figure 6.10 Typical Elution Profile from the 4 metre long x 1.5 cm i.d CBRS. Operated Isothermally (at 55°C).** A 35.3 $cm^3$ pulse of 15% w/v lactose was injected and flow rate stepped from 9.0 to 2.1 $cm^3min^{-1}$.

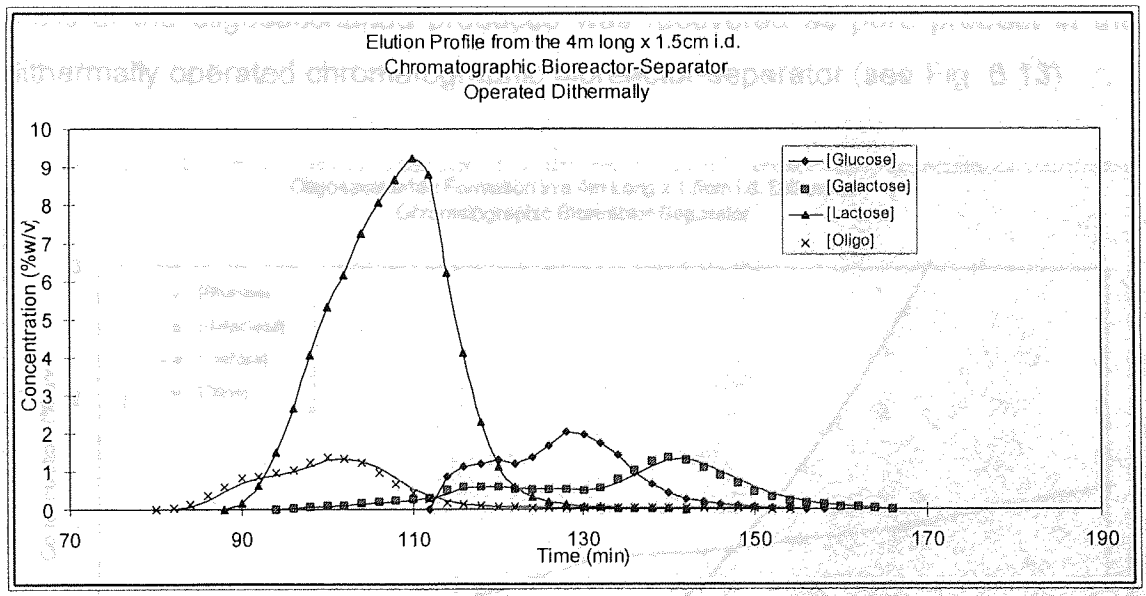



**Figure 6.11 Typical Elution Profile from the 4 metre long x 1.5 cm i.d CBRS. Operated Dithermally (at 55°C and 82°C).** A 35.3 $cm^3$ pulse of 15% (w/v) lactose was injected and flow rate stepped from 9.0 to 2.1 $cm^3min^{-1}$, as in the isothermal experiment (see Figure 6.10).

Figures 6.12 and 6.13 show a magnified view of the section: 80 to 100 minutes, demonstrating the improvement in oligosaccharide purification which was gained by operating dithermally. In the isothermally operated

chromatographic bioreactor-separator, no pure oligosaccharide was recoverable (see Figure 6.12).

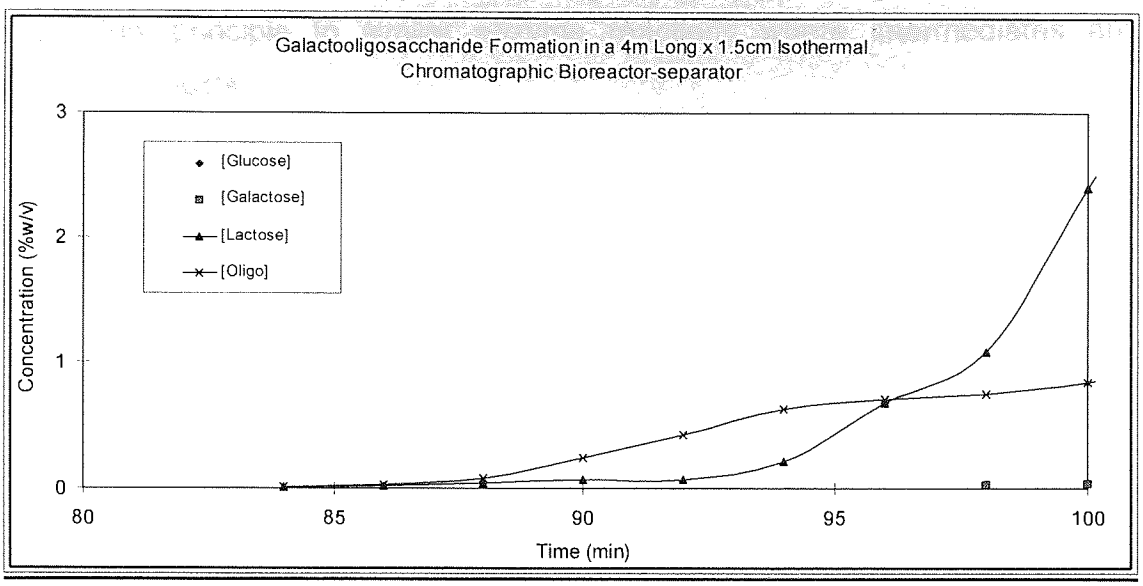



**Figure 6.12 Elution Profile for the Production of Oligosaccharides in a 4m long x 1.5 cm i.d. Chromatographic Bioreactor-Separator Operated under Isothermal Conditions**. For reasons of clarity, the profile has not been shown beyond the oligosaccharide peak. No pure oligosaccharide is visible here, due to the co-elution of lactose

8.3% of the oligosaccharide produced was recovered as pure product in the dithermally operated chromatographic bioreactor-separator (see Fig. 6.13)

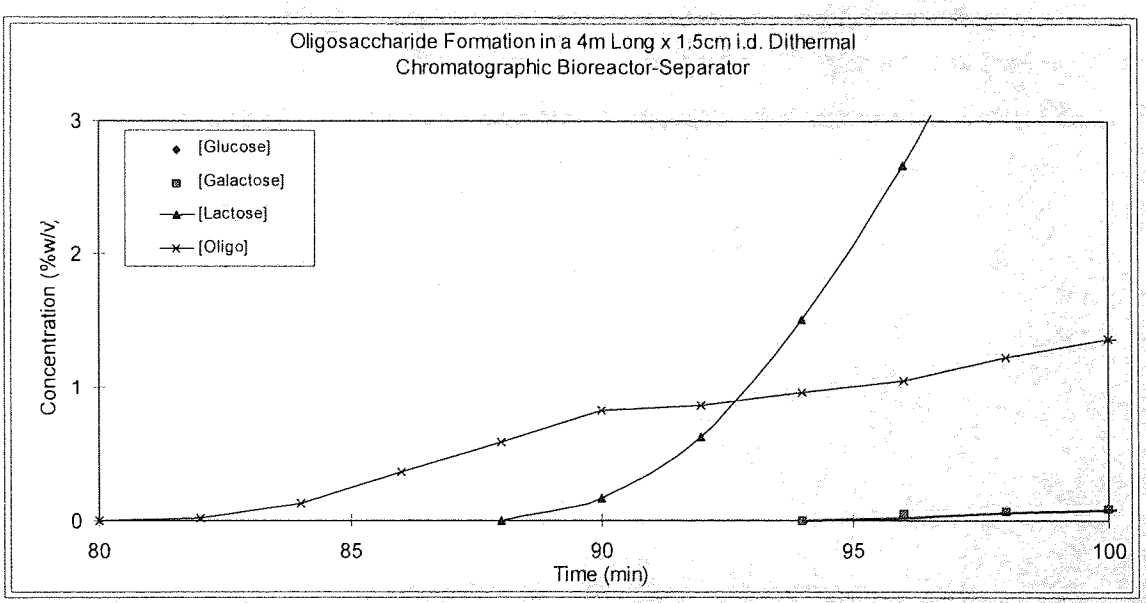



**Figure 6.13 Elution Profile for the Production of Oligosaccharides in a 4m long x 1.5 cm i.d. Chromatographic Bioreactor-Separator Operated Dithermally**. Pure oligosaccharide can be seen emerging between 83 and 88 minutes.

The Dithermal Chromatographic Bioreactor-Separator appeared to have performance advantages over a conventional, isothermally operated system in terms of its ability to produce pure oligosaccharide. It should be possible to apply this principle to similar enzyme reactions where intermediates are desired products.

## 6.5 Conclusions

Pilot experiments using a 1 metre-long x 1.5 cm i.d. CBRS enabled operating conditions to be selected for the reaction and separation sections of the 4 metre-long x 1.5 cm i.d. Dithermal Chromatographic Bioreactor-Separator.

A 3 metre-long x 1.5 cm i.d. chromatographic column was constructed and used to predict the separative capability of the 3 metre long separation section of the proposed 4 metre long x 1.5 cm i.d. Dithermal Chromatographic Bioreactor-Separator. The 3 metre long column was able to resolve the product mixture sufficiently to enable pure oligosaccharide cuts to be taken. Preparative cuts were analysed, confirming them to consist of oligosaccharides ranging from dimers to hexamers, trimers and tetramers being the dominant oligomers.

The purified oligosaccharides were shown to contain galactose and glucose in the ratio 1.7:1, so that, in terms of the dominant component, they could be classed as galactooligosaccharides.

Finally, a 4 metre long x 1.5 cm i.d. Dithermal Chromatographic Bioreactor-Separator (DTCBS) was constructed. The DTCBS appeared to have performance advantages over a conventional, isothermally operated system in terms of its ability to produce pure oligosaccharide. It should be possible to apply this principle to similar enzyme reactions where intermediates are desired products.

# Chapter Seven

## Summary Findings, Conclusions and Recommendations for Further Work

### 7.1 Summary Findings and Conclusions

***Process Optimisation***

*The preliminary aim was to optimise the performance of a Chromatographic Bioreactor-Separator (CBRS), taking the enzymic hydrolysis of lactose to glucose and galactose as the reaction of study. The processes involved in chromatographic bioreaction and separation namely: injection, mixing, reaction and separation, were examined and the individual and combined effects of these processes considered. The system was evaluated in terms of its ability to separate the hydrolysis products galactose and glucose.*

**Separative Performance**

The number of theoretical plates (N) for glucose and galactose was increased by reducing the particle size of the stationary phase, and modifying the CBRS to tolerate the resultant high back-pressures. By reducing the packing particle diameter from 200 to 75 μm, N was increased from about 500 to about 3000 for glucose and galactose in a 2 metre long x 1.5 cm i.d. CBRS.

**Inlet design**

Inlet design was considered in terms of its effect on mixing between enzyme and substrate as pulses of substrate are injected. Magnetic Resonance Imaging (MRI) was used in combination with hydrodynamic calculations for the experimental studies. MRI proved to be a valuable, non-intrusive tool for studying the flow patterns.

It was concluded that use of a needle type injector was unlikely to improve mixing between the enzyme solution and the substrate pulse at normal CBRS operating flow-rates. Also, use of a needle type injector may have detrimental effects on chromatographic separation. The sinter type injector was judged to give the best overall CBRS performance, since this gives plug-like flow during injection, while providing adequate mixing between enzyme and substrate.

**Time-Scales of Chromatographic Bioreaction and Separation**

The time scales involved in reaction and separation were compared. The approach taken by the author was to operate the CBRS at different residence times. This resulted in a range of lactose conversion values, so that the progress of reaction could be studied. Progress of reaction plots showed reasonable agreement with those predicted by an integrated rate equation, reflecting the effect of pulse volume on mixing between enzyme and substrate.

Comparison of progress of reaction plots with CBRS elution profiles revealed that there was negligible chromatographic separation during the column residence time during which enzymic reaction takes place, so that reaction and separation could be de-coupled and considered independently. To exploit potential CBRS benefits such as improved yield of desired products in product inhibited and equilibrium reactions, bioreaction and separation would need to be dynamically linked so that products are separated from the reaction zone as they are formed.

**Mixing and Mass Transfer**

Mixing between enzyme and substrate was studied. This was achieved by performing a series of tracer-type experiments which exploited the inherent conductivity of the enzyme solution, giving an estimate of enzyme concentration in the reaction zone. This gave an insight into the degree of mixing between enzyme and substrate. It appears that zones of axial mixing exist at the extremities of an injected pulse of substrate. With smaller pulse volumes, there is sufficient axial dispersion to provide adequate contact between enzyme and substrate. As pulse size is increased, the degree of axial mixing becomes a limiting factor on enzymic reaction. A study was then made of the chromatographic bioreaction and separation process, examining the relative contributions of bulk liquid mixing and internal and external mass transfer to the rate of the overall process. This involved prediction of the flow characteristics of the bulk liquid, together with the relative time scales involved in the processes of reaction, mass transfer, adsorption and desorption. The main mixing mechanism, was that of statistical dispersion of lactose due to flow of liquid through the column packing. Mass transfer processes appeared to be very rapid and not rate-controlling.

### Chromatographic Effects

Attention was also given to chromatographic effects on mixing between enzyme and substrate, brought about by retention of the substrate, lactose, relative to the enzyme. Calculations suggested that this effect would be significant in this system, resulting in penetration of the trailing edge of the lactose pulse by the enzyme solution which follows it. This would increase the amount of enzyme which comes into contact with lactose molecules. Mixing between enzyme and substrate in a CBRS is likely, therefore, to be enhanced by chromatographic effects.

### Effect of Contacting Pattern

The effect on reaction of the contacting pattern between enzyme and substrate was investigated; in particular, the difference between introducing components over a period of time (as in a CBRS) and the almost instantaneous mixing as seen in a conventional stirred batch reactor. Similar conversion levels to those seen in stirred batch reactions were reached in the CBRS, suggesting that reaction was not restricted by mixing limitations imposed by gradual introduction of the substrate; or by substrate inhibition effects due to the enzyme encountering the entire pulse of lactose at the start of reaction. So, the contacting pattern between enzyme and substrate in a CBRS, where the pulse of substrate is introduced gradually, does not confer any advantage over that of a conventional stirred batch reactor, where the substrate is introduced at the start of reaction.

***Oligosaccharide Formation***

*It was during studies concerned with factors which affect enzymic reaction in a chromatographic bioreactor-separator (CBRS), that oligosaccharides were detected. These oligosaccharides are commercially important. Consequently, emphasis was changed from the end products of lactose hydrolysis, glucose and galactose, to maximising the yield of oligosaccharides, which form as intermediates in this reaction. The approach taken was to perform a series of pilot experiments, culminating in the design, construction and evaluation of a dithermal chromatographic bioreactor-separator.*

**Reaction Control**

Because the desired oligosaccharides are intermediates in a series of reactions leading to complete lactose hydrolysis, it was necessary to control enzyme activity during product separation. If reaction was allowed to take place during separation, oligosaccharide yield would be reduced and chromatographic separation adversely affected. Pilot experiments, using a 1 metre long x 1.5 cm i.d. CBRS, enabled operating conditions to be selected for the reaction and separation sections of a 4 metre long x 1.5 cm i.d. Dithermal Chromatographic Bioreactor-Separator. These were to operate the 1 metre long reaction section at a flow-rate of 9.0 $cm^3min^{-1}$ and a temperature of 55°C; with the flow-rate being changed to 2.1 $cm^3min^{-1}$ and the temperature increased to 82°C in the remaining 3 metres of column (forming the separation section).

**Purification and Analysis of Oligosaccharides**

Consideration was then given to the separation of the components of the reaction mixture. Since it was first necessary to produce a relatively large amount of this mixture, the opportunity was also taken to generate pure oligosaccharide fractions for GPC analysis in order to determine the chain lengths of the purified oligosaccharides. Also, hydrolysis, followed by HPLC analysis, made it possible to name the oligosaccharides, in terms of the most prolific monomer unit.

The 3 metre long column was able to resolve the product mixture sufficiently to enable pure oligosaccharide cuts to be taken. Preparative cuts were analysed, confirming them to consist of oligosaccharides ranging from dimers to hexamers, trimers and tetramers being the dominant oligomers. The purified oligosaccharides were shown to contain galactose and glucose in the ratio 1.7:1, so that, in terms of the dominant component, they could be classed as galactooligosaccharides.

**Dithermal Chromatographic Bioreaction and Separation**

A 4 metre long x 1.5 cm i.d. Dithermal Chromatographic Bioreactor-Separator (DTCBS) was constructed and operated first isothermally, then dithermally. The DTCBS appeared to have performance advantages over a conventional, isothermally operated system in terms of its ability to produce pure

oligosaccharide. In particular, a greater yield of pure GOS was seen with dithermal operation (operating at 55°C for reaction and 82°C for separation), than when the CBRS was operated isothermally at a temperature of 55°C.

## 7.2 Practical Considerations

Tubular bioreactor-separator systems operated under conditions of plug flow are inherently flexible. In principle, both contacting and processing patterns can be controlled to optimise overall performance. For example, an enzyme (or a series of enzymes) could be immobilised in the tubular system with the reaction and chromatographic sections spatially separated. This approach is similar to that used by Hashimoto *et al* (1983). Such a hybrid system could also be operated in a similar way to the Dithermal Chromatographic Bioreactor-separator described in Chapter 6. The separation sections could be operated at a temperature and flow-rate which favoured separation while the reaction sections could be operated at the optimum temperature for the enzyme and with a liquid velocity high enough to minimise mass transfer effects.

The CBRS as used in the author's work has a major disadvantage: the enzyme solution is continuously fed to the column without provision for re-use. It would be possible to recycle the enzyme solution providing the rate of enzyme deactivation was characterised and fresh solution provided at suitable intervals. Also, there is the need to remove enzyme from the product stream. Enzyme immobilisation could prove to be more cost-effective. However, there are additional costs involved with the actual immobilisation process and practical problems such as the need for periodic removal of the packing for regeneration.

Advantages of simultaneous bioreaction and separation such as reduced product inhibition require dynamic linkage of the reaction and separation processes. This can be difficult to achieve, particularly, as in the case of hydrolysis of lactose using β–galactosidase, where the reaction is very fast.

## 7.3 Recommendations for Further Work

- Carry out a more detailed study of GOS formation and derive reaction rate equations.

- Perform further experiments to derive a mathematical relationship between pulse volume and enzyme concentration profile, enabling existing models of the CBRS to be developed and tested.

- Construct a mathematical model for lactose hydrolysis, which incorporates an experimentally estimated enzyme concentration for the reaction zone.

- Model the production of oligosaccharides in a DTCBS.

- Extend initial studies to confirm efficacy of dithermal chromatographic bioreaction and separation.

- Use the multi-enzyme approach to custom-synthesise oligosaccharides.

- Apply the principle of operating a CBRS with more than one temperature zone to other enzyme reactions where intermediates are the desired products. This approach could be applied to sequential reactions in a CBRS or an immobilised enzyme reactor. Use of more than one enzyme would be possible, each enzyme having a different optimum temperature for activity. Also, temperature control could be used to dynamically link bioreaction and separation, exploiting CBRS benefits such as improved yield of desired products in product inhibited and equilibrium reactions.

- Scale-up the dithermally operated chromatographic bioreactor-separator (DTCBS).

- Scale down multi-enzyme systems for potential use in chemical analysis and artificial organ construction.

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## List of Appendices

## Appendix-A1

## Chromatographic Terms Used in The Thesis

This theory treats the chromatographic bed as a series of well mixed equilibrium stages. Drawing an analogy to distillation theory, Martin and Synge (1941) defined HETP (height equivalent to one theoretical plate as the thickness of a layer (in a chromatographic column) over which the solute issuing from it is in equilibrium with the mean concentration of solute in the stationary phase throughout the layer. They also said that the value of HETP could be taken as a constant through a given column, except when the ratio of concentrations of the solution entering and leaving the plate differs greatly from unity. It was assumed that the diffusion of solute from one plate to another must be negligible. Also, it was assumed that at equilibrium, the distribution ratio of one solute between the two phases must be independent of its absolute concentration and the presence of other solutes. The column packing is visualised as a series of well-mixed equilibrium stages.

van Deemter (1956) developed an expression for plate height which takes into account the relative importance of physical factors involved in separation, such as the influence of the particle diameter of the column packing and its impact on axial diffusion. HETP can be calculated by dividing the column length by the number of theoretical plates (N):

$$HETP = \frac{L}{N}$$

where L is the length of the column and N is the number of theoretical plates.

**Number of Theoretical Plates (N)**

The number of theoretical plates is calculated using the following equation, which was developed by Gleukauf (1955):

$$N(x) = 8\left(\frac{tr}{W_{h/e}}\right)^2$$

where: N = total number of theoretical plates with regard to specified solute

tr = retention time of specified solute

h = solute peak height

e = base of natural logarithm

The peak width (W) is measured at $^{h}/_{e}$ to reduce inaccuracies due to baseline height fluctuation. N is a useful measure of column performance, indicating the degree of peak broadening. In chromatography, narrow peaks are desired, since this reduces the likelihood of overlap between neighbouring peaks.

**Resolution**

Resolution (Rs) is a measure of the degree of separation between two adjacent peaks and is given by:

$$Rs = 2\left(\frac{tr2 - tr1}{W1 + W2}\right)$$

where: tr2 = retention time of peak with higher retention time

tr1 = retention time of peak with lower retention time

W1 & W2 = width (at base) of peaks

The larger the value of Rs, the better the separation. A value of between 1.2 and 1.5 is considered ideal (Braithwaite and Smith 1996), whereas a value of 0.8 or less indicates a poor separation.

**Equilibrium Distribution Coefficient (Kd)**

The equilibrium distribution coefficient (Kd) is calculated as follows:

$$Kd = \left( \frac{Vi - Vo}{Vt - Vo} \right)$$

where: Vi = Elution Volume of solute

Vo = Elution Volume of non-retained solute

(Elution Volume = retention time x flow rate)

Vt = Volume of column

**Separation Factor**

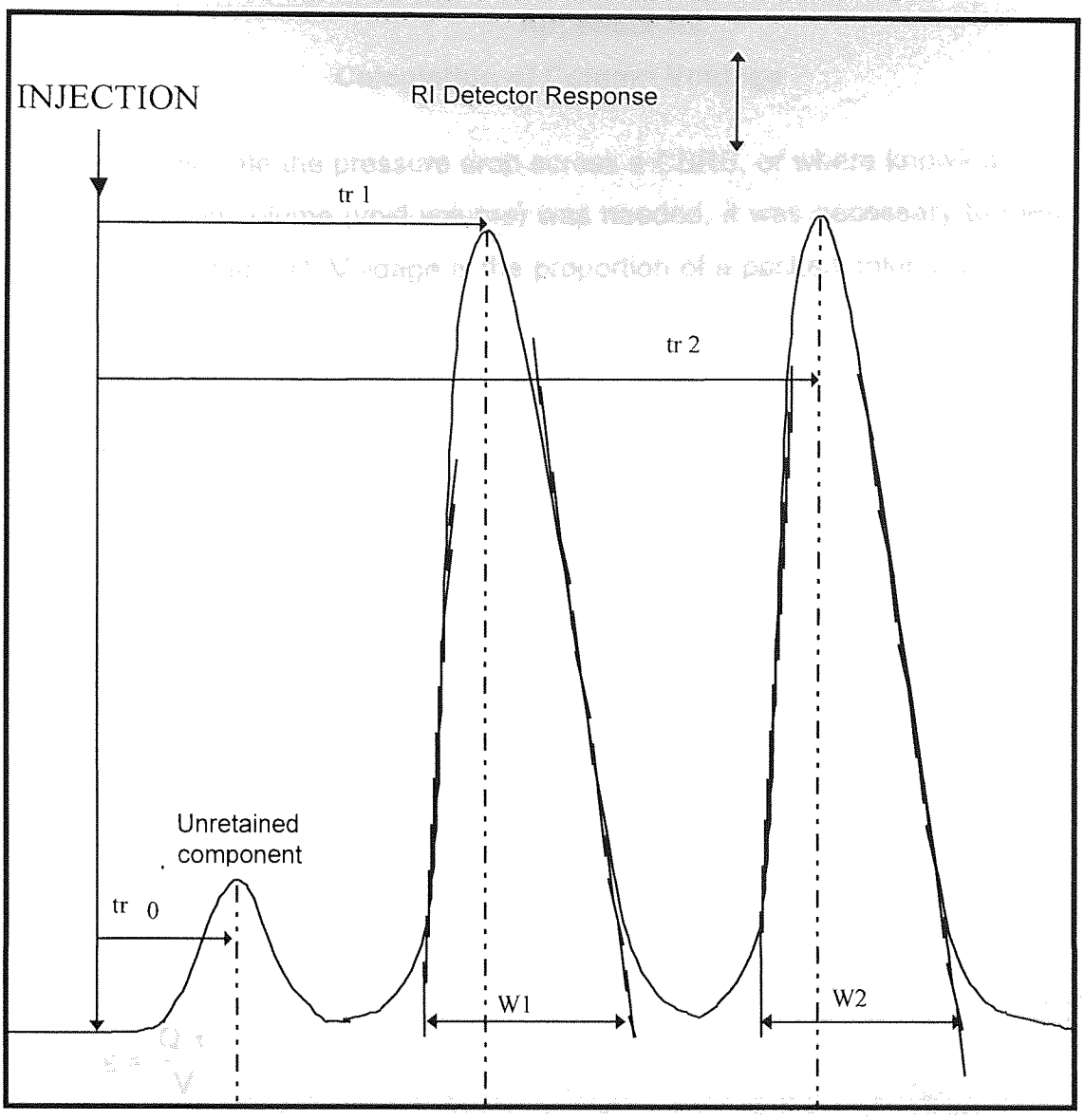
The separation factor gives an indication of the ease with which two components can be separated. The lower the separation factor, the more difficult it is to separate the two components chromatographically. It is the ratio of the Kd values of two components; here, components y and z:

$$\alpha = \frac{Kdy}{Kdz}$$

Where component y has the higher Kd value. So separation factor will always be greater than unity.

**Measurement of Chromatographic Peaks**

Measurement of chromatographic peaks is described diagramatically overleaf.




**Measurement of Chromatographic Peaks** Peak widths and retention times must be measured in the same units, either of time or length.

## Appendix-A2

## Calculation of Column Voidage

In order to calculate the pressure drop across a CBRS, or where knowledge of the effective column volume (void volume) was needed, it was necessary to measure the column voidage ($\varepsilon$). Voidage is the proportion of a packed column which is not occupied by packing.

If the volume of the column is V, the void volume can be obtained as follows:

$$\text{Void Volume} = V.\ \varepsilon$$

Residence time ($\tau$) is calculated thus:

$$\tau = \frac{V.\varepsilon}{Q}$$

So, re-arranging,

$$\varepsilon = \frac{Q.\tau}{V}$$

Volumetric flow-rate (Q) and column volume (V) are known, leaving residence time (t) to be measured experimentally. This was achieved by injecting a pulse of a component which was not retained by the column packing. In this thesis, dextran 2000kd was used, which was too large to enter the pores of the ion exchange resin used to pack the CBRS. The residence time was obtained by measuring the elution time of the resultant peak.

## Appendix-A3
## Determination of Enzyme Activity

Throughout this thesis, enzyme activity is described in terms of Units per $cm^3$ ($Ucm^{-3}$). Enzyme activity is defined as follows:

### β-galactosidase Enzyme Unit Definition

1 Unit (U) is the amount of enzyme which converts 1 μmole of lactose in 1 minute at the stated conditions of pH and temperature.

### Method of Activity Determination

(i) 2 $cm^3$ of 1% (w/v) lactose was attemperated at the stated reaction temperature.

(ii) 2 $cm^3$ of a 1:10 dilution of the test enzyme solution, previously attemperated, was added to the lactose solution (I) and whirlimixed while simultaneously starting the timer.

(iii) The reaction mixture was incubated for exactly 5 minutes before quenching.

(iv) 1 $cm^3$ distilled and deionised water was added prior to analysis by HPLC.

(v) Enzyme activity was calculated, taking dilution factors into account.

## Appendix A-4

## Quenching Efficiency

In order that samples taken at the CBRS outlet did not undergo further enzymic reaction prior to analysis, it was first necessary to quench the reaction.

Previous workers, for example, Taddei (1994) and Shieh (1994) used boiling as a means of reaction quenching during sampling. However, the apparatus required is cumbersome (requiring a boiling water bath) and there would be practical difficulties in quenching samples taken at 1 minute intervals. Also, some delay may be introduced as the samples are heated to 100°C.

For these reasons, an alkali quenching method was evaluated. This had the added benefit of eliminating the need for an additional dilution step, which would have been necessary to give sufficient sample volume for HPLC analysis. The HPLC vials held approx. 1.5 $cm^3$ and additional volume was needed for flushing the syringe used to fill the vials).

During chromatographic bioreaction-separation experiments, samples were removed from the column effluent stream by a peristaltic pump, set at a flow rate of 0.8 $cm^3min^{-1}$. Sample volumes were thus either 0.8 $cm^3$ or 1.6 $cm^3$, depending whether samples were removed at 1 or 2 minute intervals.

Samples were collected drop-wise into glass test tubes containing 1.2 $cm^3$ of 0.05m Sodium hydroxide. This meant that from the first drop of sample collected a pH of 12-13 would be encountered by the sample. Generally, samples would contain sugars and would therefore be more dense than the Sodium hydroxide solution, so that mixing was unlikely to be a limitation. As an additional precaution, the sodium hydroxide-containing tubes were chilled to around 5°C prior to sample collection, then frozen immediately afterwards.

The appropriate dilution factor was calculated and incorporated into the spreadsheet calculations used to analyse HPLC results. The efficacy of this

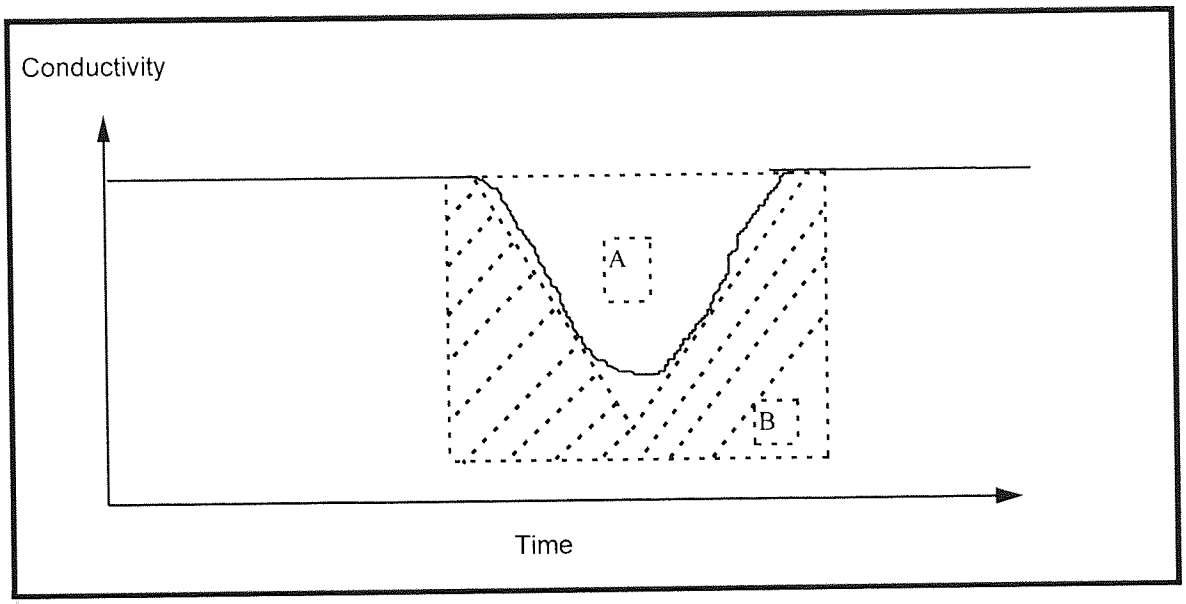
method was compared to that of the conventional boiling quenching method. A sample was taken during a stirred batch reaction and immediately divided into three portions. One portion was added to a tube immersed in a boiling water bath, another was added to an appropriate volume of chilled sodium hydroxide and frozen.

The boiled sample was analysed immediately, while after 30 minutes, the frozen alkali sample was thawed in the normal way (immersed in warm water), then analysed immediately. This sample was then left on the bench for 16 hours, simulating the effect of being the last sample on the HPLC auto-analyser carousel, before being re-analysed. An identical degree of lactose conversion was seen in all three samples, so it was concluded that the alkali quenching method was as efficient as boiling and had certain operational advantages. The alkali quenching method was consequently adopted for all following experiments.

## Appendix A-5

## Calculation of Average Enzyme Concentration

In calculating the average enzyme concentration in a pulse of lactose, it was assumed that if the enzyme was not diluted by sugars, the amount of enzyme in the area occupied by the pulse would be represented by the area of the square AB. The shape of the pulse was approximated to that of a triangle (A).




Area B = Area AB - Area A

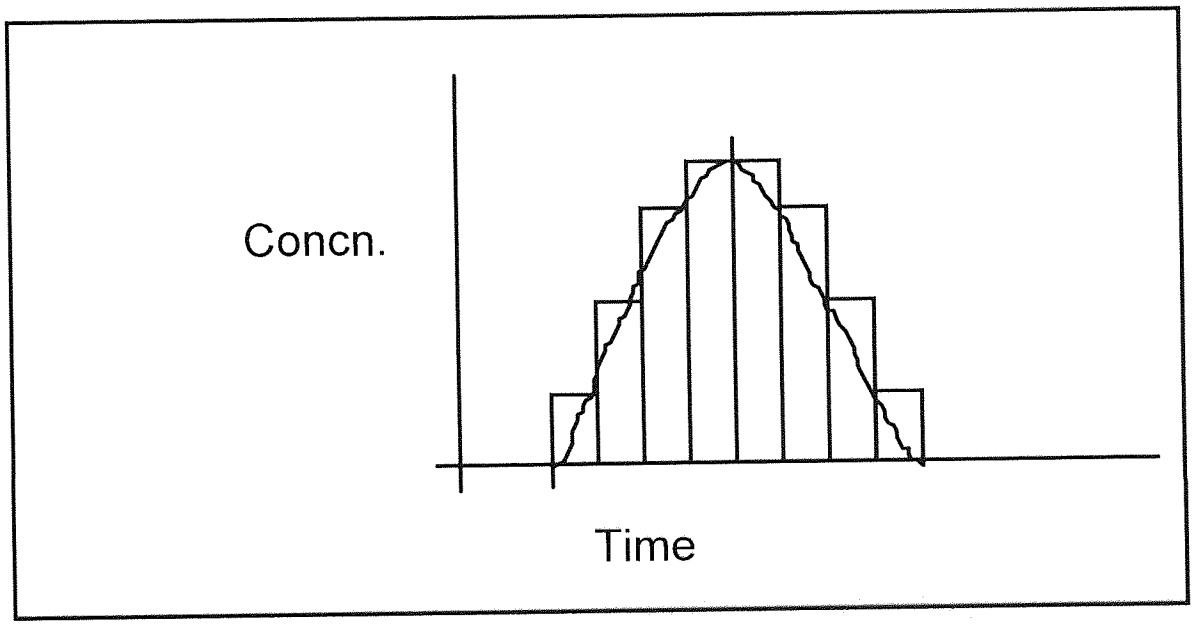
So,

$$\text{Av. Enzyme Concentration in Pulse} = \frac{\text{Area B}}{\text{Area AB}} \times \text{Enzyme Concentration in Eluant}$$

## Appendix A-6
## Spreadsheet Calculations

Spreadsheets were created in 'Excel 7-Microsoft' which enabled concentration profiles to be plotted from peak areas generated by HPLC analysis.

A histogram/Simpsons Rule-type method was used in calculating peak areas from individual points resulting from discrete samples taken at the outlet of the chromatographic reactor.




Mass balance calculations were incorporated into the spreadsheets relating the total mass of products eluted to the mass of lactose injected. Close agreement was shown generally; which suggests that this estimation method was sufficiently accurate.

## Appendix A-7

## Magnetic Resonance Imaging

Overleaf is a paper presented at the 5th. World Congress of Chemical Engineering, San Diego CA. This paper describes the Magnetic Resonance Imaging MRI techniques used (in Chapter 5) to support this thesis. This paper is followed by photographs of the MRI apparatus.

# Optimisation of a Semi-batch Chromatographic Reactor-Separator

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