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EVALUATION OF *ASPERGILLUS ORYZAE* β-GALACTOSIDASE KINETIC PARAMETERS AND IMMOBILIZATION IN A HOLLOW FIBER REACTOR SYSTEM

By

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ABSTRACT

EVALUATION OF ASPERGILLUS ORYZAE β-GALACTOSIDASE
KINETIC PARAMETERS AND IMMOBILIZATION
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The work described in this thesis was undertaken to evaluate the use of a hollow fiber enzyme reactor to hydrolyze milk and whey lactose. A thermostable enzyme, A. oryzae β-galactosidase, was selected for immobilization. To permit comparison of immobilization results with free solution results, the enzyme's kinetic parameters were determined (K_m = 153 mM, V_m = 55 $\mu Mol/mg^{-1}min^{-1}$, and K_c = 4.4 mM) at 55°C and pH 6.5. The enzyme was backflush loaded into asymmetric hollow ultrafiltration fibers incorporated in single fiber reactors (SFRs). Evaluations of two fiber materials resulted in the selection of polyamide fibers, which, unlike polysulfone fibers, permitted the recovery of enzyme activity. However, bovine serum albumin was required to enhance enzyme retention. Under the operational conditions employed, reaction rate in the SFRs was not dependent on flow rate but increased with enzyme loading. Apparent enzyme specific activity dropped with loading, and the effectiveness factors observed were less than 0.2 indicating approach to a diffusion controlled regime.

To Nancy for her loving support and unrelenting watchfulness that I complete this thesis.

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CHAPTER 1

INTRODUCTION

The purpose of the work presented in this thesis is the evaluation of an immobilized enzyme system to improve the digestibility and marketability of dairy products. While milk and dairy products are excellent sources of nutrition, a large portion of the world's population is unable to properly digest the milk sugar, lactose. Lactose content also reduces the utility of whey products. Since the monosaccharides that comprise lactose do not pose such problems, an immobilized enzyme system employing the hydrolytic enzyme, β-galactosidase has been investigated.

Dairy Products and Lactose Hydrolysis

All mammals secrete milk to nourish their young. Milk is, therefore, necessarily a very complete food. Man has taken advantage of this food by domesticating a variety of animals for milk production. In the United States, the primary dairy animal is the cow. Whole cow's milk is a complex mixture of water (88%), proteins (3.2%), lactose (4.6%), fat (3.7%), and minerals and other components (0.7%)(Harper and Hall 1976). Composition varies with a number of factors including breed of cow, individual animal, stage of lactation, age, disease, and nutrition (Banks et al. 1981).

Among the constituents of milk, lactose, a disaccharide consisting of glucose and galactose monomers, most restricts wider use of dairy products. Lactose poses problems both in digestibility and processing of dairy products.

Inability to digest lactose arises primarily from lacking the digestive enzyme that hydrolyzes the disaccharide's β bond, linking the two monosaccharides. Although 89% of adults of Northern European descent are capable of digesting lactose, populations from most other areas of the world suffer lactose maladsorption (Simoons 1979). A significant number of infants also are unable to digest lactose. In the guts of lactose maladsorbers, microbial fermentation and osmotic effects from undigested lactose often lead to various digestive problems including flatulence, diarrhea, discomfort, and protein maladsorption (Richmond and Gray 1981). Most other adult mammals also share the inability to digest lactose, thus limiting the use of dairy by-products for livestock and pet feeding.

In dairy product processing and utilization, the presence of lactose presents problems arising from its low sweetness and solubility compared with other sugars, including its constituent monosaccharides (Table 1). Since lactose is not as sweet as other sugars and more difficult to digest, it has no utility as a sweetener. Its low solubility limits the degree of concentration in milk products and syrups derived from whey. Lactose solubility is also sharply temperature dependent (Banks et al. 1981); therefore, frozen dairy products become grainy due to lactose crystallization. Unlike sugars in corn syrup, its relatively low solubility prevents shipping products containing lactose in the convenient form of a concentrated liquid.

Since an inability to break the lactose disaccharide bond impedes the consumption of dairy products by many people, availability of lactose-hydrolyzed (LH) dairy products would broaden the market for milk and other dairy products. Except for slightly enhanced sweetness due to the presence of glucose and galactose, no organoleptic differences between LH milk and standard liquid milk have been detected (Repelius

Table 1. Solubility and Relative Sweetness of Sugars[1]

Sugar	Concentration to Give[2] Equivalent Sweetness		Solubility (20° C)
	Low	High	
Sucrose	1.0%	10.0%	67.9%
Glucose	1.8	13.9	45.4
Lactose	3.5	25.9	18.0
Galactose	2.1	15.0	40.6

[1]Reference, Holsinger 1981.

[2]Weight % in solution.

1983). Since food aid sent to underdeveloped nations often includes skim milk powders, use of LH milk might substantially enhance the nutritional status of their lactose intolerant populations.

In addition to enhancing digestibility, use of LH milk is advantageous in the manufacture of sweetened, concentrated, and fermented milk products (Holsinger 1981, Burgess 1983). Since glucose and galactose are sweeter than lactose, less sweetener is required to produce sweetened LH milk products. The greater solubilities of glucose and galactose permit storage and shipment of LH milk as a 3:1 concentrate. Similar concentrates from standard milk thicken and coagulate due to crystallization of lactose. Furthermore since microorganims more quickly ferment its constituent monosaccharides than lactose, LH milk reduces the processing time for yogurt and other cultured milk products. While the increased sweetness that results from lactose hydrolysis increases the acceptance of some cultured milk products, e.g. yogurt, it detracts from others, e.g. buttermilk (Holsinger 1983).

Lactose hydrolysis also may be used to increase the utilization of whey. Whey contains approximately 50% of the nutrients in milk. The solids in whey consist of lactose (70%), protein, and soluble ions. Two forms of whey are produced, acid (pH~4) from cottage cheese production and sweet (pH~6.5) from cheeses produced with rennet. Lactose hydrolysis methods developed for milk should easily transfer to sweet whey since its pH and soluble ion composition are similar. Of the 37.9 billion pounds of whey produced in the U.S. in 1978, 60% was utilized; the remaining 40% was a waste disposal problem (Holsinger 1981).

Often protein is recovered from whey by ultrafiltration. Whey permeate, which still represents a disposal problem, is a solution of lactose and smaller quantities of minerals and other small molecules.

Uses of LH Products

Various schemes have been developed to ferment or hydrolyze the lactose in whey and whey permeate, thus transforming them from waste disposal problems to salable products. Methods involving fermentations include the production of ethanol (Dale et al. 1985, Hahn-Hagerdal 1985) and lactic acid (Linko 1985, Tuli et al. 1985) from whey permeate. Immobilized yeast cells have been used to ferment the lactose in whey to produce enhanced protein concentrates and ethanol (Kierstan and Corcoran 1984). Whey has also been proposed as a nutrient for the production of single cell proteins.

Development of low cost methods of hydrolyzing lactose will encourage the use greater amounts of whey products directly in foods. Potential and current applications for hydrolysates include beverages, frozen desserts, syrups, and confections (Holsinger 1981). Soft drinks and high protein beverages containing LH whey have been test marketed. In Europe, LH syrups are being utilized to sweeten some foods. Caramels prepared with LH whey taste and retain moisture better than those with unhydrolyzed whey (Holsinger 1981).

Since milk is a food product, any processing method to produce LH products must adhere to strict standards that maintain product purity, shelf life, organoleptic characteristics, and nutritional standards (Banks et al. 1981). Standard chemical methods of disaccharide hydrolysis, e.g. acid and cationic resins, can only be used on fairly pure lactose solutions (Poulsen 1984) and alter the product unfavorably. Treating milk with acid, for example, will precipitate casein proteins.

Unlike other methods, the enzyme, β-galactosidase or lactase, selectively hydrolyzes only the β(1-4) glycosidic bond in the lactose found in dairy products. Treatment with lactase yields a product of virtually unchanged appearance and slightly sweeter flavor. The only

chemical differences between the raw material and hydrolysate are the presence of glucose and galactose monomers and a small amount of lactase. Enzymatic hydrolysis is, therefore, the method of choice for producing LH dairy products.

Enzyme Selection

Lactases are widely distributed in nature and may be derived from a variety of animal, plant, and microbial sources (Richmond et al. 1981). Screening 62 strains of yeast, molds, and bacteria, Ramana Rao and Dutta (1978) isolated lactase activity from 27. For this study, the choice of enzyme was confined to commercial sources.

Other criteria that impinged upon enzyme selection included stability, resistance to thermal inactivation, optimum pH range, and probable acceptability by the FDA, i.e. the enzyme should be generally recognized as safe (GRAS). Since milk processing requires either high (>130°F) or low (<45°F) temperatures to inhibit microbial growth (Zall 1981), and low temperatures reduce enzyme activity, ideally the enzyme should remain active and stable at least to 130°F. The pH of milk ranges from 6.5 to 6.7 (Harper and Hall 1976). Precipitation of casein proteins at pH 4.6 and product adulteration from any substantial pH adjustment dictate the use of enzymes active at the pH of milk.

Commercially available lactases are isolated from fungi (*Aspergillus niger* and *A. oryzae*), bacteria (*E. coli*), and yeast (*Saccharomyces lactis* and *S. fragilis*). Of the available enzymes, the *E. coli* and yeast enzymes have been most extensively characterized. While the pH optimum of *E. coli* lactase lies between 6.6 and 7.5, and the enzyme is stable to 55°C, its activity is drastically reduced in milk relative to buffered lactose solutions (Morisi et al. 1973). This enzyme is also not GRAS.

The pH optima for yeast lactases are approximately 6.6, and the activity is little affected by milk and whey constituents (Morisi et al. 1973, Mahoney and Adamchuk 1980). Yeast lactases are currently used in production of LH products. Unfortunately, these enzymes rapidly lose activity at temperatures above 40°C (Mahoney and Whitaker 1977); therefore, any dairy process requiring significant holding times must take place at temperatures below 10°C (Forsman et al. 1979). Since the enzyme's activity undergoes a nearly three-fold reduction over the 25°C range between 20° and 45°C (Mahoney and Whitaker 1977), activity at 10°C is drastically reduced. Low activity can lead to either prohibitive processing times or the use of large amounts of enzyme.

The pH optima for fungal lactases lie between 3.5 and 5.0. Thus fungal lactases are generally more useful in hydrolyzing lactose in acid whey than unacidified milk products. Although the optimum pH for Takamine® fungal lactase (Miles Laboratories, Elkhart, Indiana) is 5.0., the enzyme retains approximately 50% of maximum activity at pH 6.5 (Miles, Park et al. 1979). It is derived from A. oryzae, the organism utilized for industrial production of alpha-amylase, and is GRAS. A. oryzae β-galactosidase shows negligible inactivation in 30 min. at temperatures up to 55°C and is relatively unaffected by ions found in milk. This enzyme has been selected for use in this project on the bases of temperature stability and broad pH range. Unfortunately, A. oryzae lactase is poorly characterized at neutral pH, requiring measurement of the enzyme's kinetic parameters.

Current β-Galactosidase Applications

The methods of enzymatic lactose hydrolysis fall into two broad categories: free enzyme and immobilized enzyme technologies. The use of

free enzyme inevitably entails the loss of enzyme with product; therefore, the obvious advantage of immobilization resides in permitting reuse of the enzyme. On the other hand, immobilized enzymes often suffer lower apparent activity due in part to diffusional barriers and require higher capital outlay. Whether immobilization is economically justified depends upon factors including increased usable enzyme life, activity in the immobilization matrix, and equipment and immobilization costs.

Most LH milk currently marketed is produced in batch processes with free enzyme. Free enzyme processes, simply stated, entail the addition of yeast β-galactosidase to milk either before or after pasteurization. The milk is then held at 10°C overnight or at 37-40°C for a few hours (Repelius 1983, Kligerman 1983). Lactose hydrolysis of 70% is considered sufficient. Treating 2% milk has cost $0.09/quart (Richmond 1981). Lactase is also marketed for home use.

Sundry methods of immobilizing β-galactosidase have been examined in the laboratory (Richmond et al. 1981). Three methods have found application in commercial and pilot plant use. A plant in Snamprogetti, Italy, produces up to 10 tons LH milk per day (Swaisgood 1985). This process employs yeast lactase entrapped during extrusion of cellulose triacetate fibers. The process is a batch reaction system with continuous recirculation through a bed packed with the enzyme containing fibers (Morisi et al. 1973, Pastore and Morisi 1976). A typical cycle requires 20 hours to complete hydrolysis (Repelius 1983).

Commercial systems that hydrolyze lactose in acid whey operate in the United States and Finland (Poulsen 1984). Both systems employ *A. niger* β-galactosidase. The Finnish system utilizes an adsorption resin to immobilize the enzyme. In the U.S. the Nutrisearch facility in Winchester, KY, employs lactase immobilized on porous glass and titania spheres (Swaisgood 1985). The process was developed by researchers at

Corning Glass Works (Pitcher 1978). The hydrolysate is used in fermentations as a growth medium replacement. Summation of the values of hydrolysate and whey protein concentrates and reduction of waste disposal costs provide economic justification for the process (Swaisgood 1985).

Recent reviews have noted that cost and noncompetitiveness with free enzyme currently inhibit the industrial application of immobilized lactase systems (Richmond 1981, Poulsen 1984). Poulsen found fewer than ten plants employing immobilized enzyme technology existed worldwide and listed several additional problems including:

1) Sweeteners may be produced more cheaply from starch than whey.
2) The need for milk improvement is not great enough when all factors are considered.
3) Although whey presents disposal problems, other uses including fodder and ethanol fermentation may be more economical.
4) Immobilized enzyme processes still have too many weak points, e.g. risk of contamination, slow rate of reaction, and unacceptable cost.

The outlook for immobilized enzymes is, however, considered good as processes are developed and refined (Richmond 1981).

Among the factors affecting the economic application of immobilized enzymes in the food industry is the requirement for a high standard of sanitation to prevent contamination of the product (Swaisgood 1985, Richmond 1981). Microbial growth in the reactors also shortens catalyst life and efficiency. In the immobilization schemes currently used for dairy products, the reactors, support media, and, necessarily, the enzymes require periodic chemical sanitization. Since the enzyme is inextricably bound to the support, only relatively mild sterilants that do not inactivate the enzyme may be employed. Since current systems also

employ flow around cylindrical or spherical catalyst supports, low shear microenvironments may exist where microbial attachment and growth are possible.

To ameliorate the problems listed above, a system was sought with easily reversible immobilization, in which microbes in the substrate-product stream are always separated from the immobilized enzyme. Ideally, a high shear environment is maintained in the process stream to discourage microbial attachment and fouling; and, the support and, possibly, enzyme activity may be recovered and recycled.

Hollow Fiber Immobilization

Physical immobilization of enzyme in the matrix of the outer layer of asymmetric hollow ultrafiltration (UF) fibers meets the criteria listed above. These fibers (Figure 1) consist of a thin semipermeable inner membrane, approximately 0.5 microns thick, and an outer supporting macroporous spongy layer. In normal UF operation (Figure 2), the medium to be filtered is pumped through the lumen under pressure. The solvent and other small molecules cross the membrane relatively unimpeded and are forced out the shell side ports in the permeate (ultrafiltrate) stream. Larger molecules are retained on the lumen side and emerge from the downstream port in more concentrated solution. Nominal molecular weight cutoffs for such membranes range from 3000 to 500,000 daltons. Membranes are spun from polymers including polysulfones, polyamides, and acrylics (Chambers 1976).

Enzymes may be physically immobilized in the spongy layer by either static loading (Waterland et al. 1975) or backflush loading (Breslau and Kilcullen 1978). In static loading, the shell side of the cartridge is filled with enzyme solution that diffuses into the fibers. By

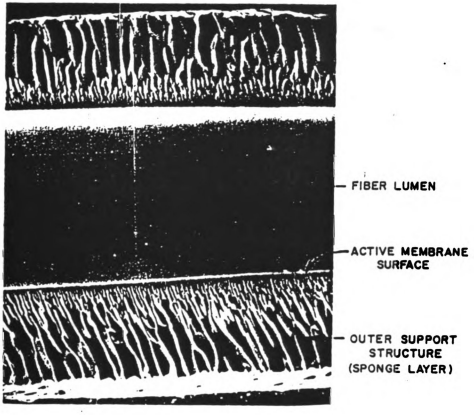

Figure 1. Photomicrographs of a Romicon hollow fiber showing the inside active membrane surface, and the outer support structure. (Breslau and Kilcullen 1978)

repeatedly filling and draining the shell side with stock enzyme solution, the concentration of enzyme in the spongy layer approaches that of the stock soltuion.

To backflush load enzyme, pressure is applied to the shell-side and enzyme stock solution is ultrafiltered from the shell-side to the lumen-side of the fibers (Figure 2). This method achieves significantly higher loadings than static loading. The enzyme forms a gel-like layer in the spongy matrix at high loadings (Chambers 1976). Subsequent cross-linking of enzyme with reagents, such as glutaraldehyde, may be utilized to retain the enzyme (Breslau and Kilcullen 1978). After loading by any of the above methods, shell-side solutions are drained from the hollow fiber reactor (HFR).

Operation of the reactor involves pumping substrate solution through the lumen at low pressure, the recycle mode (Figure 2). During operation the shell-side ports are closed, and the shell-side contains no liquid. Substrate diffuses across the filtration membrane into the spongy layer where it reacts. Products of the hydrolysis reaction then diffuse back across the membrane and exit the reactor in the lumen-side outlet stream.

Operating hollow fiber reactors in relatively high shear stress regimes may attenuate fouling problems. The lack of dead spaces for microbial attachment in the flow path may reduce fouling from microbial growth. As increased shear stresses reduce fouling from milk solids during UF operation (Yan et al. 1979), the problem of deposition of a non-microbial fouling layer on catalyst support beads (Pitcher 1978) may also be reduced by high shear.

Advantages of physical immobilization of enzymes in hollow fiber reactors include (Chambers et al. 1976):

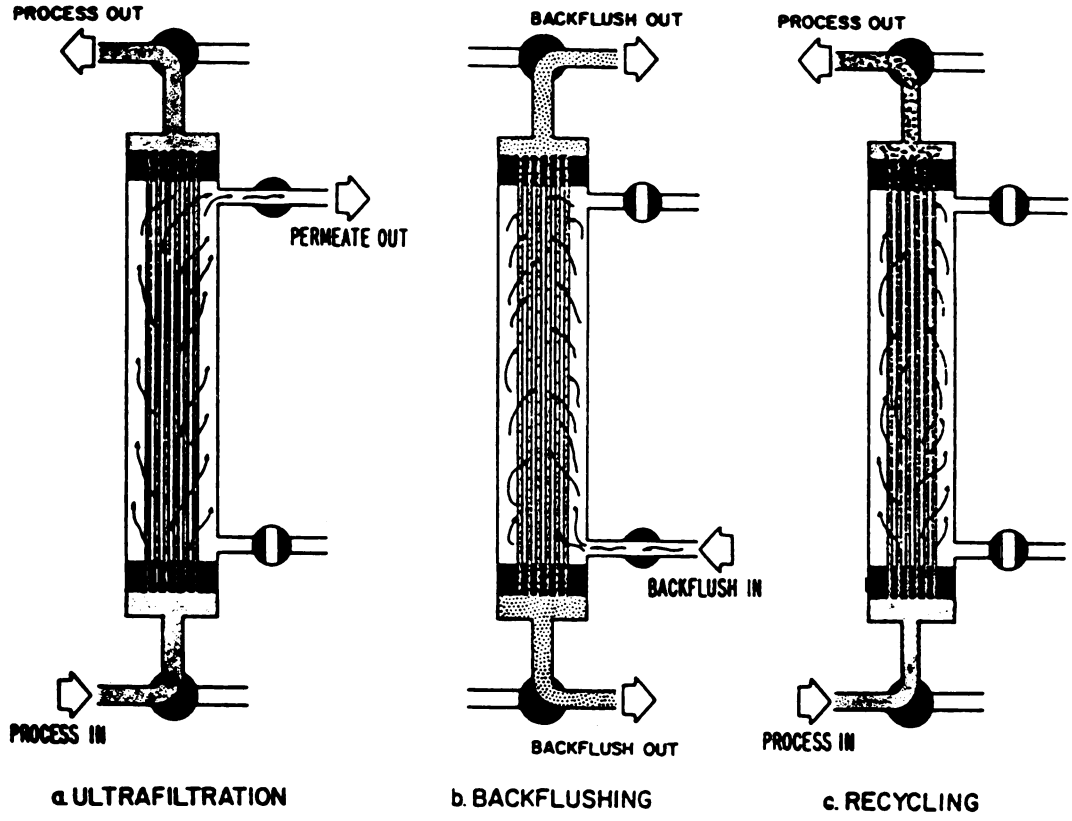

Figure 2. Schematic of hollow fiber cartridge showing the three modes of operation used in system's design. (Breslau and Kilcullen 1978)

1) Quick and easy preparation without the necessity of chemically altering the enzyme.
2) Relatively small effect on the kinetic properties of the enzyme.
3) Prevention of microbial and antibody access to the enzyme.
4) Selectivity of products and substrates through membrane selectivity.
5) Large ratio of surface area to volume.
6) Absence of enzyme leakage.
7) Continuous operation at low pressure.

While membrane selectivity limits the applicability of recycle reactors to operations with small substrate and product molecules (Strathmann 1985), it is advantageous in dairy product lactose hydrolysis since potentially interfering proteins are isolated from the enzyme. Lactose (MW 342) and its hydrolysis products easily cross UF membranes that should be impermeable to A. oryzae β-galactosidase (MW 90,000 - 110,000) (Park et al. 1979, Ogushi et al. 1980) and most milk proteins (MWs 10,000 - 300,000)(Harper and Hall 1976).

Since the enzyme is not chemically bound to the support, reclaiming the support for repeated use simply involves first flushing the enzyme from the spongy layer. Reuse of ultrafiltration fibers in dairy applications then requires employing chemical cleaning and sanitization methods that are well developed (Harper 1979, Delaney and Donelly 1977). Isolation of the catalyst from contaminants also gives rise to the possibility that enzyme flushed from the reactor may be recycled through several loadings.

Although Chambers lists lack of enzyme leakage and small effect of immobilization of kinetic properties as advantages, each proposed hollow fiber system must be evaluated to confirm the validity of these statements. Results of immobilizing β-galactosidase have thus far been

mixed. Enzyme retention and rate of inactivation in the fiber have varied with fiber composition, pore size, protein structure, and loading method.

Results of Enzyme Immobilization in HFR's

Investigators who have employed UF fibers for enzyme immobilization have developed experimental methods and have reported such problems as enzyme leakage into the lumen and inactivation in contact with fiber materials. E. coli β-galactosidase static-loaded in polysulfone membranes (molecular weight cutoff, MWC, 50,000) did not leak during loading or operation (Waterland et al. 1975). Inactivation of enzyme in contact with the fibers was not evident.

Korus and Olson (1977) backflushed alpha-galactosidase derived from Bacillus stearothermophillus into two types of hollow fibers. They observed 50% activity losses in acrylic copolymer membranes (MWC 50,000) over seven days. Since the backflush permeate had contained 14% of the enzyme activity during loading, they hypothesized leakage into the substrate solution during HFR operation to explain the loss. Alpha-galactosidase loaded on polysulfone membranes (MWC 10,000) had a half life of only 2 - 3 days. Activity losses on polysulfone seemed to result from inactivation of the enzyme. Inactivation was attenuated by treating fibers with bovine serum albumin (BSA). Similarly, Korus and Olson (1975) found that yeast β-galactosidase rapidly lost activity on polysulfone fibers. They reported a half life of 4 days.

Huffman-Reichenbach and Harper (1982) found that polysulfone (MWC 10,000 and 50,000) and acrylic copolymer (MWC 50,000) retained 36% and 10%, respectively, of A. oryzae lactase with single pass backflush loading. Recycling lumen-side effluent for additional passes through the UF membrane further reduced total activity retained. Retention did not seem

to be a function of polysulfone fibers' nominal MWC. Enzyme leakage also occurred during operation at high flow rates. The half life of enzyme in contact with the membranes was 2 hours, and treatment with BSA reduced the apparent half life of the enzyme.

Other investigators achieved high retention of lactase on UF fibers by backflush loading. Polysulfone (MWC 10,000) fibers retained 99% of yeast β-galactosidase loaded, and acrylic copolymer retained 81% (Kohlwey and Cheryan 1981). Contact with polysulfone fibers shortened yeast lactase half life to 34.7 hours at 20°C compared with 352 hours for free enzyme in buffer. Pretreating with BSA, however, increased the half life of immobilized enzyme to 1990 hours. Breslau and Kilcullen (1978) also were able to achieve high loadings, 5.45 g/ft^2, with A. niger β-galactosidase on acrylic copolymer (MWC 50,000).

Thus far, investigators have not operated hollow fiber reactors with β-galactosidase to produce data directed toward scaling-up HFRs to dairy conditions. Several investigators (Waterland et al. 1975, Kohley and Cheryan 1981) have utilized dilute solutions of o-nitrophenyl-β-D galactopyranoside (ONPG) as a substrate. Since enzyme kinetic parameters with different substrates vary widely and ONPG's hydrolysis product o-nitrophenol (ONP) adsorbs to some hollow fiber materials, ONPG may not be a realistic substrate for simulation of lactose results. ONPG is, however, a convenient substrate for assaying enzyme activity since ONP concentration can be measured by spectrophotometry without chemical modification.

Since neither glucose nor galactose may be directly determined by spectrophotometry, reaction with lactose is not as easily assayed as with ONPG. Data available for lactose conversion in HFRs tends to be more limited than for ONPG. Breslau and Kilcullen (1978) reported 22% conversion of a 10% lactose solution over 3 hours operation of their

system. They have not, however, specified total fluid volume, actual residence time in the reactor, or flow rate. Korus and Olson (1975) reported good agreement of their data for yeast lactase conversion of lactose with the model developed by Waterland et al. (1974).

Ultrafiltration in the Dairy Industry

Although UF membranes have not yet been used to immobilize enzymes in the dairy industry, UF technology has recently been increasingly utilized. Over 100,000 m^2 of membrane has been installed for use in the dairy industry (Bembaris and Neely 1986). Delaney and Donnelly (1977) and Harper (1980) reviewed current and potential applications, UF membrane structures and materials, cleaning, and process problems and considerations. While cellulosic membranes were the first utilized, newer synthetic materials - polysulfone, polyamide and polyimide - have gained increasing acceptance due to their superior heat and chemical resistance. Although wide bore tubes have been the predominant configuration, thin channel, laminar flow systems have been increasingly accepted in the dairy industry (Harper 1980). Hollow fiber devices are also employed by the dairy industry (Bemberis and Neely 1986).

UF is now the method of choice for producing whey protein concentrates (Horton 1982). In cheesemaking, UF concentrates milk and thus enhances the recovery of protein from milk by 16-19% (Chandan 1982, Horton 1982). Commercial installations in Europe employ UF to prepare milk for the production of a variety of soft cheeses. Approximately one hundred such plants were operating in 1982. Use of UF to fortify milk for yogurt production is also being considered.

The two main problems that arise in ultrafiltering dairy products are fouling and sanitization. Modern synthetic materials permit sanitization with a variety of common chemical sterilants (Harper 1980,

Delaney and Donnelly 1977). The newer materials also resist temperatures above 50°C, permitting operation at temperatures that impede microbial growth (Horton 1982).

Fouling presents the most troublesome problem in whey UF (Horton 1982). Sweet wheys tend to foul non-cellulosic UF membranes. Such fouling results from a complex interaction of the membrane material, calcium phosphate, and protein. While pretreatments and pH adjustment relieve fouling problems, they also reduce the quality of the protein concentrate. Acid whey and milk do not foul UF membranes as readily as sweet whey. Since the HFR operates at neutral pH, chemical fouling, as well as microbial growth, could present a problem.

Hollow Fiber Reactor Models

Several models have been developed to predict conversions and effectiveness factors in HFRs (Kleinstreuer and Poweigha 1984). The most complete models consider mass transfer in the lumen, membrane, and porous spongy layers (Figure 3), axial laminar flow and radial diffusion in the lumen (Region 1), radial diffusion across the UF membrane (Region 2), and radial diffusion and reaction in the spongy layer (Region 3). Other mass transport mechanisms including bulk flow across the membrane due to pressure gradients and axial diffusion are assumed insignificant.

Waterland et al. (1974) have presented the first and most complete model to predict conversions in a hollow fiber reactor using steady state assumptions. The governing equations for the three regions are

$$\frac{D_3}{r}\frac{\partial}{\partial r}\left[r \frac{\partial c_3}{\partial r} \right] = R \qquad (1)$$

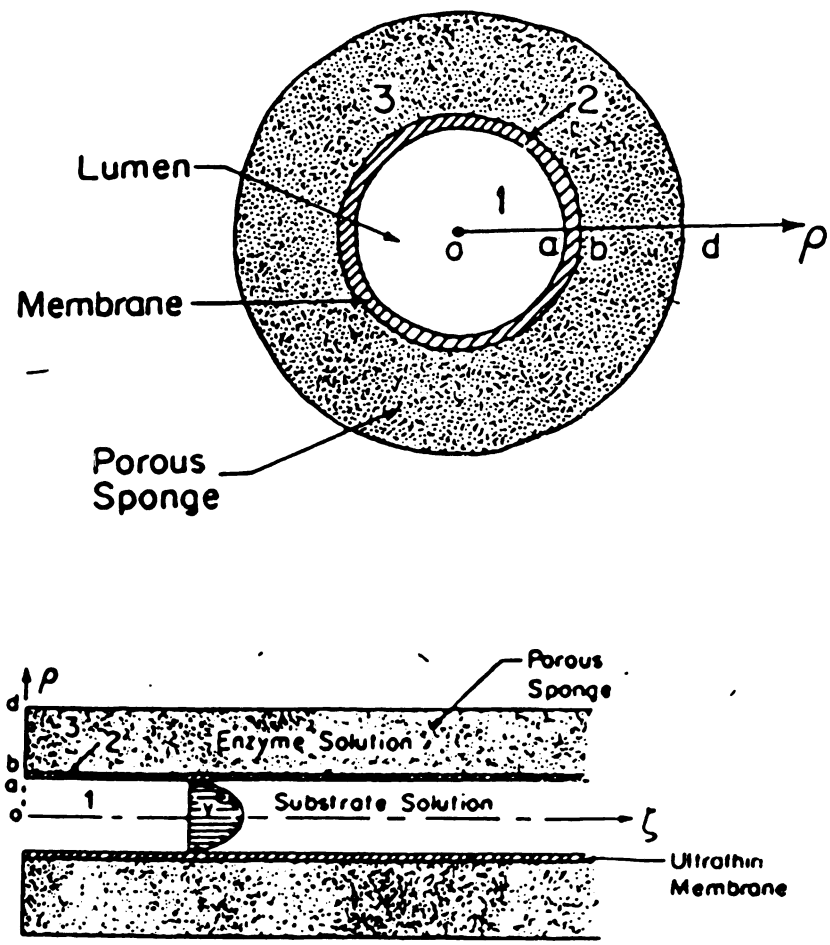

Figure 3. Cross sectional and axial schematics of the regions used in modelling hollow fiber reactors. (Waterland et al. 1974)

$$\frac{D_2}{r} \frac{\partial}{\partial r} \left[r \frac{\partial c_2}{\partial r} \right] = 0 \qquad (2)$$

$$\frac{D_1}{r} \frac{\partial}{\partial r} \left[r \frac{\partial c_1}{\partial r} \right] = 0 \quad v_z \frac{\partial c_1}{\partial z} \qquad (3)$$

where D = diffusivity, c = substrate concentration, r = radial dimension, and z = axial dimension. $v_z(r)$ is assumed to follow a Poiseuille type radial velocity profile:

$$v_z = v_o \left[1 - \frac{r^2}{a^2} \right] \qquad (4)$$

where v_o = center-line fluid velocity.

The rate of reaction is described by simple Michaelis-Menten kinetics:

$$R = \frac{V_{max} \, c_3}{K_m + c_3} \qquad (5)$$

Since use of the full Michaelis-Menten expression precludes an analytical solution to (1), the expression has been simplified by assuming $K_m \gg c_3$ to approximate a first order form:

$$R = \frac{V_{max}}{K_m} c_3 \qquad (6)$$

Boundary conditions include no flux across surface d:

$$\frac{\partial c_3}{\partial r} = 0 \Big|_{r=d} \qquad (7)$$

and

$$\gamma c_3(b,z) = c_2(b,z)$$

$$c_1(a,z) = c_w(z) \quad \text{where } z>0$$

$$c_2(a,z) = \gamma c_w(z)$$

$$D_3 \frac{\partial c_3}{\partial r}\bigg|_{r=b} = D_2 \frac{\partial c_2}{\partial r}\bigg|_{r=b}$$

$$D_2 \frac{\partial c_2}{\partial r}\bigg|_{r=a} = D_1 \frac{\partial c_1}{\partial r}\bigg|_{r=a}$$

$$\frac{\partial c_1}{\partial r}\bigg|_{r=0} = 0$$

with the initial condition:

$$c_1(r,z) = c_o \quad z<0 \tag{8}$$

The symbol γ represents the membrane partition coefficient.

The analytical solution to the above model includes an expression for lumen-side concentration at the wall that requires a numerical solution. Waterland et al. (1974) also have described an iterative numerical method to predict results with non-linear kinetics. The results are presented in terms of a Thiele modulus:

$$\lambda^2 = \frac{V_{max}\, a^2}{K_m D_3} \tag{9}$$

and dimensionless length:

$$Z = \frac{z}{a\alpha} \tag{10}$$

where

$$\alpha = \frac{V_o a}{D_1} \tag{11}$$

is the Peclet number, and concentration is in dimensionless form:

$$c_1 = \frac{c_i}{c_o} \tag{12}$$

Several of the assumptions employed in developing the model's predictions are worth noting. The enzyme solution in the spongy layer is regarded as homogeneous. Also since the solvent entrapped in the spongy layer is the same as that in the lumen, and, given the macroporous character of the spongy layer, free solution diffusivity of substrate is assumed the same as in the spongy layer. Lacking data to describe diffusion across the ultrafiltration layer, a tenfold higher resistance is assumed across the UF membrane. Since the ultrafiltration layer and spongy layer are inextricably bound, assuming diffusivity may be the only method to separate the resistances presented by the two layers.

Solutions of the model for first order kinetics predict rapidly increasing conversions at constant dimensionless length (Z) as the Thiele modulus is varied from 10^{-2} to 10. As Z decreases, outlet conversions decrease and approach the asymptotic conversion more slowly as

the Thiele modulus increases; i.e. the shift to a completely diffusion-controlled regime occurs at higher values of the Thiele modulus. Transition from kinetic to diffusion control also occurs over a wider range of Thiele modulus values as Z decreases.

Numerical solution for non-linear kinetics involved the additional parameters:

$$\theta = \frac{K_m}{c_o}$$

$$\Psi = \lambda^2 \theta$$

Using the above expressions, a nondimensional form of the Michaelis-Menten equation is

$$R = \frac{\Psi c_3}{\theta + c_3} \qquad (13)$$

Results for non-linear kinetics, where $\psi = 100$, were similar to the first order solutions. As θ decreased, i.e. as the reaction approached 0th order kinetics, the transition from kinetic control to diffusion control occurred more rapidly and shifted to higher modulus values. Experiments designed to test the model's predictions have generated data that correspond well with predictions (Waterland et al. 1975).

While this solution accurately predicts conversion in the above case, its calculations are quite cumbersome (Kim and Cooney 1976). The model may also be unnecessarily rigorous in its consideration of the UF membrane since varying the assumed ratio D_1/D_2 between 5 and 20 yielded negligible changes in predicted conversions (Waterland et al. 1974).

Kim and Cooney (1976) employed the same modelling equations and assumptions as Waterland et al. Using again the first order limit of the Michaelis-Menten equation (6), they developed a simpler method to solve for first order kinetics. Since the method of Kim and Cooney is easier to use and is as accurate as Waterland et al., it may be preferable for first order kinetics; however, the solution method does not have the same capacity to allow for axial variation in the rate constant (Kleinstreuer and Poweigha 1984).

Another approach treats hollow fiber reactors as CSTRs rather than plug flow type reactors (Webster and Shuler 1978, Webster et al. 1979, Davis and Watson 1985). A CSTR model is used when a recycle loop with a high recirculation rate yields nearly constant concentration in the lumen. While the model was developed for fibers surrounded by a cartridge filled with catalytic solution, it is also applicable to HFRs as system geometry is similar. By using the CSTR simplification, the model eliminates consideration of axial and radial concentration gradients in the lumen. The descriptive equations for the ultrafiltration fiber and surrounding medium are the same as Equations (1) and (2) above, and the boundary conditions are similar except concentration at interface a (Figure 3) is constant. As above, analytical solution requires simplification to first or zeroeth order kinetics. Since product-inhibited enzymes operate at the lowest catalytic reaction rate in a CSTR, employing this type of operation may not be feasible for lactases, which are generally inhibited by the product, galactose.

Webster and Shuler (1981) have modelled transient responses in hollow fibers to changes in the inlet concentration of substrate. As above the model ignores lumen concentration gradients. The model also disregards resistances from the ultrafiltration membrane and predicts rapid approach to steady state in response to changes in lumen concentration.

The solution shows concentration at the outer edge of the fiber reaching 80% of the ultimate concentration within 3 s after a step concentration change for a fiber whose lumen diameter is 0.13 mm and outer diameter is 0.18mm.

Lewis and Middleman (1974) simplified the descriptive equations (1), (2), and (3) by again assuming first order kinetics, slow kinetics (Thiele modulus <0.1), and negligible resistance from the ultrafiltration layer. These assumptions permit incorporating equations (3) and (4) and the condition of radial flux continuity into the expression:

$$D_3 \left.\frac{\partial c_3}{\partial r}\right|_R = \frac{v_o R}{4} \frac{\partial c}{\partial r} \qquad (14)$$

Equations (1) and (14) are then amenable to analytical solution. Using static-loaded urease in the HFR, Lewis and Middleman tested their model at Thiele moduli of 10^{-1} and 4.4×10^{-2}. Experimental results conformed with the model particularly well at the lower Thiele modulus value, and a small but consistent error was observed at the higher value.

Davis and Watson (1985) presented a numerical solution for a diffusion limited regime in a hollow fiber reactor from the modelling equations presented by Waterland et al.(1974).

The above models necessarily employ a number of simplifying assumptions to develop the descriptive equations and analytical solutions. Attempting to use such models with data obtained from the experiments reported in this thesis may be complicated by kinetics and the method of β-galactosidase immobilization. While the models consider simplified cases for Michaelis-Menten kinetics, investigators have found that A. oryzae lactase is product inhibited (Miles 1978, Park et al. 1979,

Ogushi et al. 1980). Inhibition complicates the kinetic expressions and may require considering product concentration distribution in the model.

The models also assume evenly distributed catalytic activity in the spongy layer. Backflush loading may, however, yield enzyme concentrated around the inner membrane in the spongy layer (Chambers 1976). The existence of such a layer greatly increases the possible rate of reaction in the region surrounding the lumen and reduces the mean diffusion path required for reaction.

Experimental

The experimental program described in this thesis is designed to obtain data for hollow fiber reactors hydrolyzing lactose relevant to dairy application. The objectives were to evaluate enzyme, fiber materials, and immobilization technique, as well as to obtain information necessary for modelling. The experiments were designed to simulate conditions for handling dairy products whose pH is approximately neutral, i.e. milk and sweet whey products.

The following chapters describe methods and results in experiments that determine:

1) Enzyme kinetics - To obtain the parameters needed for future modelling and to assay the behavior of the enzyme in free solution; end point assays and conversion over time were used.
2) Residence time distribution - The experiment was designed to compare residence time of lactose in HF cartridge with that of a non-diffusing species.
3) Retention of enzyme - Methods were developed to enhance the retention of backflush-loaded β-galactosidase in a HFR and assay fiber material compatibility with enzyme.

4) Reactor performance - Single fiber reactors were operated with different enzyme loadings and flow rates to assess performance.

Since this thesis describes the first experiments in a continuing study, the results are inherently incomplete. Additional data are required for modelling and conclusive indications of the system's applicability. The results and techniques presented herein are useful for the continuing evaluation of the proposed system.

CHAPTER 2

ENZYME KINETICS

Introduction

Models of catalyst behavior in a reactor require knowledge of intrinsic reaction kinetics. Unlike *E. coli* and yeast lactases, the kinetics of *A. oryzae* β-galactosidase have not been extensively studied. Thus the experiments described in this chapter were conducted to determine the enzyme's kinetic parameters under temperature and pH conditions similar to dairy applications.

Enzyme kinetics often may be represented by the Michaelis-Menten equation:

$$V = \frac{\partial c}{\partial t} = \frac{ke_o c}{c + K_m} \qquad (15)$$

where c is substrate concentration, k is the first order constant for the conversion of substrate to product from the enzyme substrate complex, e_o is enzyme concentration, and K_m is the Michaelis constant (Cornish-Bowden 1976). The quantity ke_o is commonly lumped into a single term, V_m, which represents the 0th order limit for the reaction rate.

The simple form of the above equation does not, however, adequately describe the reaction rates of many enzymes. Since β-galactosidases generally undergo product inhibition by galactose, the experiments were designed to obtain the data required to derive inhibition constants. The Michaelis-Menten equation may be modified to a more general form to describe inhibition:

$$V = \frac{V_m c}{c(1+i/K_u) + K_m(1+i/K_c)} \qquad (16)$$

where i is inhibitor concentration, K_u the uncompetitive inhibition constant, and K_c the competitive inhibition constant. The above equation describes mixed inhibition. Either or both the inhibition constants may have very high values permitting the use of reduced forms of the equation. When K_u approaches infinity, the following rate equation describes competitive inhibition:

$$V = \frac{V_m c}{c + Km(1+i/K_c)} \qquad (17)$$

Conversely as K_c becomes very large, the limiting case is uncompetitive inhibition:

$$V = \frac{V_m c}{c(1+i/K_m) + K_m} \qquad (18)$$

While not all enzymatic reactions are adequately described by the above equations and the underlying mechanisms are more complex than implied, the equations frequently provide a convenient framework to model for enzyme reactions (Laidler and Bunting 1973, Cornish-Bowden 1976).

While several investigators have published kinetic parameters for A. oryzae lactase (Table 2), none have studied its kinetics at neutral pHs. Each investigator also has apparently used lactase from a different fungal strain, none of which may match the product used in this study. While each of the studies cited found galactose inhibits β-galactosidase, the kinetic parameters and description of the inhibition

mechanism required for this study's application have not been reported. The other product of lactose hydrolysis, glucose, apparently is not an inhibitor.

Since lactose and its hydrolysis products cannot be assayed directly during the enzymatic reaction, an end point assay method was used. The reaction was stopped after 10 minutes, and the amount of the product, glucose, was measured. Assuming the appearance of glucose to be linear over time, the rate of reaction was simply determined. The linearity assumption was confirmed experimentally. Batch reactions confirmed the predictive value of the experimentally determined kinetic parameters.

Mathematics and Data Treatment

The Michaelis-Menten equation (1) may be linearized by inverting the equation:

$$\frac{1}{V} = \frac{K_m}{V_m}\frac{1}{c} + \frac{1}{V_m} \qquad (17)$$

A plot of 1/V versus 1/c (Lineweaver-Burk plot) of kinetic data yields convenient estimates of the kinetic parameters, as the intercept on the ordinate is $1/V_m$ and the slope is K_m/V_m. The generalized equation (16) for enzyme reaction with inhibition may also be linearized by taking its inverse (Laidler and Bunting 1973).

$$\frac{1}{V} = \frac{K_m}{V_m c}\left[1 + \frac{i}{K_c}\right] + \frac{1}{V_m}\left[\frac{1}{K_u} + 1\right] \qquad (18)$$

Furthermore, linearization reveals that at constant inhibitor concentration the intercept and slope are described respectively by the following expressions:

$$\frac{1}{V_{mapp}} = \frac{1}{V_m}\left(i/K_u + 1\right) \tag{19}$$

$$\frac{K_{mapp}}{V_{mapp}} = \frac{K_m}{V_m}\left(1 + i/K_c\right) \tag{20}$$

If K_m and V_m have been determined by assays without inhibitor, K_u and K_c may be calculated from K_{mapp} and V_{mapp} in assays with inhibitor. Equations (5) and (6) may be solved for K_u and K_c:

$$K_u = \frac{i}{\left(\frac{V_m}{V_{mapp}}\right) - 1} \tag{21}$$

$$K_c = \frac{i}{\left(\frac{V_m}{K_m}\right)\left(\frac{K_{mapp}}{V_{mapp}}\right) - 1} \tag{22}$$

While the parameters K_m and V_m may be conveniently estimated from plots of experimental data and the above relationships, more accurate estimates may be derived from statistical treatment of the data. Wilkinson (1961) developed a weighted non-linear regression method to determine K_m and V_m. The method yields good estimates for kinetic parameters compared with other statistical methods (Atkins and Nimmo 1975). Wilkinson's method was developed into a BASIC computer program

(see WILKIN) to evaluate the kinetic parameters of enzymes (see Appendix).

The accuracy of the calculated kinetic parameters was confirmed by predicting batch conversions over time. Predictions were obtained from the integrated form of equation (16):

$$t = \frac{1}{V_m}\left[\left(1 + \frac{c_o}{K_m} + \frac{K_m}{K_c}\right)\left(c_o - c\right) + K_m\left(1 + \frac{c_o}{K_c}\right)\ln\frac{c_o}{c} + \frac{c^2 - c_o^2}{2K_u}\right] \qquad (23)$$

where the inhibitor is a stoichiometrically produced reaction product, e.g. galactose in lactose hydrolysis, $i = c_o - c$. Solutions for product concentration versus time were accomplished by a linear interpolation method in the KINDET program (see Appendix). A simple method of determining how well the predictions of the integrated model fit sample data is the Chi-square test:

$$\chi^2 = \Sigma\frac{(o-e)^2}{e} \qquad (24)$$

where o is the observed value, e the expected value from the model. The better predictions are those that minimize the chi-square values. KINDET evaluates kinetic constants from Equations (21) and (22), finds solutions for predicted conversions at experimental sampling intervals, and determines chi-square values.

To ascertain whether observed variations in the results of the kinetics experiments fall within the range of predictable experimental

errors, the data were subjected to a propagation of error analysis (see Appendix for program). Predicted variance of results was calculated from (Crandall and Seabloom 1970):

$$\text{var}(f) = \left[\frac{\partial f}{\partial a} \times \text{sdev A}\right]^2 + \left[\frac{\partial f}{\partial b} \times \text{sdev B}\right]^2 + \ldots$$

$$+ \left[\frac{\partial f}{\partial n} \times \text{sdev N}\right]^2 \qquad (25)$$

The analysis assumes random errors. The propagation of error analysis was performed using finite difference approximations to determine the expected magnitude of each identified source of error.

Materials and Methods

The enzyme was donated by Miles Laboratories (Takamine Fungal Lactase 30000, manufacturer's assay 32,130 LU/g)(1 LU yields 1 μMole lactose/min). Glucose concentration was determined by the peroxidase-glucose oxidase method (PGO)(Sigma Diagnostics, St. Louis, MO, Procedure #510). Assay results were read on a Perkin Elmer, Lambda 3A UV/VIS spectrophotometer. Lactose (cat.# L3625) and galactose (cat.# G0625) were obtained from Sigma. All other chemicals were reagent or analytical grade.

Enzyme assays were conducted in 0.05 M potassium phosphate buffer (pH 6.5, 1.0 mM MgSO4, 0.1 mM MnCl2). The ions in the buffer were at approximately the free solution concentrations in milk. Manganese and magnesium were included since they have been identified as activating ions for other lactases. Lactose solutions for assays at different concentrations were prepared by diluting 277.8 mM (9.5%) stock solution

in buffer with additional buffer. In determinations of inhibition kinetics, 200 mM galactose solution in buffer was also added to the assay mixture. Enzyme stock solution in buffer was added to initiate reaction in samples.

Kinetic Parameters

Data to determine kinetic parameters were obtained by preparing lactose solutions at concentrations (Table 2) bracketing the expected K_m. In preliminary experiments the expected K_m was estimated from literature values (Table 3). Results of the preliminary experiments permitted refinement of the expected K_m and experimental substrate concentrations.

Experimental solutions measured into 16x150 mm test tubes, which were placed in a constant temperature water bath shaker (New Brunswick, Model G76D) at 54.5°C at least 10 minutes before adding enzyme. To initiate reaction, enzyme was pipetted into each tube. Solutions were immediately vortexed and placed in the water bath. Experiments were conducted with enzyme concentrations of 0.0125 and 0.0083 mg/ml, adjusted by changing the volume of the experimental solution. Each treatment was incubated between 2.5 and 5 minutes, the time varying with enzyme and inhibitor concentration. Following incubation, the enzyme was inactivated by placing the test tube in a boiling water bath for 5 minutes. A blank control tube was prepared parallel with each experimental concentration. Each blank was treated exactly as the corresponding experimental solutions, except it was placed in boiling water immediately after the addition of enzyme.

Following inactivation, product (glucose) concentration was measured. The assay consisted of 0.5 ml analyte and 5.0 ml of PGO

Table 2. Lactose Solutions used for Kinetic Parameter Determination

Final[1] Lactose Conc. (mM)	Volume[2] Lactose Stock Sol'n (ml)	Volume[3] Buffer (ml)
18.52	0.53	7.37
27.78	0.80	7.10
37.04	1.07	6.83
55.56	1.60	6.30
74.08	2.13	5.77
111.12	3.20	4.70
138.9	4.00	3.90
185.2	5.33	2.57
260.4	7.5	0.40

[1]Not all lactose concentrations were used in each repetition. Total volume was 8 ml in 16 mm x 150 mm test tubes.

[2]Lactose stock solution was 277.78 mm prepared by dissolving 100 g lactose memohydrate in 1.01 l buffer.

[3]Volume of buffer was reduced 0.2 ml, and 0.2 ml 400 mM galactose solution added to yield a galactose concentration of 10 mM for inhibited cases.

Table 3. Kinetic Constants for *A. oryzae* β-galactosidase with Lactose

Assay Conditions			Constants		
pH	Temperature (°C)	Strain	V_{max} ($\mu g\ min^{-1} mg^{-1}$)	K_m (mM)	Reference
4.5	30	RT102	12.19	18	Tanaka et al. 1975
3.0	60	NS*	24	50	Park et al. 1979
4.8	37	YU22B	44	38	Ogushi et al. 1980
4.8	37	Y22	29	35	Ogushi et al. 1980

*Not specified.

enzyme-color reagent analytical solution. Since the PGO assay appeared to become non-linear above absorbance (OD) values of 0.8 (i.e. glucose concentrations of approximately 0.8 mM), experimental samples were diluted to hold the expected OD below 0.8. When the expected OD was below 0.8, the experimental solution was analyzed without dilution. In determining kinetic parameters, the maximum required dilution was 2:1. Results were analyzed using the WILKIN program.

Batch Conversion

The concentration of lactose in the batch conversion experiments was set at 138.9 mM (4.75 % w/v). This concentration is within the range of lactose concentrations observed in dairy products and is used to determine units of enzyme activity. Experimental solutions were prepared by diluting 10 ml stock lactose solution with an equal total volume of buffer and enzyme solutions. As in the kinetic parameter determinations, reaction tubes containing stock lactose and buffer were held in the constant temperature bath at least 10 minutes prior to adding enzyme. To initiate reaction, between 0.25 and 2.0 ml of 1.0 mg/ml stock enzyme solution was added to each tube yielding experimental lactase concentrations of 0.0125 to 0.1 mg/ml. Immediately following addition of enzyme each tube was vortexed and a sample withdrawn with a Pasteur pipet to serve as a blank control. Each tube was then returned to the constant temperature bath. Samples were withdrawn at 10, 30, 60, 120, and 240 minutes in experiments conducted in parallel with the kinetic parameter determinations.

Dilutions of up to 100:1 were required to hold glucose concentrations below 0.8 mM. Dilutions of 25:1, 50:1 and 100:1 were prepared using disposable micropipets to measure aliquots of the samples into

buffer measured with an Eppendorf Digital Pipet to yield an analyte volume of 0.5 ml assuming no volume change on dilution. Sample volumes for lesser dilutions were measured with the digital pipet.

Additional batch experiments were conducted in conjunction with the HFR operation described in Ch. 5. The volume of experimental solution was 40 ml in a 125 ml Erlenmeyer flasks with enzyme concentrations of 0.025 or 0.0125 mg/ml. Additional treatments were prepared with a buffer sans magnesium and manganese to test the effect of these cations on enzyme activity and a buffer containing 0.5 mg/ml BSA in 0.5 mg/ml enzyme stock solution to test the effect of this protein on enzyme activity.

Results and Discussion

The first attempts to determine the kinetic parameters of A. oryzae β-galactosidase yielded non-linear Lineweaver-Burk plots and values that did not adequately predict conversion in batch reactions. To identify the sources of these deviations, a series of short experiments were conducted to check the effects of enzyme concentration, time, and volume. The methods employed were identical to those used to determine kinetic parameters.

Use of the Michaelis-Menten equation and end point assays require that conversion be a linear function of enzyme concentration and time over the range of values assayed. As shown in Figure 4, a linear relationship holds for the plot of product versus enzyme concentration from 1.25 to 50 μg/ml β-galactosidase both with and without galactose in the reaction mixture. The data show no apparent trends toward non-linearity.

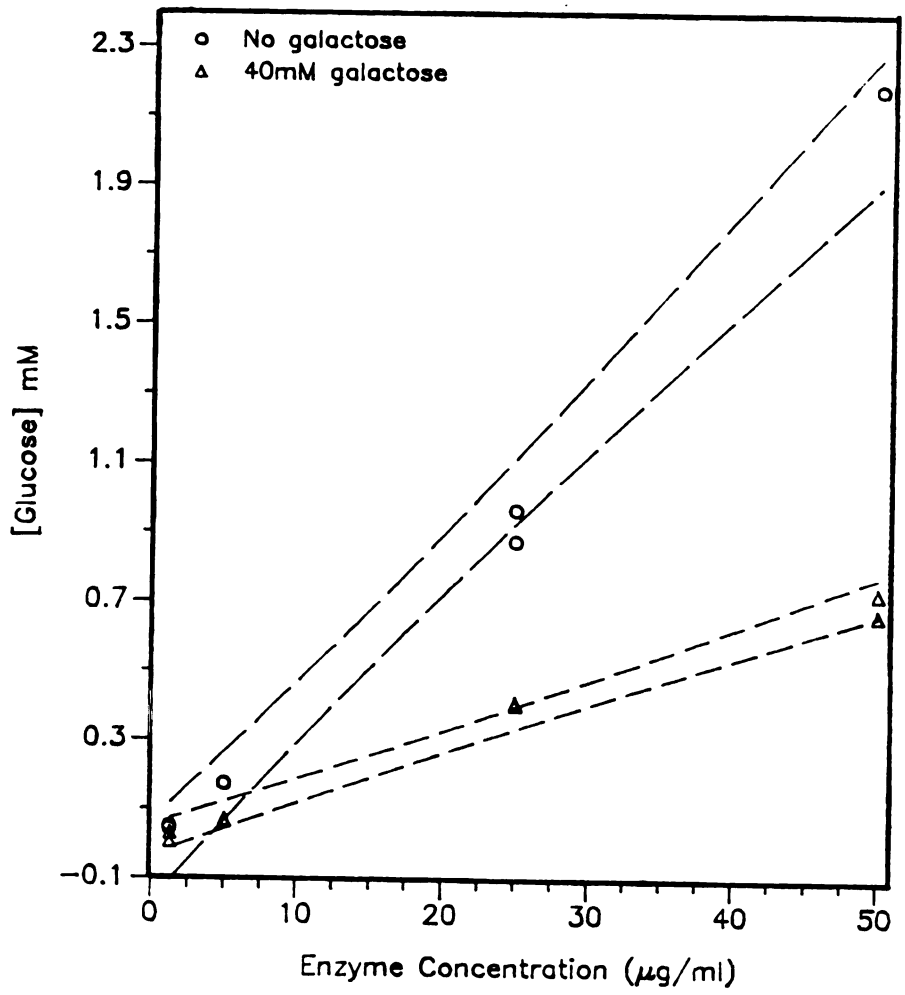

Figure 4. Product concentration versus enzyme concentration. Product concentration in 138.9 mM lactose following 5 minutes incubation with galactose and 2 minutes without galactose. Dashed lines indicate 0.95 confidence limits.

Figures 5 and 6 show that a linear relationship also holds between product concentration and time over the interval from 2 to 16 minutes. The greater than zero ordinate intercepts, however, demonstrate the necessity of carefully treating the blanks exactly as the experimental solutions. The intercept values indicate that product was appearing in the assay mixtures after the cessation of timing. Hypotheses to explain this phenomenon include: 1) the existence of a time interval after the solution was placed in boiling during which the enzyme was still active, and 2) non-enzymatic lactose hydrolysis resulting from heating in the boiling water bath.

To eliminate these potential sources of inaccuracy in subsequent assays, the procedures were changed so that each sample's residence time in the boiling water bath was carefully timed, and enzyme was added to the blank solutions. Implementation of these and other procedural refinements, described below, permitted the determination of kinetic constants usable for predicting batch results.

In addition to handling controls and experimental treatments in an analogous manner, other refinements in conducting the experiment included:

1. While apparent glucose levels increased with time in the boiling water bath, the rate appeared highly variable after 5 minutes. Thus the inactivation step for each tube was timed at 5 minutes.
2. Glucose oxidase also reacted with galactose, albeit at a far slower rate than with glucose. Analytes containing high galactose concentrations (i.e. those used to obtain K_{mapp} and V_{mapp} with inhibition), therefore, required blanks that had been developed the same amount of time as the experimental analyte.

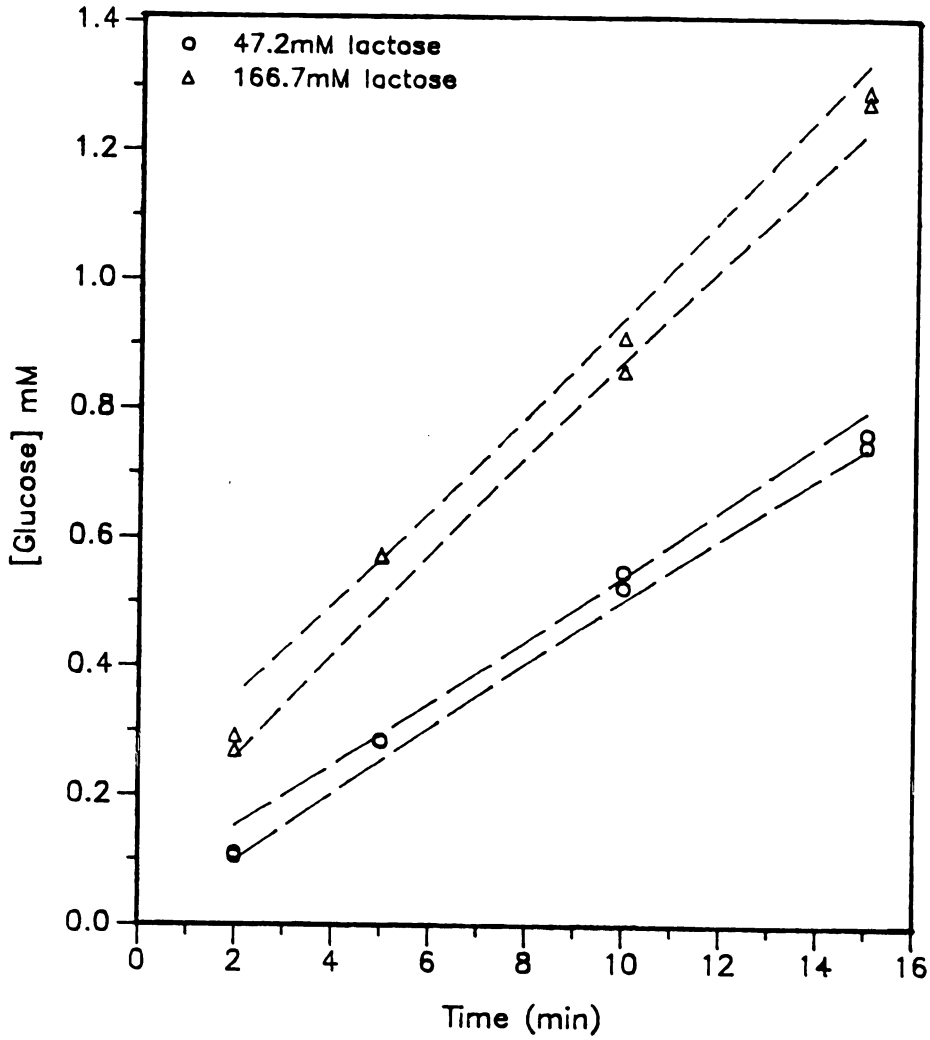

Figure 5. Product concentration versus time without galactose. Lactase concentration = 0.005 mg/ml. Dashed lines indicate 0.95 confidence limits.

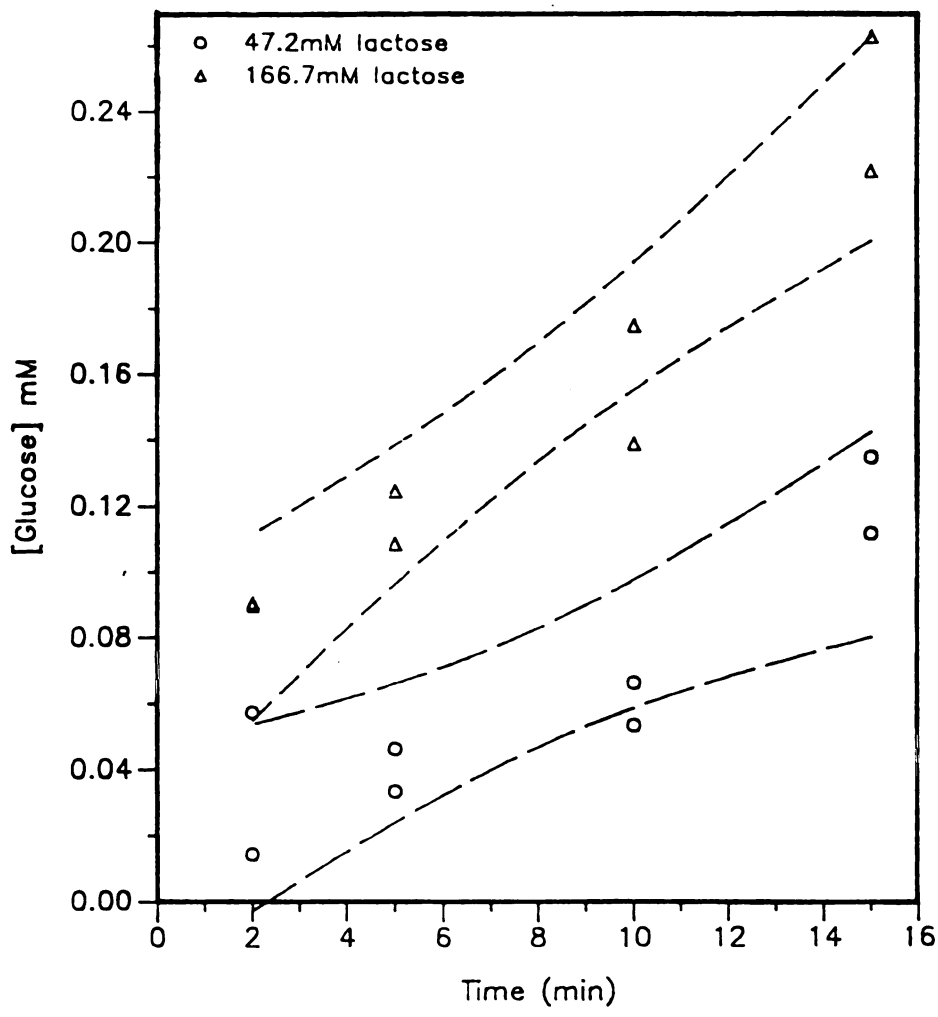

Figure 6. Product concentration versus time with 40 mM galactose. Lactase concentration = 0.005 mg/ml. Dashed lines indicate 0.95 confidence limits.

3. The response of the PGO analysis to glucose deviated unacceptably from linearity at high glucose concentrations (~1 mM). Thus analyte solutions were diluted to hold the expected glucose concentrations below 0.8 mM.
4. The apparent unit rate of enzymatic reaction decreased at low volumes of reaction mixture. Conversion of lactose in 10 ml experimental volume exceeded conversion in 0.5 ml by 45% (Table 4). The hypothesis that this might result from enzyme adsorption to glass was not supported by experiments using disposable polypropylene centrifuge tubes and Triton X-100 to reduce adsorption. Neither affected the rate of reaction. Also transferring 50 ml of stock enzyme solution (0.5 mg/ml) through a series of 5 glass flasks with 10 minutes residence time in each flask did not yield a significant reduction in enzyme activity. Thus adsorption to glass was not considered a likely explanation. The effect was ameliorated by increasing reaction volume to more than 5 ml., where no change in rate with volume was observed.

Kinetics Results

Double reciprocal plots of velocity versus substrate concentration show good linearity both without (Figure 7) and with (Figure 8) inhibition. Statistical analysis of the results by the WILKIN program yields the following mean parameter values with standard deviations:

No inhibition

$K_m = 153 \pm 7.4$ mM

$V_m = 51.2 \pm 1.4$ μMoles $mg^{-1}min^{-1}$

10 mM galactose

Table 4. Product Yields in Different Reaction Volumes

Volume of[1] Reaction Mixture (ml)	Composition[2] of Container	Mean[3] Product Concentration mM
0.5	glass	0.515
0.5	plastic	0.527
10	glass	0.745
10	plastic	0.769

[1]Reaction mixture final composition was 138.9 μM lactose, 0.005 mg/ml β-galactosidose in buffer.

[2]Glass containers were 16 mm x 150 mm pyrex test tubes for the 0.5 ml reaction volume and 25 mm x 200 mm for the 10 ml reaction volume. Plastic containers were 20 ml polypropylene disposable centrifuge tubes.

[3]N = 2

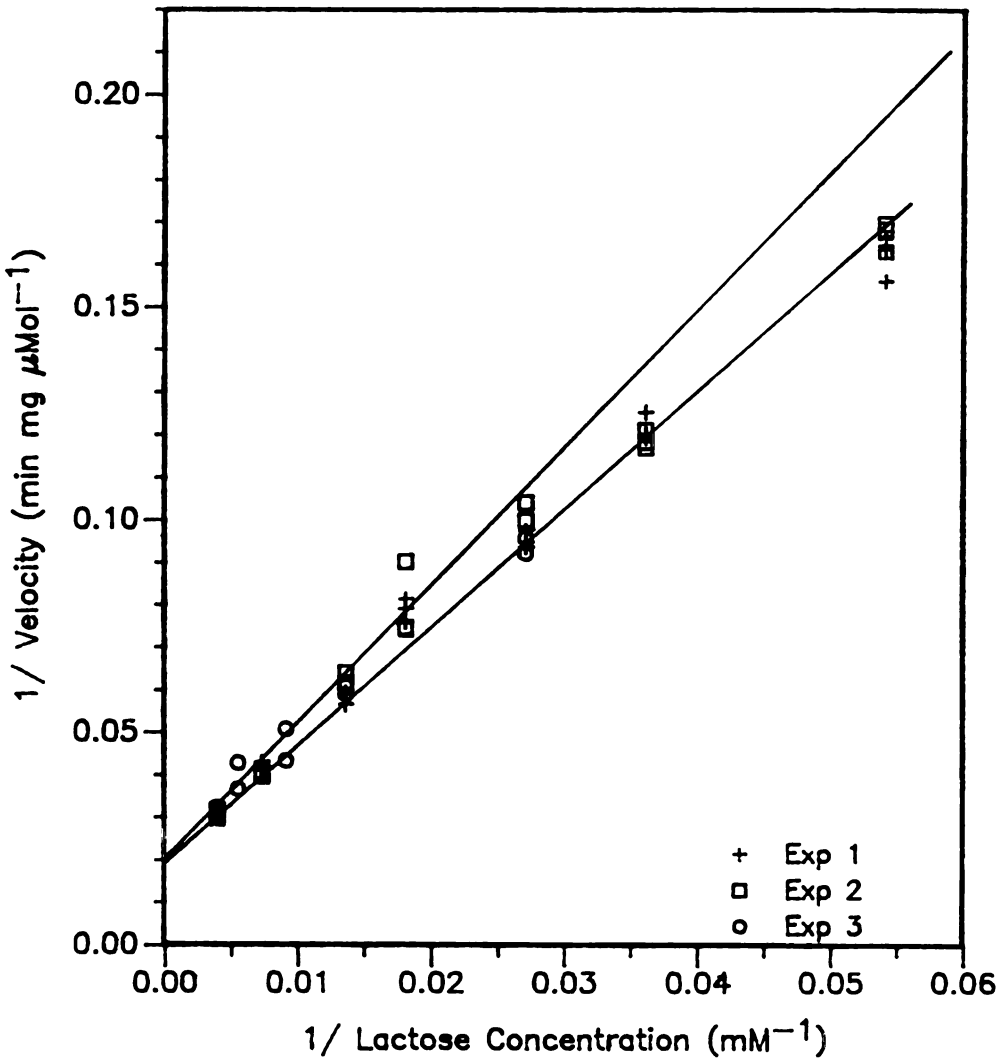

Figure 7. Double reciprocal plot of velocity versus substrate concentration without galactose. Lines indicate upper and lower standard deviation bounds predicted by K_m and V_m given in text.

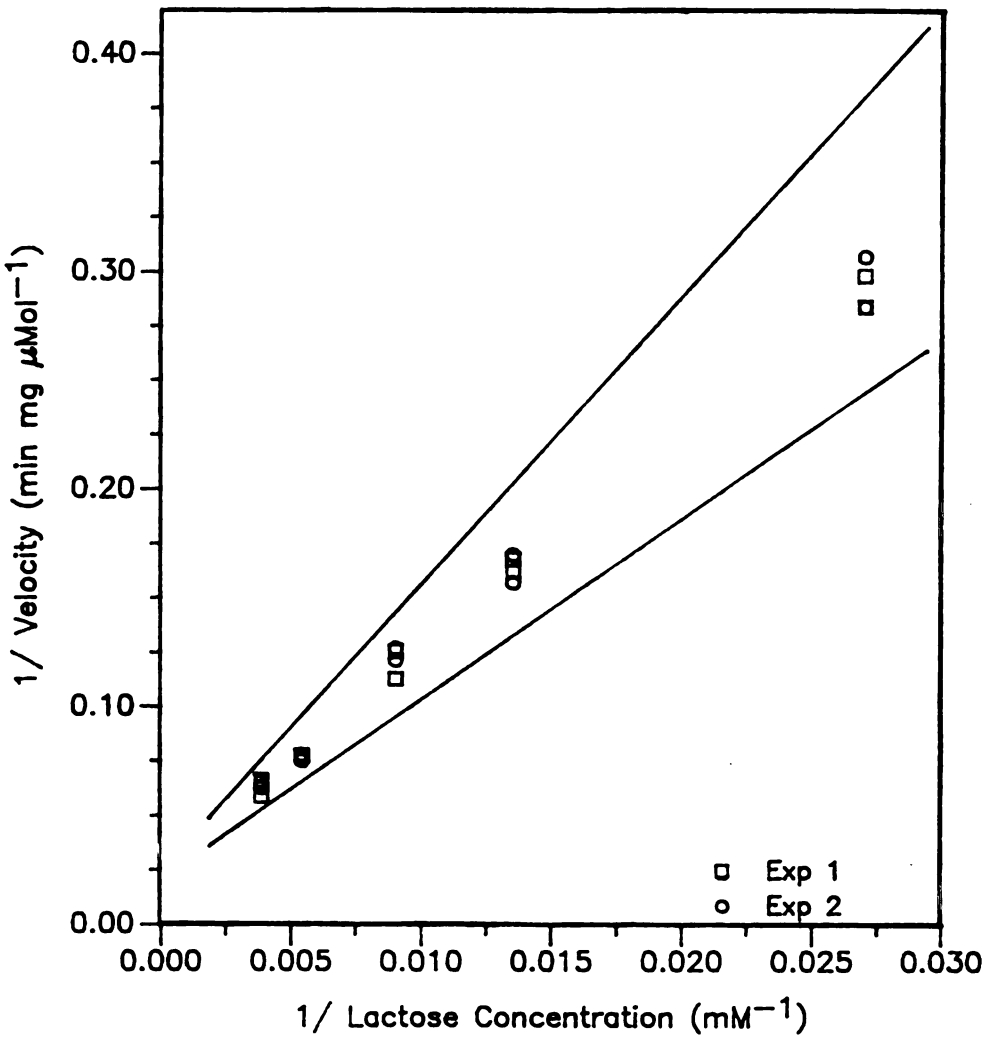

Figure 8. Double reciprocal plot of velocity versus substrate concentration with 10 mM galactose. Lines indicate upper and lower standard deviation bounds predicted by K_{mapp} and V_{mapp} given in text.

$K_{mapp} = 487 \pm 65$ mM

$V_{mapp} = 46.3 \pm 4.5$ μMoles $mg^{-1}min^{-1}$

The high value for K_{mapp} with galactose indicates that galactose strongly inhibits the enzymatic reaction. It also tends to increase the standard deviation of the K_{mapp} parameter, for kinetic parameter values may be most precisely determined statistically when substrate concentrations bracketing K_m are assayed. The low solubility of lactose prevents assay at higher concentrations.

Using the values reported above, Equations (19) and (20) yield competitive and uncompetitive inhibition constants in the following range:

K_u = 39 to infinity

K_c = 2.7 to 6.4

Since low values indicate higher levels of inhibition, and the values of K_m and substrate concentration are initially similar, the results of this determination suggest that competitive inhibition model may adequately describe product inhibition of A. oryzae lactase.

Propagation of Error

To check whether observed variability among product concentrations might result from recognized sources of error in measurement and stability, experimental procedures for determining kinetic parameters were subjected to a propagation of error analysis (see Appendix). Predicted standard deviations ranged from 17% of product at a low lactose concentration to 10% at a high concentration. Observed standard deviations were 19% and 12%, respectively. The largest estimated sources of error were pH, reaction timing, volume of enzyme solution in the reactor, and preparation and volume of standard solutions. Estimated

variance of the control blanks, a source of error not identified when the analysis was conducted, was obtained by calculating the variance for experimental blanks and was approximately 10^{-3}. Incorporation of this value slightly increases the predicted values above. The propagation of error analysis was conducted only for reaction without inhibition.

Batch Results

The program, KINDET, compared batch reaction conversions with predicted conversions at kinetic parameter values throughout the ranges obtained by analysis of the end point assay results. The best fit to the data for batch reaction with 0.0125 mg/ml (Figure 9) and 0.1 mg/ml (Figure 10) β-galactosidase was obtained where:

V_m = 55 μMol mg^{-1} min^{-1}

K_m = 153 mM

V_{mapp} = 55 μmol mg^{-1} min^{-1}

K_{mapp} = 500 mM

yielding K_c = 4.4 and K_u very large.

Thus a competitive inhibition model adequately describes the reaction of the enzyme.

The constants reported above also yield predictions that correspond well with the results of additional batch experiments (0.025 mg/ml enzyme)(Figures 11, 12, and 13) that were conducted to confirm the free enzyme reaction rate during reactor experiments (Chapter 5). The model predicts glucose concentration accurately for the first 90 minutes but diverges about 10% from actual conversions at 2 hours. While the parameters for the predicted curve are calculated from experimental data obtained in solution compositions similar to those of Figure 11, the addition of BSA (Figure 12) and exclusion of divalent cations (Figure

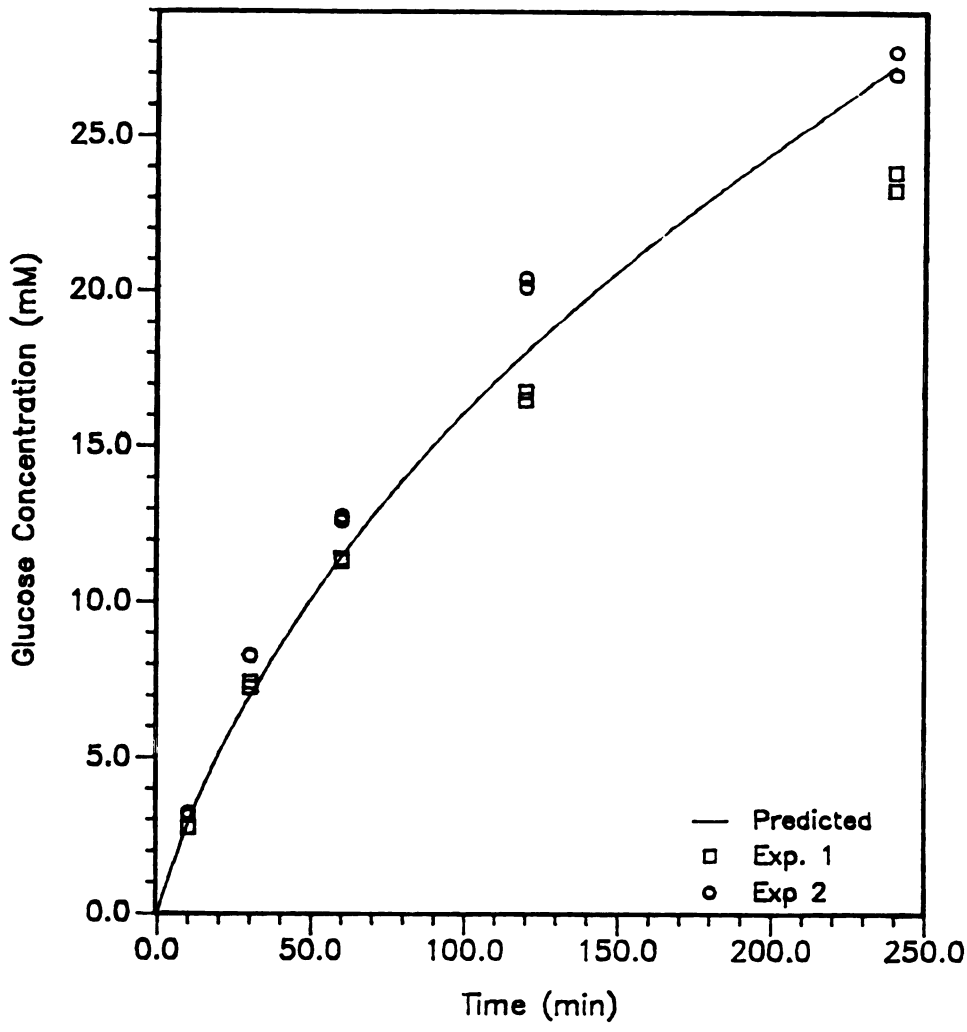

Figure 9. Product (glucose) concentration versus time with 0.0125 mg/ml β-galactosidase.

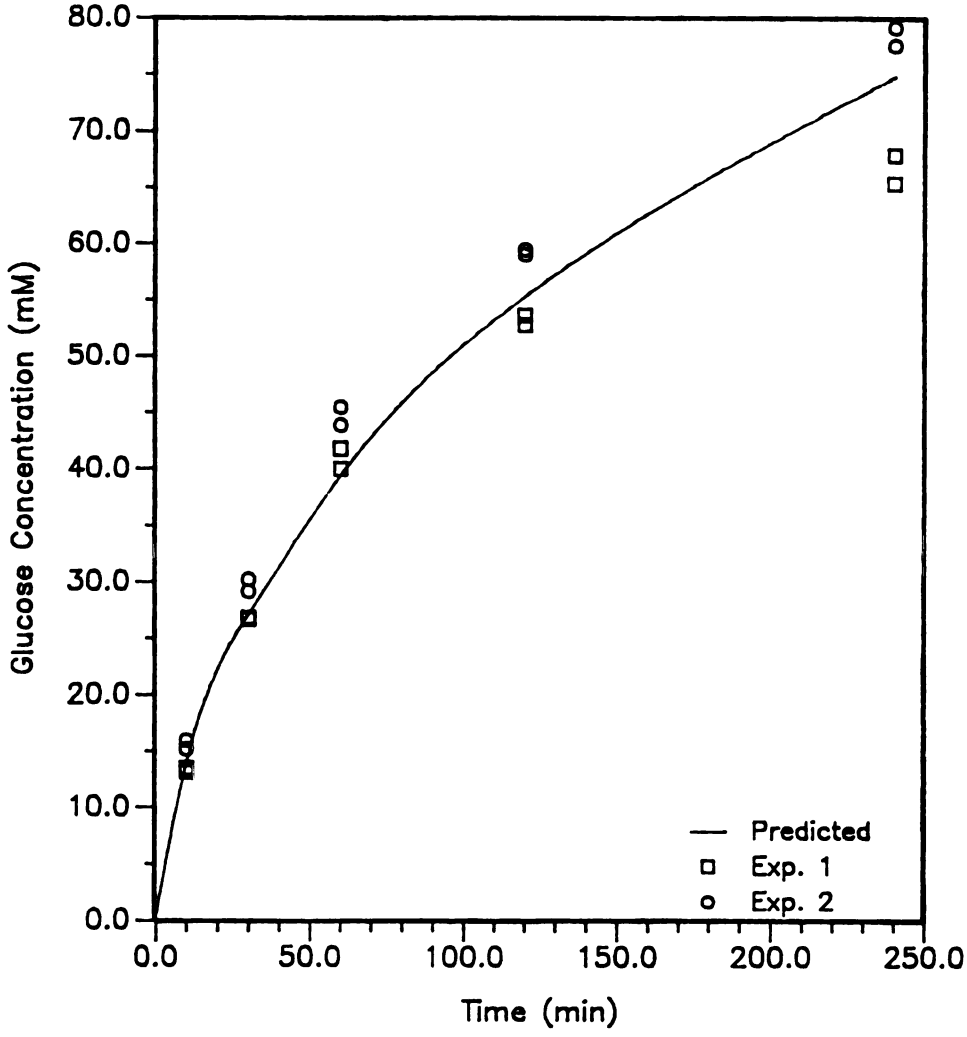

Figure 10. Product (glucose) concentration versus time with 0.1 mg/ml β-galactosidase.

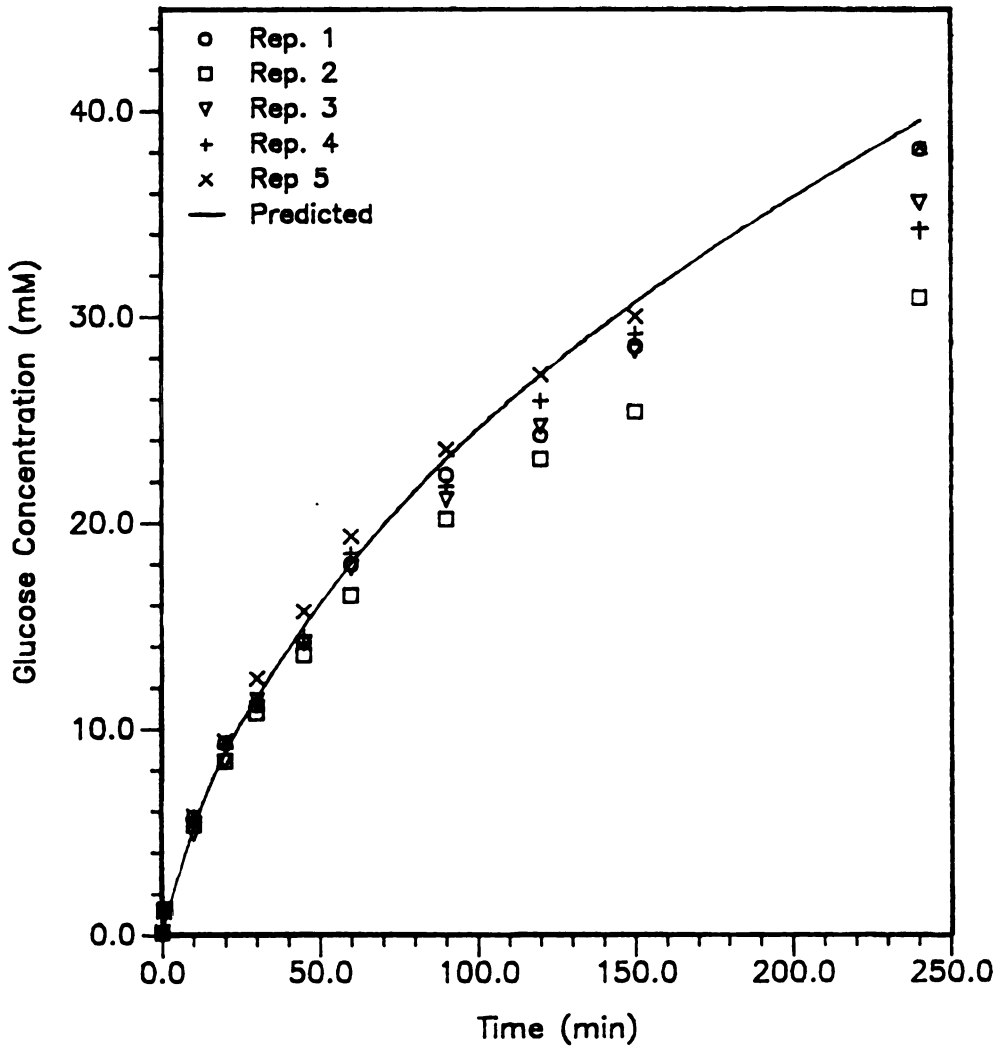

Figure 11. Product (glucose) concentration versus time with 0.025 mg/ml β-galactosidase where reactant solution contains magnesium and manganese, but no BSA.

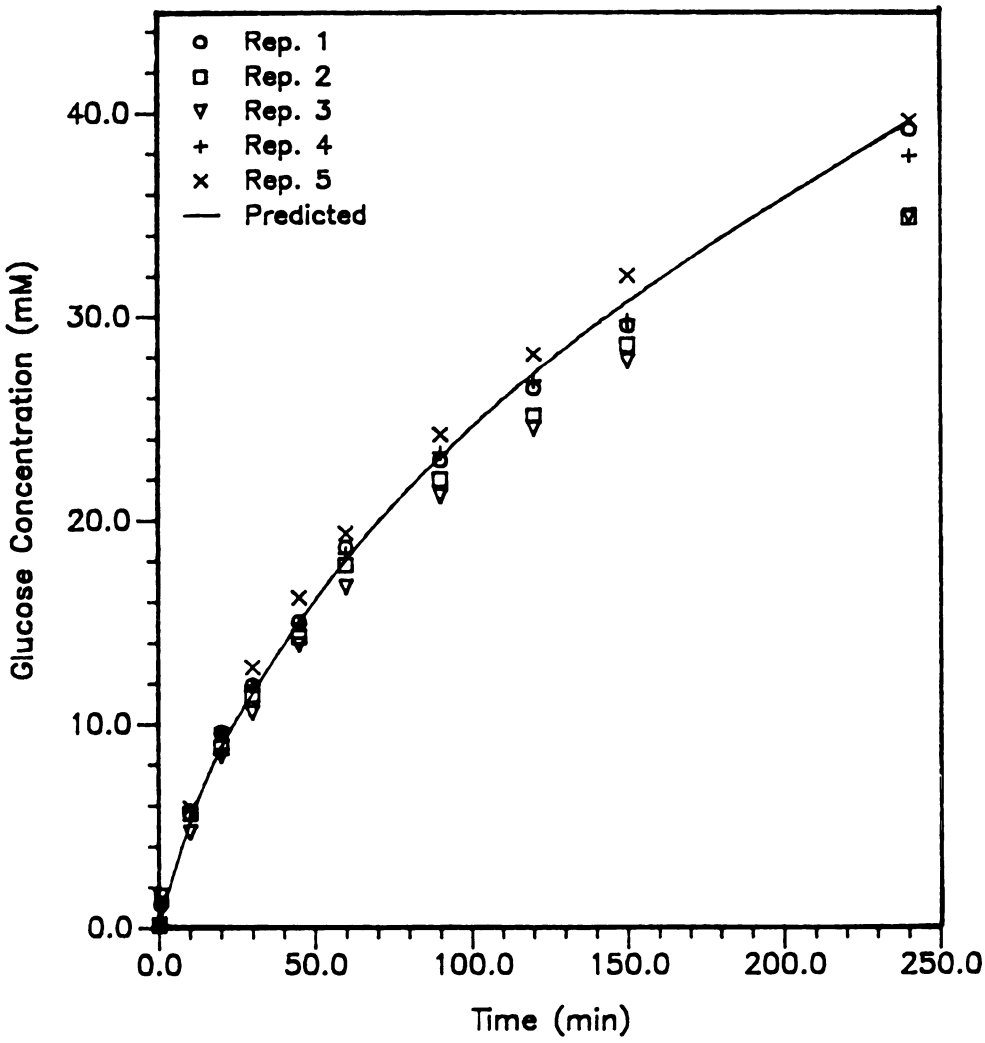

Figure 12. Product (glucose) concentration versus time with 0.025 mg/ml β-galactosidase where reactant solution contains BSA, magnesium, and manganese.

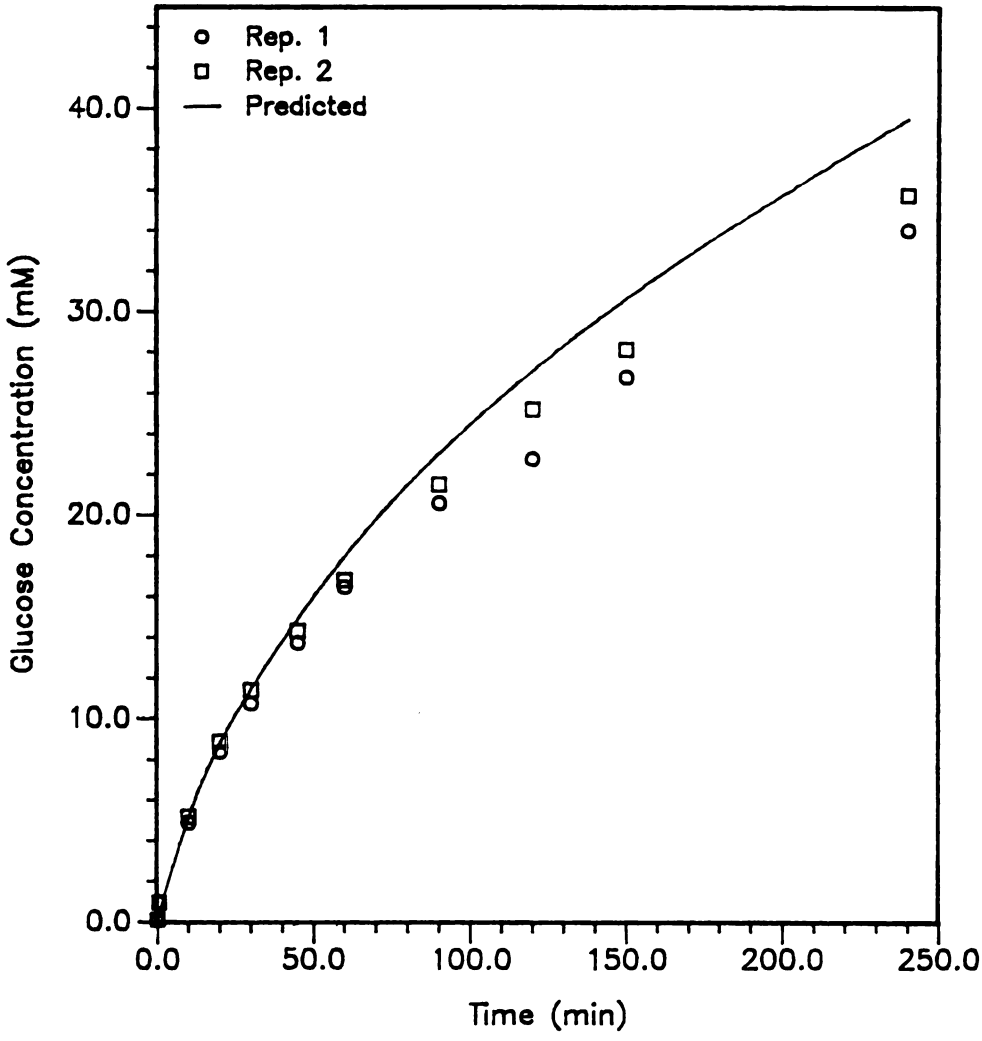

Figure 13. Product (glucose) concentration versus time with 0.025 mg/ml β-galactosidase where reactant solution contains no BSA, magnesium, and manganese.

13) do not substantially reduce the accuracy of the predicted values. Comparisons of simultaneous batch reactions also show no consistent differences in the rate of product appearance among three different solutions (Figure 14). The predicted rate of reaction (Equation 16) also corresponds with a first derivative plot of batch reactor data in solutions containing BSA (Figure 15).

A few batch experiments with 0.0125 mg/ml β-galactosidase were also conducted at the same time as the experiments described in Chapter 5. Product concentration and rate data from the first repetition of the batch experiment with 0.0125 mg/ml β-galactosidase and BSA did not correspond well with predicted values (Figures 16 and 17), possibly due to experimental errors. Subsequent repetitions, however, yielded data that better fit the predicted curves.

Since the above experiments were conducted under different temperature and pH regimes than previous studies (Table 3), V_m and K_m values differed from previously reported values. Variation of enzyme activity with pH may be described by a relationship analogous to Equation (16) (Laidler and Bunting 1975):

$$V = \frac{k_2 e_o c}{K_m\left(1+\frac{K_a}{[H^+]}+\frac{[H^+]}{K_b}\right)+c\left(1+\frac{K'_a}{[H^+]}+\frac{[H^+]}{K'_b}\right)} \qquad (25)$$

where $[H^+]$ is hydrogen ion concentration, and K_a, K_b, K'_a, K'_b are experimentally determined constants. Depending on the values of the various constants, pH variation may change the observed K_m, V_m, or both. Temperature effects on any of the constants that comprise K_m or V_m may be described by an Arrhenius relationship. The experiments described in this study were not designed to describe pH or temperature effects on

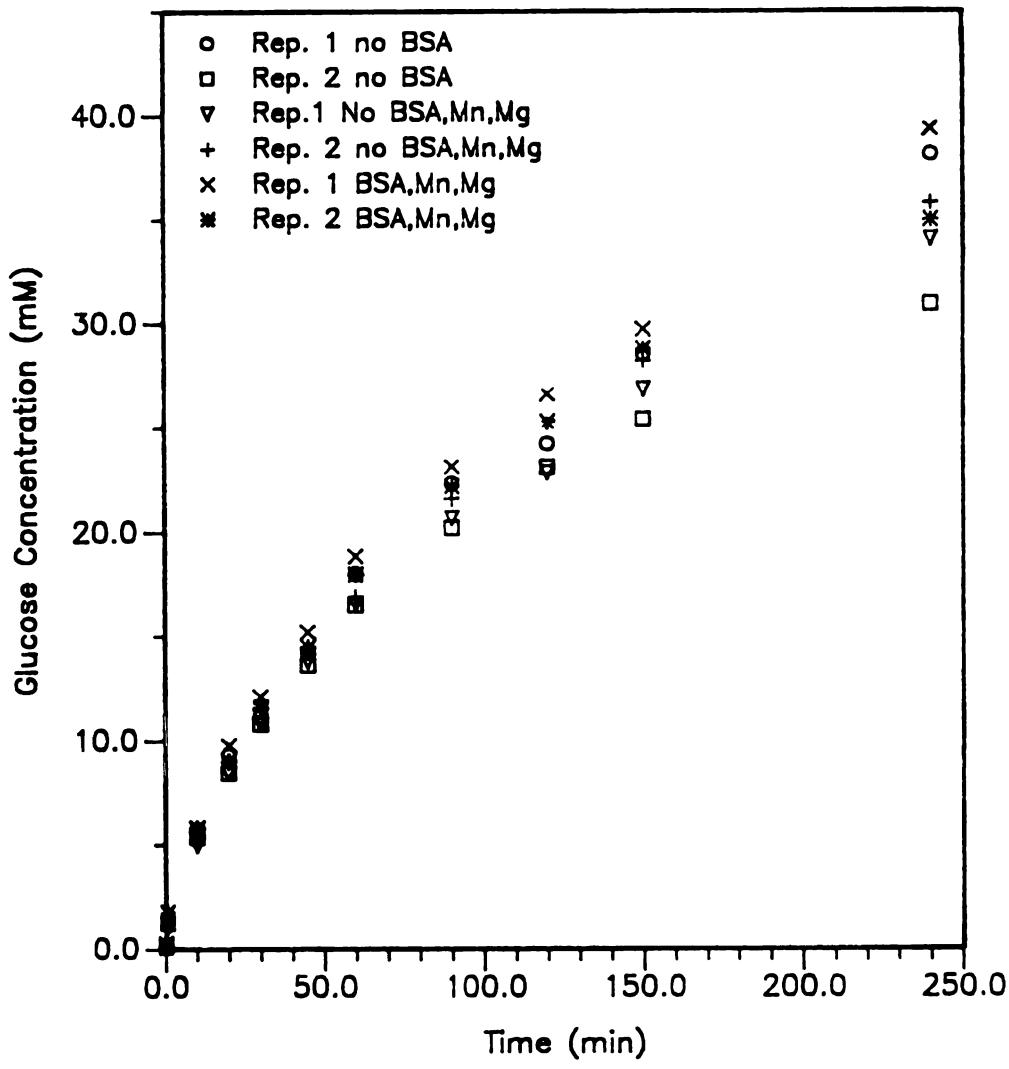

Figure 14. Comparisons of product (glucose) concentration versus time with 0.025 mg/ml β-galactosidase among reactant solutions with and without BSA and divalent cations.

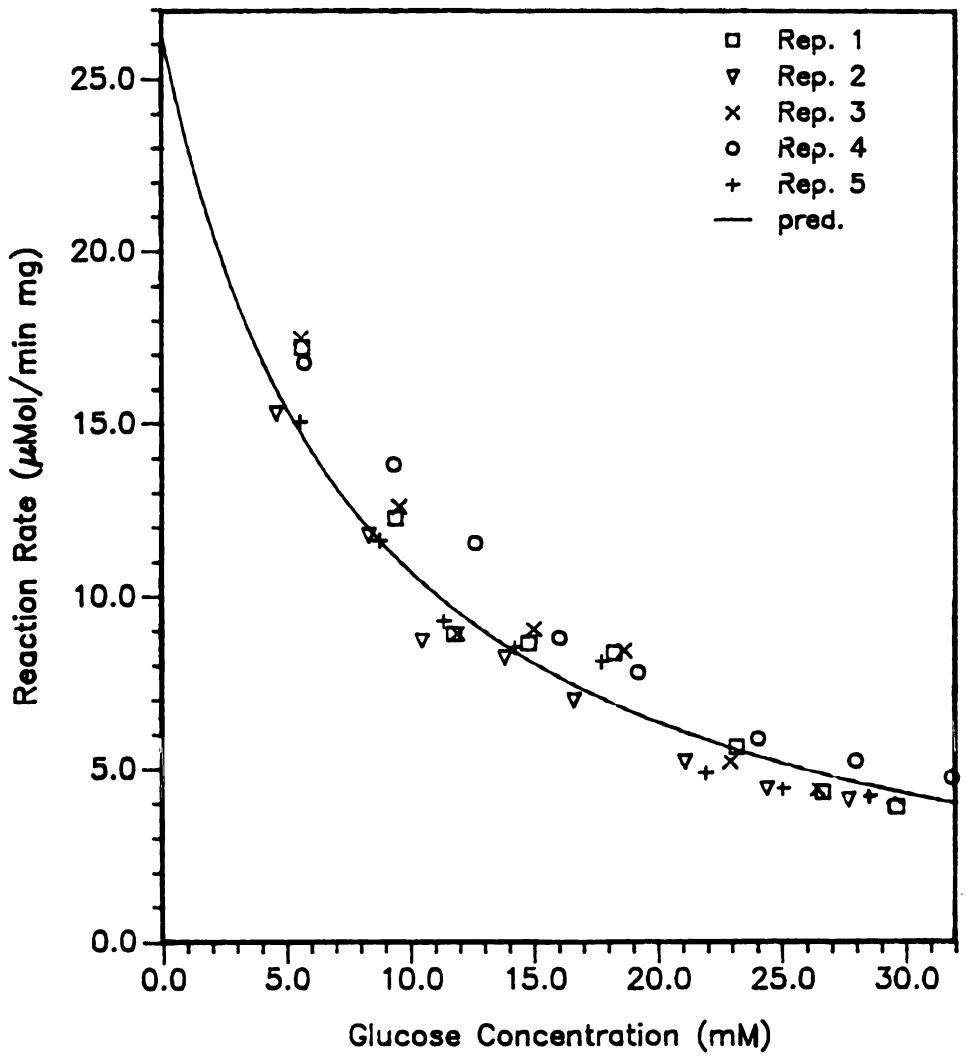

Figure 15. Calculated and predicted rates of reaction versus substrate concentration with 0.025 mg/ml β-galactosidase, with BSA.

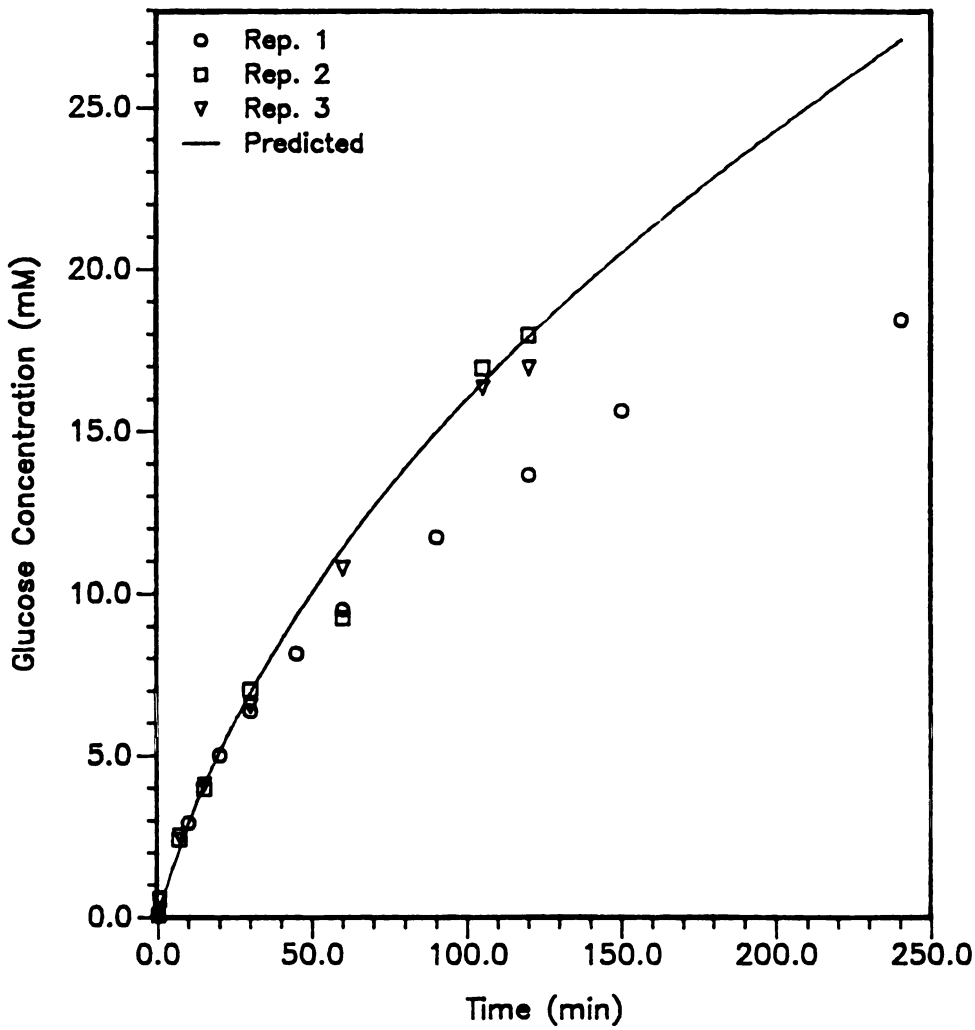

Figure 16. Product (glucose) concentration versus time with 0.0125 mg/ml β-galactosidase where reactant solution contains BSA, magnesium, and manganese.

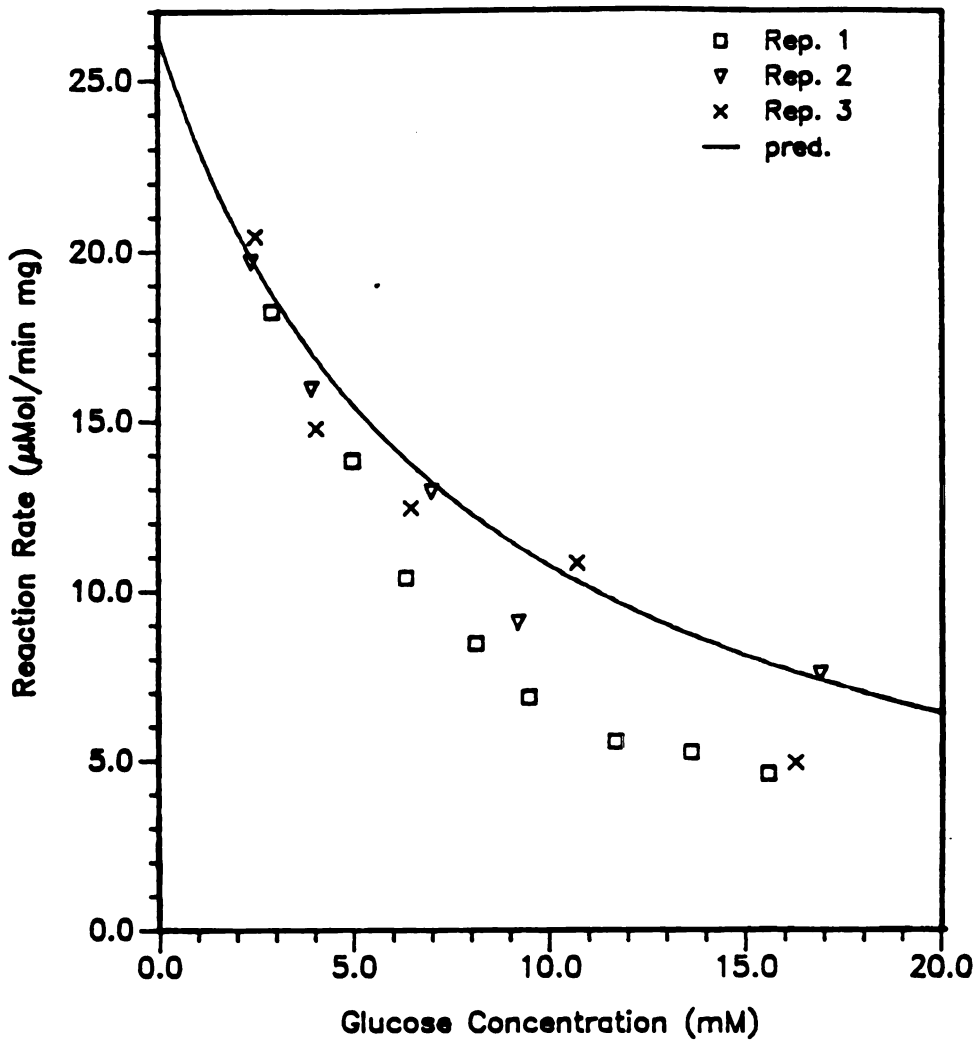

Figure 17. Calculated and predicted rates of reaction versus substrate concentration with 0.0125 mg/ml β-galactosidase.

the enzyme's kinetics. In addition to differences arising from assay conditions, enzyme purity and strain of A. oryzae may also have affected the kinetic parameters compared with previous studies.

A. oryzae β-galactosidase kinetic constants reduce the utility of the simplifying assumptions and analytical solutions for the models listed in Chapter 1. Since the reported K_m (153 mM) approximates the initial concentration of lactose in dairy products (140 mM), first order and zeroeth order approximations for the rate equation are not appropriate initially. None of the models consider radial distribution of product. Product distribution should be considered since the A. oryzae β-galactosidase reaction rate will vary with product concentration due to inhibition.

Strong galactose inhibition is a negative factor for commercial application of A. oryzae lactase in HFRs. With an initial substrate concentration of 140 mM, the rate of reaction for A. oryzae β-galactosidase drops from 26.3 μMol/min-mg initially to 0.64 μMol/min-mg at 70% conversion. The rate for an enzyme with the same K_m and V_m but no product inhibition drops to 11.9 μMol/min-mg at 70% conversion. Although galactose inhibition has been observed among lactases from various sources, the degree of inhibition for A. oryzae β-galactosidase is greater than for the other lactases that may be used in milk and sweet whey (Table 5). The relatively high values of K_c and low values of K_m for bacterial lactases indicate their reaction rates approach uninhibited rates over a broad range of conversions.

The relative insensitivity of A. oryzae β-galactosidase reaction to BSA and divalent cations may mitigate the disadvantage of product inhibition. A. oryzae lactase activity was assayed in the presence of BSA

Table 5. Kinetic Parameters for Lactases with Neutral pH Optima

Organism	T (°C)	Vmax (μmole / min mg)	Km (mM)	Ki[3] (mM)	Reference
S. fragilis	25	12.5[1]	9.5		Morisi et al. 1973
S. fragilis	23	1.9[2]	6.0	12.0	Korus and Olson 1973
S. lactis	25	15.5[1]	24.3	8.55	Forsman et al. 1979
S. thermophillus	37	295[2]	6.9	60	Greenberg and Mahoney 1982
Bacillus stearothermophilis	65		2.06	20	Griffiths and Muir 1978

[1]Assay using lactose in milk as substrate

[2]Assay using lactose in buffer as substrate

[3]Galactose competitive inhibition

primarily because BSA was used in immobilizing the enzyme. Other investigators have also found the A. oryzae enzyme relatively insensitive to a variety of common cations (Park et al. 1978, Miles 1978). On the other hand, magnesium and manganese have been reported as essential for maximum activity in lactases from yeast (Mahoney and Adamchuk 1980, Pastore and Morisi 1976), B. stearothermophillus (Griffiths and Muir 1978), and S. thermophillus (Greenberg and Mahoney 1982). Some other common cations also inhibit or enhance activity. Proteins also affect the activity of other lactases. Mahoney and Adamchuk (1980) found that heat labile whey proteins activate yeast lactases. Since milk and whey products vary in their ionic and protein compositions, the insensitivity of A. oryzae lactase to ionic and protein composition may enhance its usefulness in processing a variety of dairy products.

CHAPTER 3

RESIDENCE TIME DISTRIBUTION

Introduction

Modelling of HFR dynamics requires consideration of the actual residence time of substrate in the catalytic portion of the reactor. Since reaction occurs exclusively in the spongy matrix of hollow fibers, the bulk fluid residence time does not directly translate to residence time in the catalytic portion of the reactor.

To determine the mean residence time of substrate in the spongy matrix of the hollow fibers, a series of tracer experiments were performed. Differences between the residence time distribution (RTD) of the diffusing species, lactose, and that of the nondiffusing species, blue dextran, served as a measure of the mean residence time (τ_1) of lactose in the spongy matrix.

Materials and Methods

Measurement of outlet concentration change with time after imposing a step concentration change in lactose or blue dextran input to the cartridge yields an F distribution (Levenspiel 1972):

$$F = \frac{C}{C_o} \tag{26}$$

where C_o is the magnitude of the step change from a 0 inlet concentration and C is the outlet concentration. Integration of the data yields τ:

$$\tau = \int_o^\infty (1-F)dt \tag{27}$$

RTD and τ at four different flow rates were determined for both lactose and blue dextran.

Experimental

Reagents were analytical or reagent grade, except lactose (Sigma cat. #L3625), sucrose (Sigma cat. #S8501) and blue dextran (M.W. $2x10^6$, Sigma cat. #D5751). Spectrophotometric assays were performed using a Perkin Elmer, Lambda 3A UV/VIS instrument. All solutions were prepared in distilled water.

Hollow Fiber System

All experiments described in this chapter employed a UF cartridge (serial# 4PA106) donated by Romicon. The UF fibers (PA30) were composed of a polyamide with a nominal molecular weight cutoff of 30,000. Manufacturer's specifications list fiber dimensions at 1.12 mm inner diameter, 2.007 mm outer diameter, and thickness of the inner membrane between 0.1 and 1.0 μm. These values could not be verified without destroying the cartridge and were, therefore, assumed sufficiently accurate for the purposes of this study. The cartridge contained 68 fibers with an effective length of 39.4 cm and area of 1.0 ft^2. Specified upper limits for fiber operation were 55°C and 25 psig (transmural) across the membrane.

The cartridge was installed in a laboratory reactor system (Figure 18). The fluid conducting elements of the system consisted of Tygon tubing (3/16 in i.d.), with junctions and connections of polyethylene T, Y, and quick disconnect connectors. Pinch clamp valves at junctions determined the circulation pattern, and screw clamp valves were used to increase back-pressure. Both the reservoir and cartridge were held at 54.5+/-1°C in a glass tank by an immersion circulator-heater

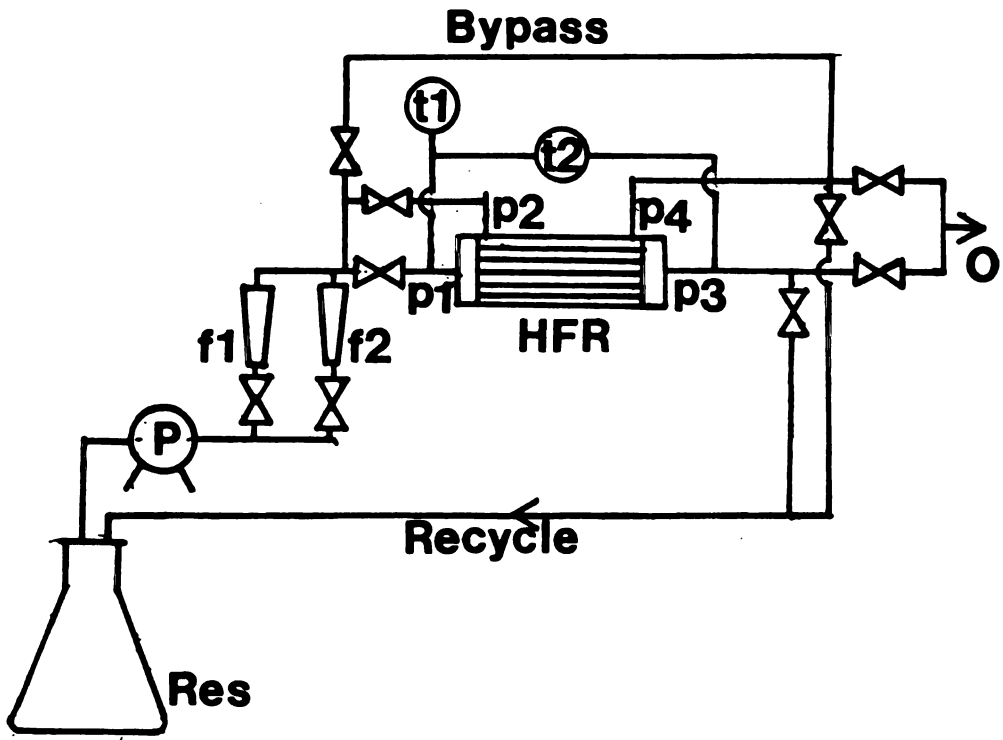

Figure 18. Laboratory hollow fiber reactor system.

Key: Res = Reservoir flask

P = Gear pump (Cole Parmer 7520-25 drive, micropump #8121 head)

f1,f2 = Flowmeters (Cole-Parmer FM044-40 and FM102-5)

p1 = Lumen side inlet port

p2 = Shell side inlet port

p3 = Lumen outlet port

p4 = Shell outlet port

t1 = 15 psig pressure transducer (Omega #px142-015 G5V)

t2 = 5 psi differential pressure tranducer (Omega #px142-005 D5V)

0 = Outlet/sample port

(Polyscience, Polytemp Model 73). Both the system tubing and tank were wrapped with foam insulation.

Before and after each use, the cartridge was cleaned and sanitized in accordance with the manufacturer's instructions. Cleaning consisted of three cycles: an acid cycle (pH 2-3, 0.045M H_3PO_4, 0.10M KH_2PO_4), a caustic cycle (1% NaOH), and a sanitizer cycle (200 ppm NaOCl). The protocol for each cycle was as follows: 1) pumping approximately two column volumes of washing fluid through the lumen side to the outlet port, 2) through the shell side to the outlet, 3) ultrafiltering and recirculating several column volumes (P1, P4, recycle). Cleaning often included backflushing acid and sanitizer solutions (P2, P4). Washing solution was also pumped through the bypass loop during each cycle. After each cycle all loops of the system were flushed with approximately two system volumes of distilled water.

During RTD experiments the shell side of the system, bypass loop, and recycle loop were all closed. The shell side of the reactor was drained of liquid except that which was retained in the spongy layer of the hollow fibers. Fluid was pumped from the reservoir through the lumen, then to the outlet port, where samples were collected. For these experiments, two fluid reservoirs were used. A valve permitted quick switching from one reservoir to the other. One reservoir contained 138.9 mM sucrose for equilibrating the system; the other contained the experimental solution used to impose the step change - either 138.9 mM lactose or 0.8 g/l blue dextran in 138.9 mM sucrose. Initial equilibration of the system was necessary to prevent flow of fluid from the spongy layer into the lumen. Sucrose was selected for initial equilibration of the tube side and spongy layer of the system as its molecular structure and diffusivity closely resemble lactose, and it is not detected by reducing sugar assays.

At the beginning of each experiment, the system was equilibrated by slowly pumping 500 ml sucrose solution through the lumen and out the outlet port. The system was then opened at a connector 20 cm upstream of the tube side inlet port. Experimental solution was pumped from the reservoir and exhausted at the connector to fill the system's tubing with the experimental solution up to that point. The step change was imposed by closing the connector, resuming flow through the lumen-side with experimental solution.

Blue dextran samples were collected at the outlet port at approximately 0.6, 0.8, 1.0, 1.2, 1.4, 1.6, 2.0, 2.4, and 3.0 bulk fluid residence times, calculated from the measured sucrose solution flow rate and estimated volume of the system downstream of the opened connector. Lactose effluent was sampled beyond three bulk fluid residence times. Volume of the system downstream of the the opened connector was estimated at 42.3 ml from the calculated 26.3 ml lumen volume of the fibers, the length and diameter of tubing, and estimated volume of cartridge fittings.

After the completion of sample collection, the exact flow rate (q) was determined by timing collection of fluid in a graduated cylinder. Since blue dextran in solution affected the rotameter reading and since reclosing the system at the connector changed the flow rate due to the back pressure from the cartridge and additional tubing, it was necessary to determine the flow rate with each repetition. The upper limit for flow was approximately 110 ml/min, set by the experimenter's ability to accurately time samples.

Blue dextran was assayed by spectrophotometry at 620 nm. Since the cuvettes required approximately 3 ml sample volumes, the reported point

concentrations of blue dextran in the outlet stream represent the concentration in a 5 ml sample collected over a period centered about the given time.

Lactose was assayed by the Park Johnson method for reducing sugars (Cooper 1977). Since the maximum concentration of lactose that may be accurately assayed by this method is 0.07 mM, samples were diluted 1750:1 in two steps. To compensate for the resulting variability in sample assays, each sample was assayed three times and the mean used to determine the F value. The quantity, 1-F, was integrated over time by the trapezoidal method.

Results and Discussion

Integration of the blue dextran F-distributions (Figure 19, 20, 21, and 22) yields values for the blue dextran mean residence times (τ_d) (Table 6). The RTD for blue dextran fits neither the simple plug flow nor CSTR models (Levenspiel 1972). Since calculation of Reynolds numbers in all sections of the system yields a maximum value of 440 for the experiments described in this section, a laminar flow regime describes the fully developed flow pattern throughout the system. Laminar flow without radial dispersion would have yielded F values of approximately 0.5 at one τ_d. The mean residence time for blue dextran, however, consistently coincides with an F value of approximately 0.62 indicating some degree of radial dispersion. Likely radial dispersion mechanisms include diffusion and mixing in discontinuities at connections and in the end caps of the columns. Since radial dispersion due to diffusion should decrease with increasing flow rate, mixing is probably the more important mechanism in this case.

At all flow rates, the mean value for the apparent volume ($q \times \tau_d$) is 45.8 +/- 2.5 ml, approximately the calculated volume of the system.

Table 6. Integral Results of Residence Time Distribution Experiments with Blue Dextran

q (ml/min)	$\bar{\tau}_D$ [1] (sec)	q x $\bar{\tau}$ (ml)
15.5	156.5	40.5
15.2	182.8	46.3
31.8	87.2	46.2
31.8	89.6	47.5
59.5	47.1	46.8
55.8	48.8	45.4
99.2	29.0	48.0

[1] $\bar{\tau}_D$ = mean residence time for blue dextran.

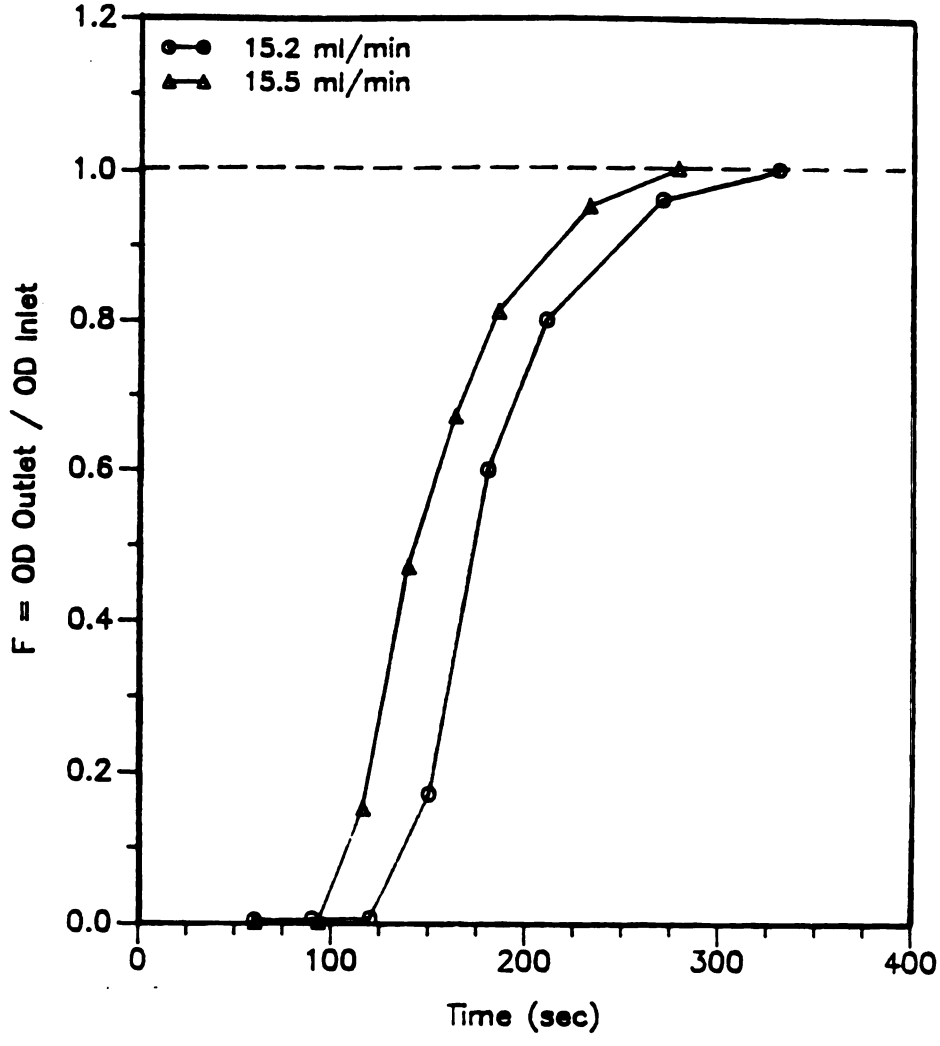

Figure 19. F distribution following step input of blue dextran at flow rate of approximately 15 ml/min.

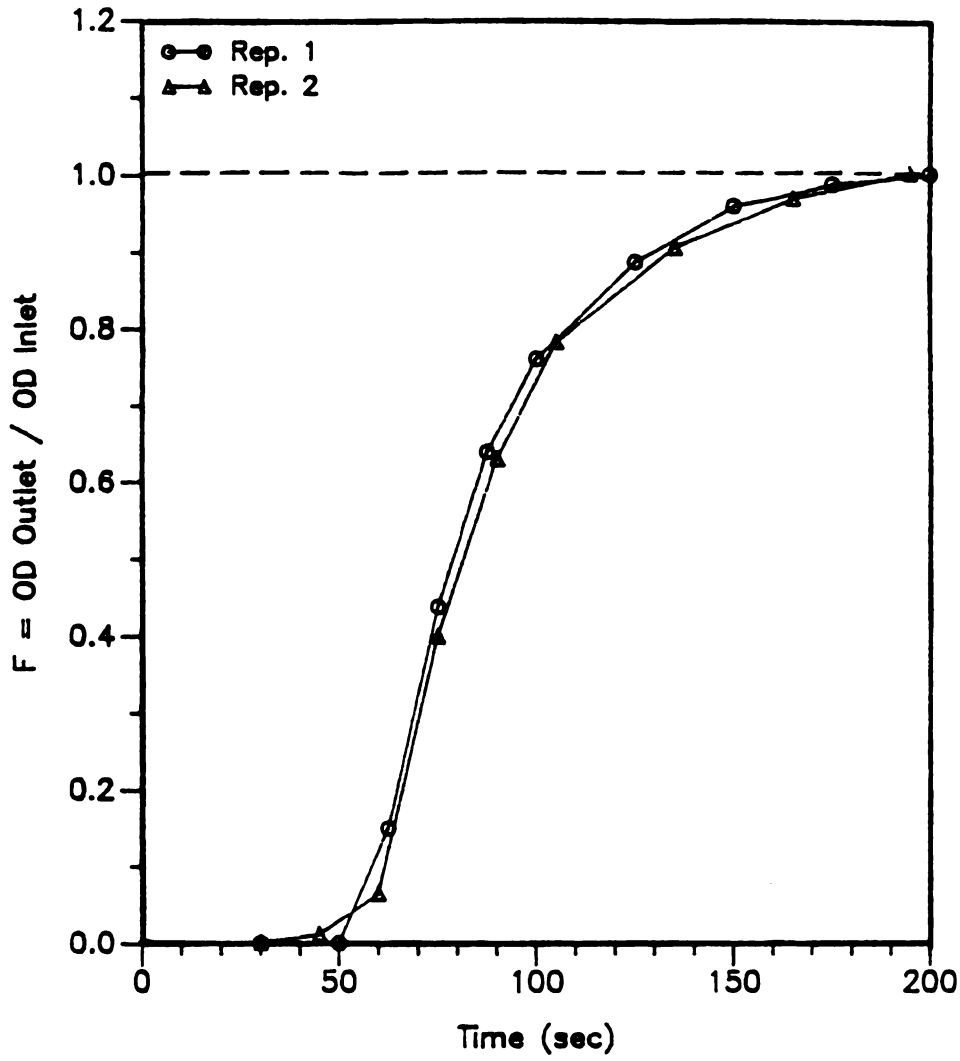

Figure 20. F distribution following step input of blue dextran at flow rate of approximately 32 ml/min.

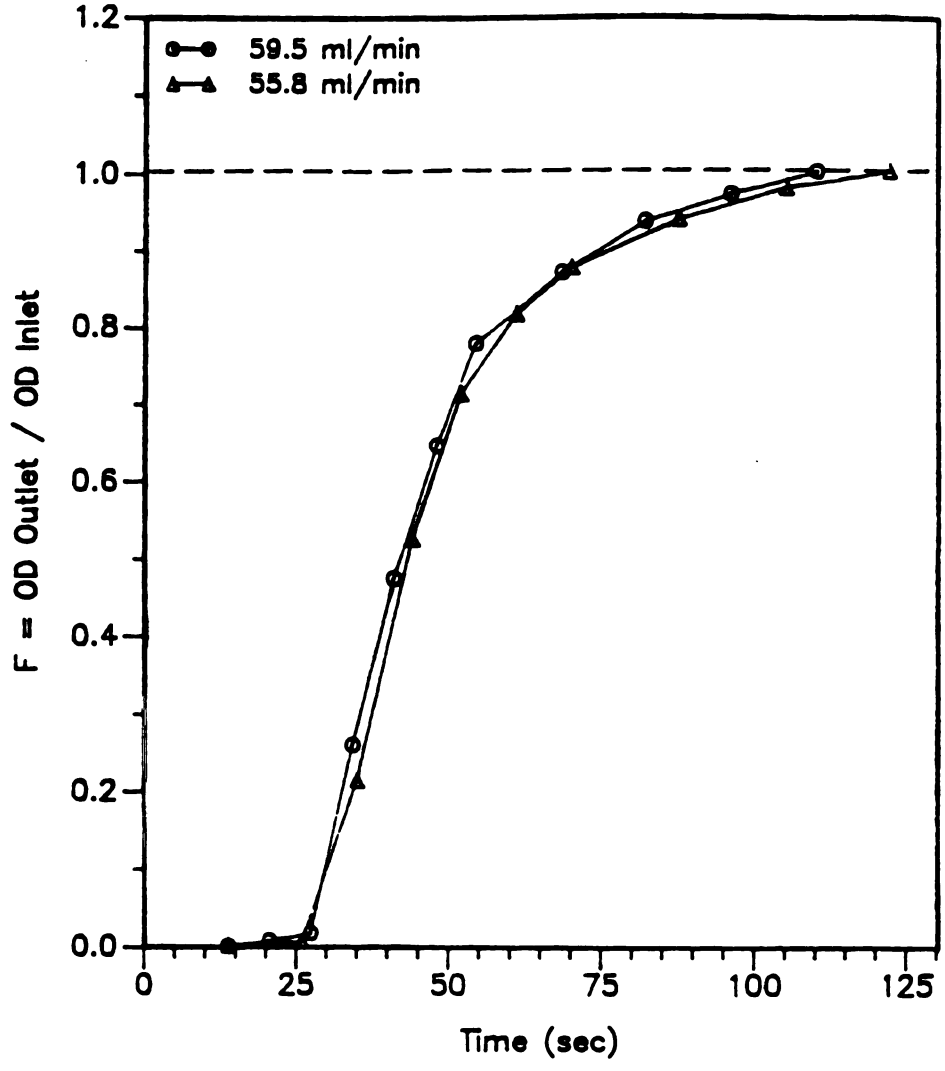

Figure 21. F distribution following step input of blue dextran at flow rate of approximately 55 ml/min.

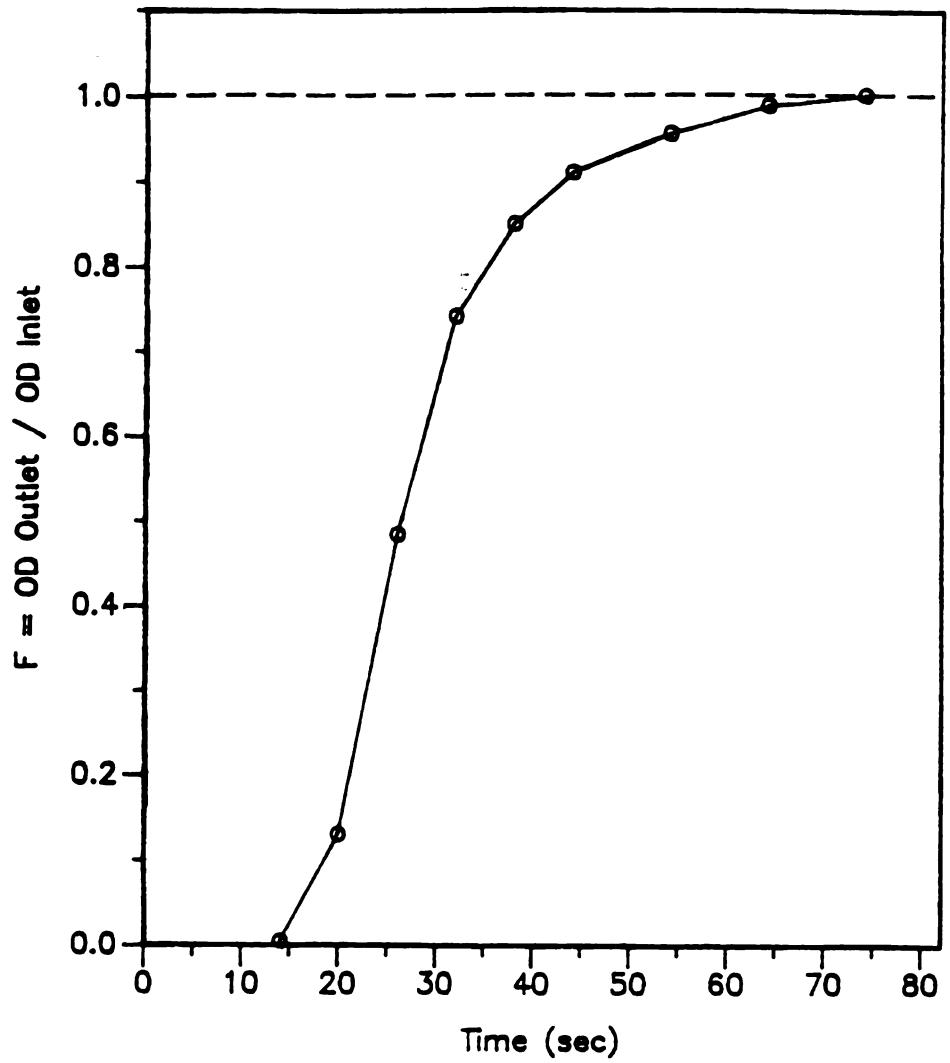

Figure 22. F distribution following step input of blue dextran at flow rate of 99.2 ml/min.

Thus, although conducted at different flow rates, comparison of lactose and dextran RTD experimental results is convenient since expected τ_d is easily calculated. Blue dextran mean residence time is assumed to represent bulk fluid mean residence time in calculating the dimensionless time described below.

Lactose RTD data is plotted versus a dimensionless time, sampling time divided by predicted τ_d (Figures 23, 24, 25, and 26). Despite repetitions of concentration determinations, considerable noise appears in assay results as F approaches 1. Integration of the mean values for each repetition yields mean residence times for lactose (τ_ℓ) (Table 7). The apparent volume ($q \times \tau_\ell$) consistently exceeds the apparent volume for blue dextran. The increased apparent volume for lactose is believed to result from diffusion into the spongy layer.

As q increases, the apparent volume decreases and appears to approach the volume predicted from $q \times \tau_d$ (Table 6). If lactose diffusion across the ultrafiltration membrane is primarily responsible for lactose and blue dextran RTD differences, T values over 1 (Table 7) reflect the lactose residence in the spongy layer:

$$T = \frac{\tau_\ell - (V_E/q)}{V_L/q} \qquad (28)$$

where V_E = volume of system not including fibers, and V_L = lumen volume of fibers. Excluding one anomalous value, values for T fit the curve, shown in Figure 27 ($r^2 = 0.93$):

$$T = 1 + 2.409\ e^{-1.115\ q/N} \qquad (29)$$

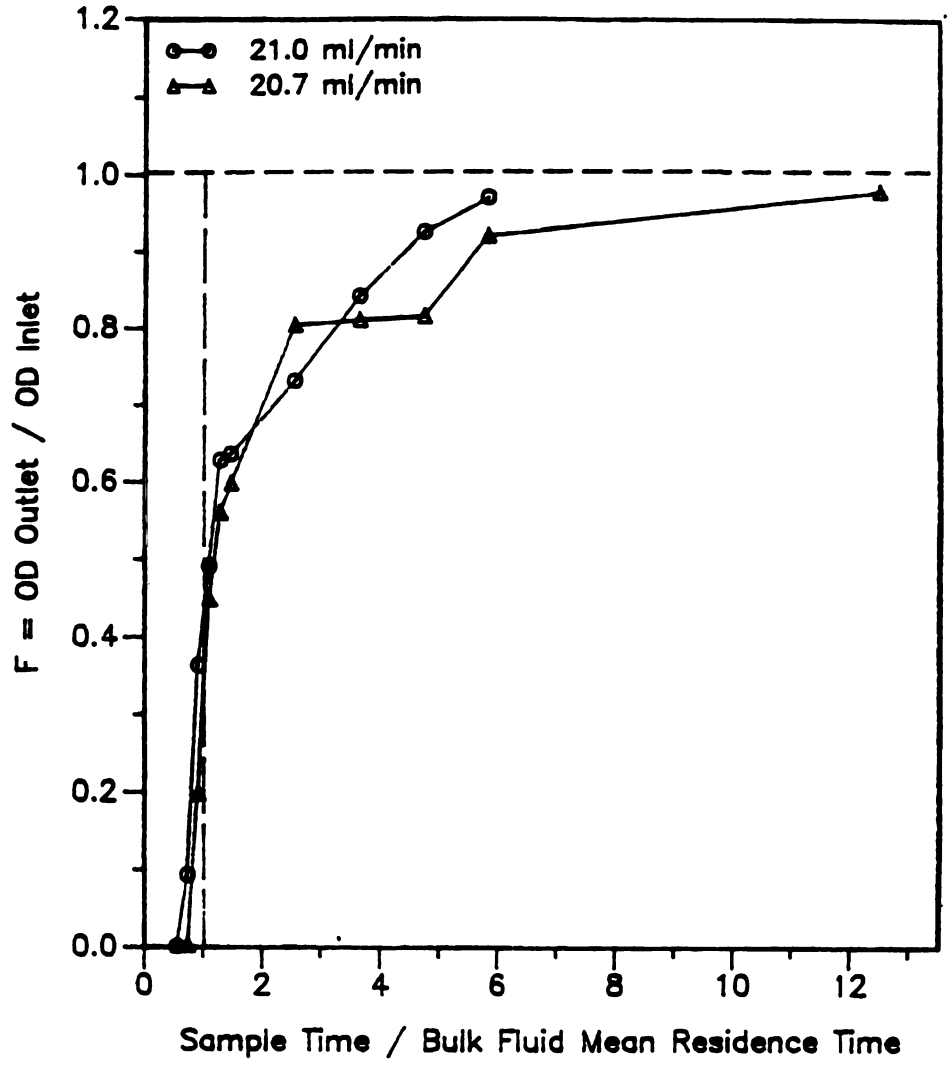

Figure 23. F distribution following step input of lactose at flow rate of approximately 20 ml/min.

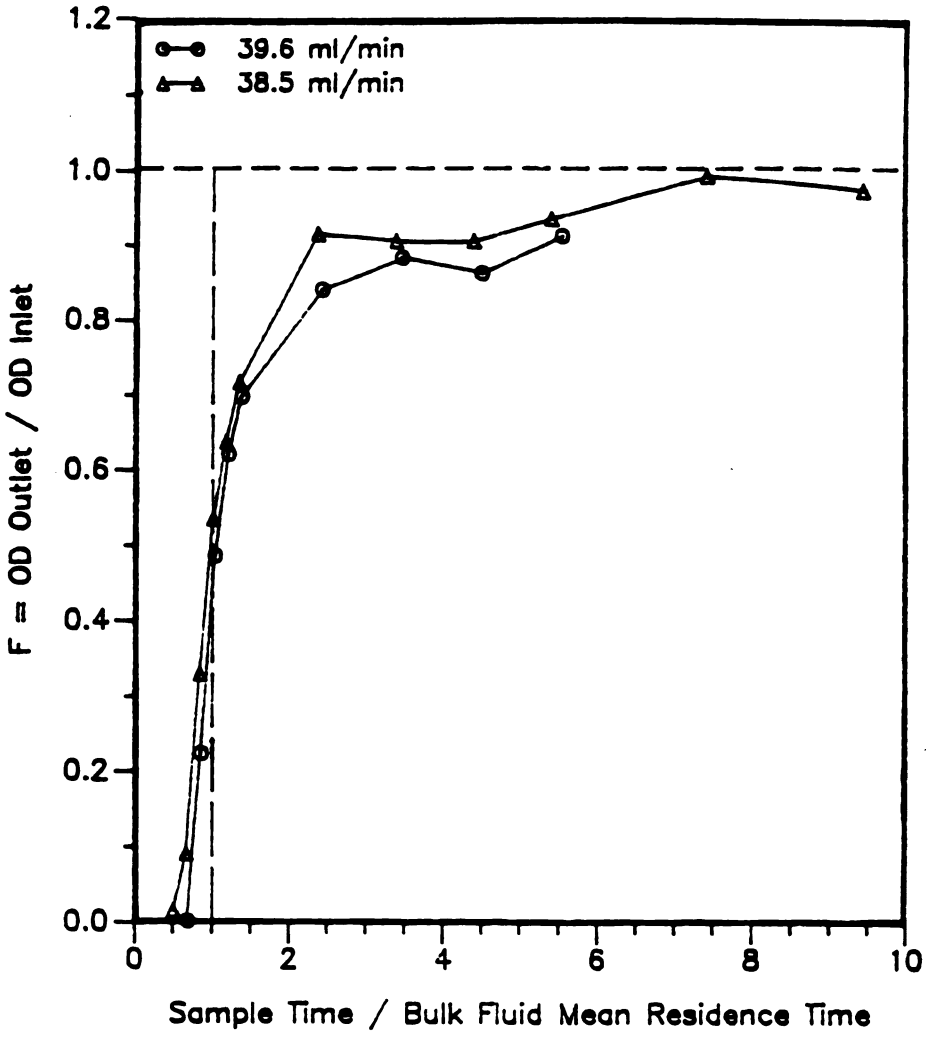

Figure 24. F distribution following step input of lactose at flow rate of approximately 40 ml/min.

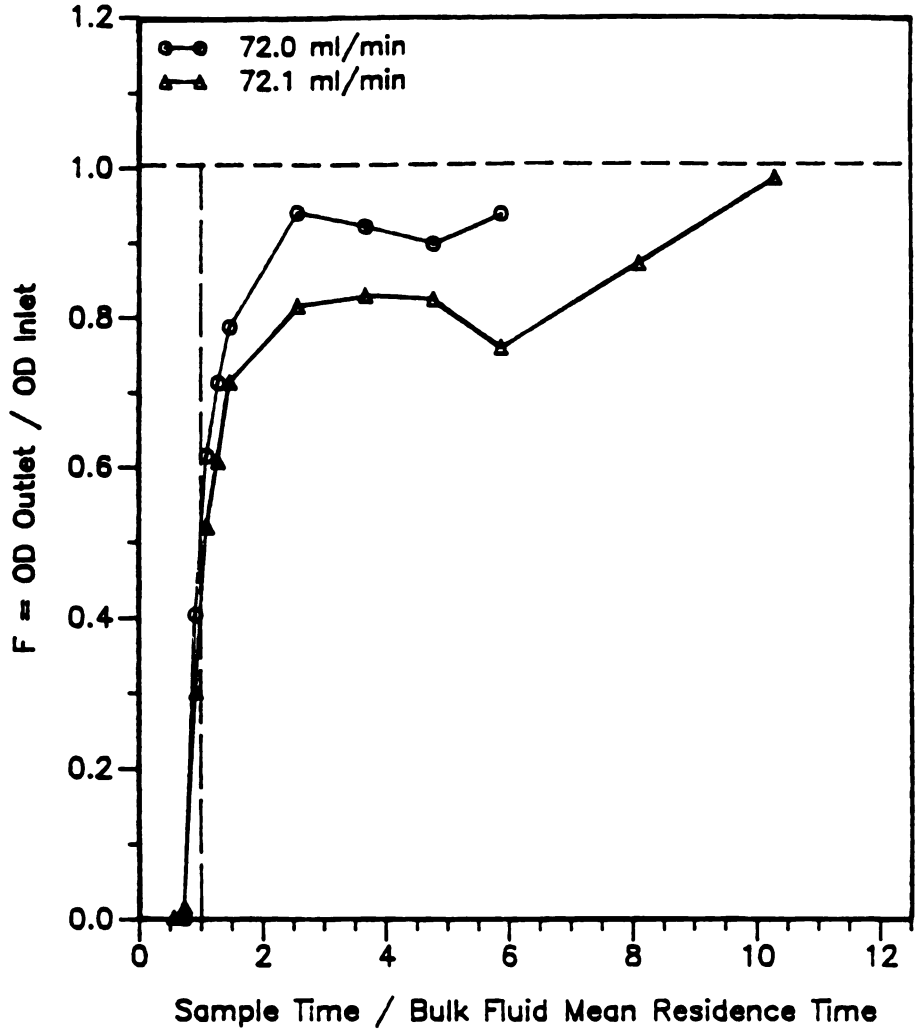

Figure 25. F distribution following step input of lactose at flow rate of approximately 72 ml/min.

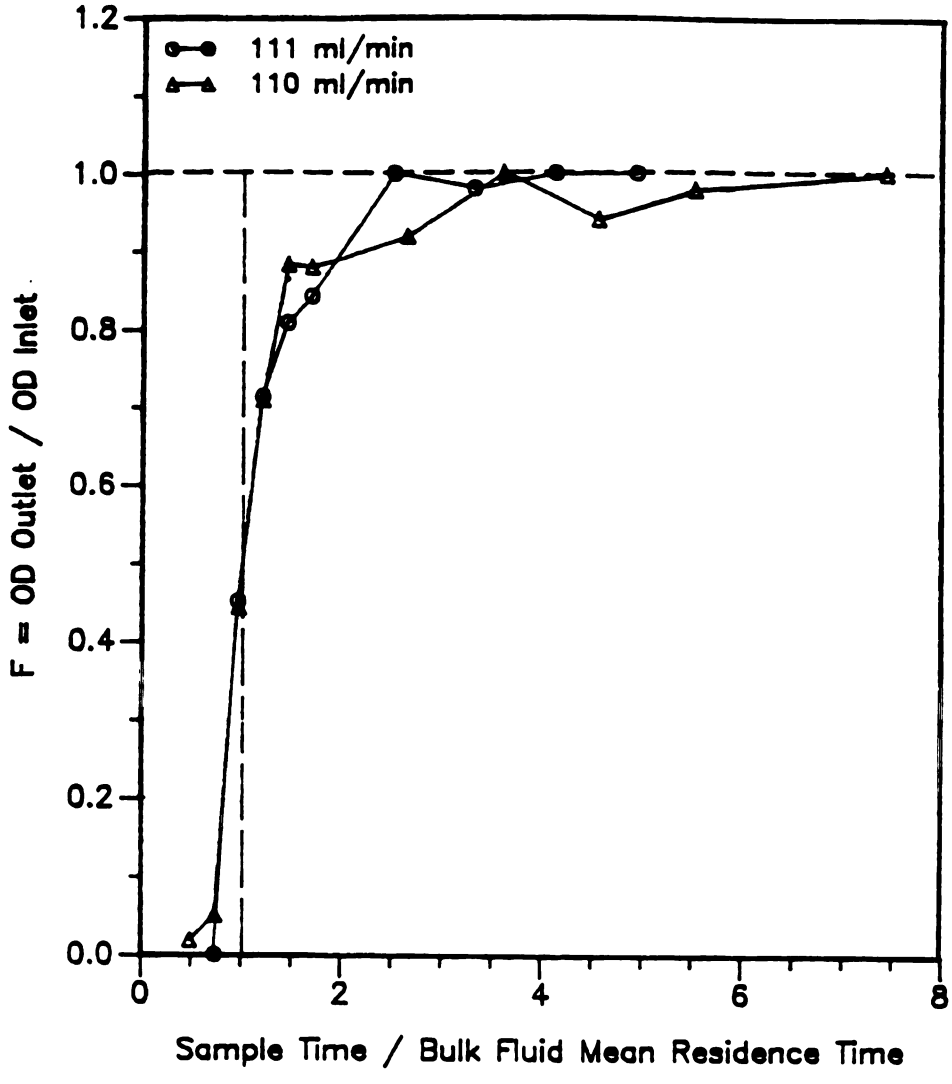

Figure 26. F distribution following step input of lactose at flow rate of approximately 110 ml/min.

Table 7. Integrated Results of Residence Time Distribution Experiments with Lactose

q ml/min	$\bar{\tau}_\ell$[1] sec	$q \times \bar{\tau}_\ell$ (cm^3)	T[2]
20.7	288.7	99.7	3.05
21.0	247.6	86.6	2.55
38.5	113.8	73.1	2.04
39.6	120.1	79.1	2.27
72.0	57.3	68.8	1.87
72.1	99.2	119.3	3.78
109.8	31.8	58.3	1.47
111.1	247.6	53.8	1.31

[1] $\bar{\tau}_\ell$ = Lactose residence time in system.

[2] T = Ratio of lactose mean residence time in the lumen of the hollow fibers to the dextran mean residence time in the fiber lumen, assuming blue dextran equals bulk fluid residence time in fibers. Equation 28 in text.

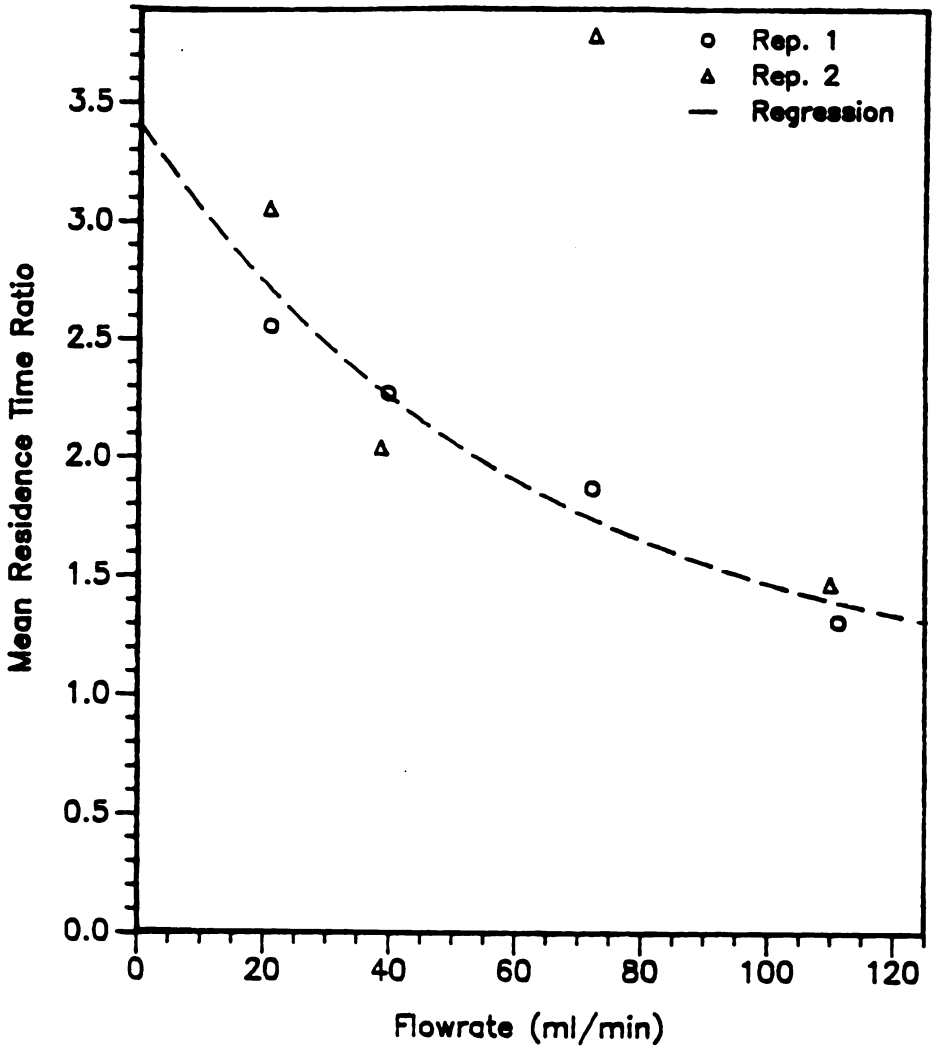

Figure 27. Ratio of lactose mean residence time in hollow fiber cartridge to bulk fluid mean residence time versus flow rate.

where N is the number of fibers. Equation (29) predicts residence time ratios for lactose to bulk fluid for fibers with radial dimensions and permeability identical to the PA30 fibers.

The asymptotic values for T approximate the values that are expected from a physical model of the system. As q approaches 0, T approaches 3.4; this compares with the ratio of total fiber volume to lumen volume. Although the spongy layer void fraction is not specified, the manufacturer states that it is large. Thus this asymptotic volume prediction corresponds fairly well with the actual volume of the fiber.

As q increases, T approaches 1. As r_ℓ approaches r_d, lactose spends relatively less time in the catalytic portion of the reactor, i.e. the rate of axial convective mass transfer in the lumen increases relative to radial diffusive mass transfer. Thus less conversion may be expected over a specified reactor length, given a constant reaction rate. The apparent rate of reaction may, however, increase with flow rate if lumen-side resistances significantly impede the reaction. More rapid flow rates will tend to reduce lumen-side resistance, and may increase conversion at a given residence time.

The recommended minimum flow rate through the lumen of a hollow fiber cartridge, identical to the one used in these experiments, is 750 ml per minute to prevent fouling. This is well above the maximum flow rate employed in this study. If the observed relationship for T holds at higher flow rates, the mean residence time for lactose in the spongy layer is 1.1×10^{-5} times the bulk fluid residence time. This prediction indicates very short residence times for lactose in the catalytic portion of the reactor.

CHAPTER 4

ENZYME RETENTION

Introduction

Backflush loading was selected as the most efficient method of achieving high enzyme concentrations in HFRs (Breslau and Kilcullen 1978). Compared with static loading, it is a more rapid method and permits higher enzyme concentrations. Since enzymes are not chemically cross-linked or bound to the support, recovery of enzyme activity and reuse of the hollow fibers are possible.

Attempts to backflush load enzyme in the PA30 cartridge described in the Chapter 3 proved unsuccessful. Backflushing, whether single pass or multiple pass, yielded virtually no enzyme retention in the membrane. It is unlikely that enzyme leakage resulted from damage to the fibers since neither air nor blue dextran, a macromolecular species, leaked across the fibers even with 15 psig transmembrane pressure. It was, therefore, necessary to consider other fiber types for immobilizing the enzyme.

The first experiments described in this section compared polysulfone (PM10 and PM30) and polyamide (PA10 and PA30) UF fibers. The numbers 10 and 30 in the fiber labels specify nominal MWCs of 10,000 and 30,000, respectively. The fibers were evaluated for retention of protein, retention of enzyme activity, recoverability of enzyme, and enzyme inactivation. Following the selection of PA10 fibers, a subsequent experiment examined the addition of BSA to the enzyme stock solution to enhance activity retention in the fibers during operation. Enzyme retention and recovery from reactors prepared for lactose hydrolysis were measured.

Some potential sources of variability in these factors were identified. The operation of the HFRs to hydrolyze lactose is described in the Chapter 5.

Materials and Methods

Reagents used for β-galactosidase activity assays, o-nitrophenyl-β-D-galactopyranoside (ONPG) (cat #N-1127) and o-nitrophenol (ONP), were obtained from Sigma. Folin phenol reagent for the Lowry protein assay was manufactured by Fisher Scientific. Other reagents and analytical equipment were described in the preceding chapters. Protein and ONPG solutions were prepared in the buffer described in Chapter 1.

Single fiber reactors

Single fiber reactors (SFR) (Lo et al. 1978) were prepared using PA10, PA30, PM10 and PM30 UF fibers for the experiments described in this and the following chapter (Fig. 28). Shell material was borosilicate glass, 20.5 cm long, 0.8 cm o.d.. The ends were tapered to 0.5 cm o.d., 1 cm long. All UF fibers were donated by Romicon. The SFR was assembled by pushing 3 cm sleeves of silicone rubber tubing (3/16 in i.d.) over the ends of the glass reactor shell. The ultrafiltration fiber was then fed through the shell. Male Luer fittings were then slid onto the fiber. Before the fittings were pushed into silicone sleeves, sufficient sealant to fill the void between the sleeve and the UF fiber was placed on the fiber. The fitting was then pushed into the sleeve. Several sealants and adhesives were employed to hold the UF fibers in the reactor, including fast and slow curing epoxies and Silicone Rubber General Purpose Sealant (Dow Corning). A combination of cyanoacrylate adhesive (Elmer's Wonder Bond Plus) in the Luer fitting and the silicone rubber sealant in the sleeve appeared to work best.

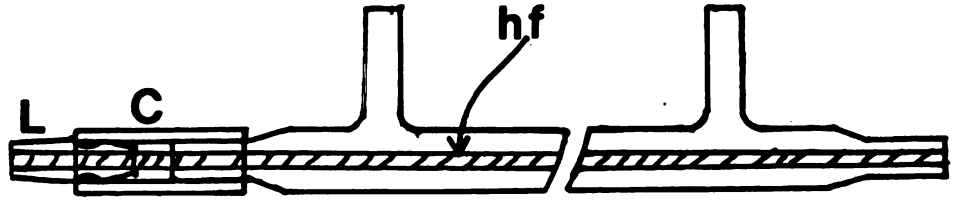

Figure 28. Single fiber reactor.

Key: L = Male Luer-Fitting
hf = Hollow ultrafiltration fiber
c = Sleeve of silicone rubber tubing

The SFRs were cleaned and sanitized by the same protocol described for the cartridge in Chapter 4. Before operating and after cleaning, SFRs were examined for leakage by pressurizing to 15 psig with air from a syringe.

The system was the same as described in Chapter 4 (Fig. 18) except the SFR replaced the cartridge and 1/8" dia. tubing was used to carry liquids. Luer fittings were substituted for the quick-disconnect connectors around the reactor. The reservoir and SFR were held at 54.5+/-0.5°C in a shaker water bath (Fisher Labline).

Analytical Techniques

β-galactosidase weight was determined by the Lowry method (Cooper 1977). Standards to obtain protein concentration in mg/ml consisted of known concentrations of β-galactosidase in buffer. The Lowry method performed well on single protein samples; however, mixtures of lactase and BSA were not conveniently assayed by this method. Pure BSA yielded approximately three times the absorbance of β-galactosidase on a weight basis; absorbances were not necessarily additive in analyses of mixtures of the proteins, and samples from solutions that had crossed the membrane could not be assumed proportionally the same in constituency as the solution applied.

Activity assays were performed in all experiments to measure enzyme distribution following backflush loading and flushing from the UF fibers. Activity of β-galactosidase in samples was determined by the rate of reaction in 10 mM ONPG. Assay mixtures consisted of 4 ml of 12.5 mM ONPG stock solution in buffer with 0.1 to 1.0 ml of sample solution and buffer added to yield a total volume of 5 ml. Prior to activity determinations, solutions were permitted to equilibrate at room temperature one hour or more. Experimental sample solution was pipetted

into a mixture of ONPG stock solution and buffer in a test tube. The assay mixture was vortexed immediately, quickly transferred to a cuvette, and inserted in the spectrophotometer. Activity was determined by monitoring the rate of change in absorbance at 420 nm. Rates were determined relative to the enzyme stock solution. The standard solution for determination of product consisted of 0.4 mM ONP in buffer.

Enzyme activity was measured in units of μMoles of product, ONP, produced per minute per ml in experimental solution. Total activity in experimental solutions was determined by multiplying measured activity by solution volume.

Experimental Procedure

The experiments described in this section were conducted to determine which fiber material performs best for immobilizing A. oryzae β-galactosidase and to measure immobilization on PA10 fibers. In all experiments, the system was flushed with buffer before loading. The shell-side of the system was then filled and quickly flushed with an additional 50 ml enzyme stock solution. The shell-side loop was then closed to recirculate enzyme solution to the reservoir and throttled at the shell-side outlet to yield a back pressure of approximately 10 psig. Backflush effluent from the lumen-side outlet was collected in a graduated cylinder and assayed for enzyme activity by the ONPG assay.

Fiber Comparisons

To compare PA10, PA30, PM10, and PM30 fibers, backflush effluent and ultrafiltrate solutions were analyzed both for activity and by the Lowry method. Thus protein and activity retention in and recovery from the fibers could be compared. After enzyme loading, the reactor was drained, and the tube-side was rinsed with buffer. The following day,

buffer was forced through the fiber in the ultrafiltration mode. The ultrafiltrate was collected in four 3.5 ml fractions.

Specific activity was measured in μMoles of ONPG converted per minute per mg protein, measured by the Lowry assay. Material and activity balances around the fibers were used to determine loss of enzyme:

$$L = C_o V_o - (C_B V_B + C_F V_F) \qquad (30)$$

where C_o is concentration of enzyme stock solution; V_o is volume of volume of stock solution; V_B is volume of backflush loading effluent; C_B is concentration of protein or activity in backflush loading effluent; V_F is volume of ultrafiltrate; and C_F is concentration of protein or activity in ultrafiltrate. Comparing loss of activity with loss of enzyme protein permitted assessing enzyme inactivation on the fibers.

Enzyme Retention in PA10 Fibers

In experiments to measure retention of enzyme in PA10 fibers after loading with and without BSA, the shell-side of the SFR was drained and closed after loading. The tube-side was then flushed with 40 ml of buffer, flowrate 3-5 ml/min collected in four 10 ml fractions. The tube-side was left filled with buffer approximately 12 hours. The buffer was drained and collected the following day. Buffer was then circulated at 7 ml/min through the lumen-side of the system for 3 hours to determine leakage of enzyme into the lumen during operation. All solutions from the reactor were analyzed for enzyme activity. To determine recovery of enzyme from the reactor, the reactor was operated in the ultrafiltration mode with fresh buffer. The ultrafiltrate was collected in four 10 ml fractions and assayed for enzyme activity.

Reactor Loading

In operating the system as a reactor, enzyme loading was assumed to equal enzyme activity backflushed into the fiber and not detected in the backflush effluent, the buffer rinses after loading, and the buffer equilibrated in the lumen 12 hours. To confirm negligible enzyme leakage into substrate solution during SFR operation, half of each reaction sample was incubated in the water bath until the following sampling interval. Lack of significant additional conversion confirmed the paucity of leakage.

Results and Discussion

Fiber Comparisons

Far more activity was found in the effluent from the PA than the PM fibers after backflush loading (Table 8). The PA30 fibers entrapped virtually no activity and the effluent from PA10 fibers still contained 62% of stock activity. Backflush effluent from PM10 and PM30 fibers converted ONPG at 37% and 19% the rate of enzyme stock solution, respectively.

While backflush effluent from PA fibers contained approximately equal proportions of enzyme activity and protein mass, effluent from PM fibers contained a greater proportion of protein than activity. Thus the specific activity of PM fiber effluent is less than than stock specific activity. This suggests that PM fibers either selectively retain β-galactosidase and permit impurities to cross or inactivate some enzyme during backflushing.

Despite having a higher molecular weight cutoff, the PM30 fibers apparently retained more activity than the PM10 fibers. Since it is not known whether the fiber compositions are exactly the same, this

Table 8. Leakage[1] of A. oryzae β-galactosidase Activity and Protein with Backflush Loading

Fiber[2] Type	Activity[3] %	Protein[4] Weight %	Specific[5] Activity %
PA10	62 ± 7	61 ± 17	102 ± 10.7
PA30	93 ± 6	84 ± 9	112 ± 1
PM10	37 ± 13	44 ± 11	87 ± 18
PM30	19 ± 14	37 ± 15	76 ± 18

1 Results of analysis of tube side effluent, mean values for 0.1 and 0.5 mg/ml enzyme stock solution. Intervals are Mean ± 1 sdev. Backflush pressure = 15 psig.

2 PA = polyamide; PM = polysulfone; 10 = 10,000 nominal MWC; 30 = 30,000 MWC

3 Rate of ONPG conversion per ml backflush effluent ($mm.ml^{-1}min^{-1}$) as percent of stock solution activity. N=3

4 Protein concentration mg/ml in effluent as % of stock protein concentration. N=2

5 Activity of enzyme per mg protein in the effluent as % of stock unit activity.

anomalous result may have arisen from differences in adsorption due to chemical differences between the fiber types.

Specific activity results, derived from protein and activity analyses, show the protein recovered from the PM fibers to be less active than that recovered from the PA fibers (Table 9). Specific activity for permeate from the PM30 fibers is lowest. Permeate from PA fibers shows specific activity somewhat higher than the stock solution. Possibly, the retention of some impurities on the fibers during washing yields a more pure enzyme, and thus higher specific activity than applied.

Material and activity balances (Table 9) indicate the recovery of approximately 85% of both protein mass and total activity applied to PA10 fibers either in the backflush effluent or UF permeate. The apparently negligible losses of material and activity from PA30 fibers results from their retaining virtually no activity during loading. Their inability to retain significant activity on loading precludes consideration of their use in HFR applications with the A. oryzae lactase.

Both protein and activity loss on the PM fibers are greater than on the PA fibers, indicating a significant portion of the enzyme remained entrapped in the fibers. The PM fibers also appear to inactivate enzyme. The specific activities for protein in both the backflush effluent and UF permeate are less than the stock solution, and the mass and activity balances show greater recovery of protein than activity.

Huffman-Reichenbach and Harper (1982) also observed leakage and inactivation of A. oryzae β-galactosidase backflushed into polysulfone fibers. Other investigators reported inactivation of yeast lactase (Kohlwey and Cheryan 1981) and alpha-galactosidase (Korus and Olson 1975) on polysulfone fibers. Both also found that pretreating the

Table 9. Recovery of A. oryzae β-galactosidase Activity and Protein

Fiber Type	Specific[2] Activity %	Mass and Activity Balances % Loss[3] Protein Mass	Total Activity
PA10	117 ± 28	14.8 ± 0.3	17 ± 2
PA30	120 ± 42	4 ± 6	4 ± 6
PM10	85 ± 5	33 ± 10	48 ± 9
PM30	58 ± 5	48 ± 19	66 ± 10

[1] Results of analysis of ultrafiltration (UF) washing of enzyme from fibers on which enzyme had been backflush loaded 24 h. before, with both 0.1 and 0.5 mg/ml enzyme, N=2.

[2] Activity of enzyme per mg protein in ultrafiltrate as % of stock solution unit activity.

[3] Amount of enzyme, protein or activity, not recovered either by leakage into backflush effluent or by ultrafiltration.

fibers with BSA greatly reduced inactivation. On the other hand, BSA reduced the half-life of A. oryzae β-galactosidase in contact with polysulfone (Huffman Reichenbach and Harper 1982). In contrast to the results for A. oryzae lactase, both A. niger (Breslau and Kilcullen 1978) and yeast (Kohlwey and Cheryan 1981) lactases were successfully backflush loaded in UF fibers.

PA10 fibers were selected for further study over the PM fibers. Although PA10 fibers retained only one third the activity backflushed, the enzyme was not significantly inactivated in contact with the fibers for 16 hours. Recovery of enzyme from the fibers was also superior, supporting the manufacturer's (Romicon's) assertion than PA fibers tend to adsorb less protein than PM fibers.

Enzyme Retention in PA10 Fibers

Before employing PA10 fibers to hydrolyze lactose in the SFR, enzyme retention in the spongy layer over time and during operation were measured. Substantial enzyme leakage into the lumen was detected overnight and during operation with recirculating buffer when enzyme stock solution with no BSA was loaded onto the reactor (Table 10). BSA was then added to the stock solution in an attempt to improve the retention of β-galactosidase. While adding 0.5 mg/ml BSA apparently did not increase retention during loading, very little leakage from the fiber was detected either during overnight equilibration or into the recirculating buffer. Thus subsequent experiments were conducted using BSA in the enzyme stock solution. Reactor operation confirmed that little β-galactosidase leaks into the lumen when BSA is used in immobilization.

To determine whether BSA affects activity, reaction rates have been determined using enzyme stock solutions with and without BSA. β-galactosidase activity without BSA was 1.04+/-0.05 times enzyme activity with

Table 10. Retention of A. oryzae β-galactosidase in PA10 Fibers with and without Bovine Serum Albumin

Cycle[1]	Flow Pattern	% Total Stock Activity in Effluent Without BSA	With[2] BSA
Backflush loading	P2-P3-0	50.6	49.8
Rinses	P1-P3-0	2.7	4.4
Drain	P3-0	6.2	0.2
Recirculating buffer	P1-P3-Res	4.2	0.6
Drain 2	P3-0	3.9	0.2
Ultrafiltration Cleaning	P1-P4-0	22.0	47.4
Totals		89.6	102.6

[1] Cycle descriptions: (Reference Figure 18 for flow pattern):

1) Backflush loading - 0.5 mg/ml enzyme solution - 15.7 ml without BSA and 10.1 ml with 0.5 mg/ml BSA in enzyme solution.

2) Rinses - Pumped buffer at approximately 5 ml/min through the lumen to rinse any residual enzyme.

3) Drain - Buffer in the reactor overnight was drained.

4) Recirculating buffer - Buffer was recirculated through the system 3 h to check leakage during operation.

5) Ultrafiltration cleaning - Enzyme was flushed from the reactor by ultrafiltering buffer.

[2] Activity with BSA for backflush loading effluent and rinses was determined against stock solution without BSA.

BSA (N = 6). This, along with results of the batch enzyme experiments (Chapter 2), suggests that BSA has little effect on the rate of enzymatic reaction. However, since BSA immobilized in the fiber is presumably more concentrated than the assay mixtures, the effect of BSA on A. oryzae β-galactosidase is not definitively known. However, qualitative evaluations seem favorable.

Since shell-side solution was recycled during backflush, the activity of shell-side solution was compared with uncirculated stock solution. The purpose of this determination was to assure backflushed enzyme activity had remained in the spongy layer rather than diffusing back into the shell-side recycle stream. Assay of the recycled solutions in early experiments showed a drop in activity to 94.1 +/-2.5% of stock activity. Turbidity also developed downstream from the pump during loading and an in-line filter fouled rapidly. It was, therefore, suspected that the gear pump was denaturing some of the enzyme. Replacing the gear pump with a peristaltic pump after the third experiment (Table 11) alleviated the problem. Recycle enzyme activities subsequently were the same as stock solution activity (100.2 +/-2.5%).

Reactor Loading

Enzyme loading (Table 11) for experiments in which the SFR was used for lactose hydrolysis was estimated by subtracting the enzyme activity detected in the tube-side effluent from total enzyme backflushed through the reactor. Enzyme activity recovery by ultrafiltering buffer after operation varied from 35 to 95% with a mean of 66% of the enzyme retained during loading. No apparent relationship between recovery and loading was noted.

Due to problems with sealants, only one SFR survived more than one experiment. Retention of enzyme appeared to improve in that SFR after

Table 11 - Retention of *A. oryzae* β-galactosidase and Reactor Loadings in PA 5-10 Fibers

Exp/ Status[1]	Loading Pressure (PSIG)	Backflush[2] Volume (ml)	Leakage Distribution[3] % BF	R	Dr	Estimated Retention[4] (mg)	Recovery on UF (mg)
1/N	10	15.3	59.8	2.8	0.1	2.85	2.13
2/N	10	3.6	49.3	7.9	0.3	0.77	0.62
3/N	10	27.0	50.6	0.6	1.1	6.44	2.25
4/N	10	43.5	65.7	0.4	0.1	7.35	4.48
5/P	9	2.6	17.1	1.7	0.4	1.05	0.52
6/P	9	1.05	21.4	4.3	0.3	0.39	0.37
7/P	9	8.2	24.3	1.1	0.2	3.05	nd

[1] Exp/Status - Experiment and whether SFR was first used for this repetition (N) or used in previous repetition (P)

[2] β-galactosidase stock solution - 0.5 mg/ml β-galactosidase and 0.5 mg/ml BSA in buffer

[3] Percent total enzyme activity detected in backflush effluent (BF), rinses (R) and fluid drained from reactor after overnight equilibration (Dr)

[4] Amount of enzyme reported present in reactor

first loading (Table 11). While that SFR failed to retain a mean of only 24% of the activity applied after the first loading, mean leakage was 60% for new fibers. Since slightly lower backflush pressure was used and only one SFR endured more than one experiment, the increased retention with repeated loading may not be a repeatable result.

CHAPTER 5

REACTOR OPERATION

Introduction

The experiments described in this chapter were conducted to obtain operational data on the SFRs described in the previous chapter. Operation of SFRs at the temperature and pH for processing milk and sweet whey yielded preliminary data on the effects of enzyme loading and flow rate on reactor performance. Storage life of A. oryzae β-galactosidase applied to PA10 fibers in a SFR and denaturation by sodium hypochlorite, commonly employed as a sanitizer for hollow fibers in the dairy industry, were also evaluated.

Flow rates generally were less than those recommended by one manufacturer, Amicon, to prevent fouling during ultrafiltration. The recommended flows yield shears of approximately 2 N/m^2, which translate to an average velocity of 19 cm/s in the fibers used in this study, assuming viscosity of 1.5 cp and laminar flow. Since fouling during ultrafiltration is partly pressure driven, and, by contrast, reactor operation yields very low cross-membrane pressures, lower flow rates than recommended were used in order to increase single-pass residence time in the reactor. An average velocity of 11.5 cm/s (6.5 ml/min) was employed in most experiments described in this section.

Materials and Methods

Stock solutions, analytical methods, and reagents for determination of the lactose hydrolysis product, glucose, were as described in Chapter 2. Preparation of the SFR was described in Chapter 4, and a schematic for the system was presented in Chapter 3 (Figure 18). The reactor and fluid reservoir were held at 54.5+/-0.5°C in a Fisher Labline shaker bath. To assure constant temperature in the reactor, all external

tubing was insulated, and fluid was passed through a 50 cm x 5 mm outer diameter glass tubing loop immersed in the bath immediately upstream of the reactor.

Operation

Since the SFRs used in the operational experiments were also used to evaluate enzyme retention and recoverability (Table 11, Chapter 4), stock solution concentration and volume loaded were, in large part, selected to permit the determination of retention and recovery in the experimental protocol described in the previous chapter. The stock solution concentration was held constant to reduce the number of variables in enzyme retention determination. Consequently, loading amounts were constrained on the lower end by an inability to accurately measure backflush volumes of less than 2 ml due to droplets of liquid retained in the system and the method of recovery of liquid in the SFR, i.e. blowing from the fiber into a graduated cylinder with air from a syringe. At the upper limit, the rate of cross-membrane flow seemed to slow with increasing loading; therefore, seven hours were required to backflush 7 ml enzyme stock solution.

The general method of assembling and operating the reactor was similar for all experiments described in this chapter. One day after the reactor was loaded as described in Chapter 4, the buffer that was left in the system overnight was drained, and the reservoir was filled with 150 ml of 138.9 mM lactose solution. After all ports to the reactor were closed to isolate the reactor, lactose solution was circulated through the recycle and bypass loops for approximately 10 minutes. The reservoir was emptied by opening the sample port and subsequently refilled with fresh lactose solution. Both the recycle and bypass loops were closed, and the lumen-side ports of the SFR opened. Approximately

100 ml of lactose solution was then pumped through the lumen (rotameter reading 15, flow rate ca. 6.5 ml/min) to bring the reactor to steady state. Solution was exhausted through the sample port into a graduated cylinder. The rate of filling the graduated cylinder was measured to determine precise flow rate. Except in the experiment examining the effect of flow rate on reactor performance, all operations were conducted at approximately 6.5 ml/min.

Operation commenced with opening the recycle loop and closing the sample port. Samples (ca. 3 ml) were collected during operation by simultaneously closing the recycle loop and opening the sample port. Sample volumes were recorded. Each sample was then divided in two equal portions in disposable polypropylene centrifuge tubes. One portion was immediately placed in a hot (90+/-5°C) water bath for 5 minutes to inactivate any enzyme that might have leaked into the lumen. The other portion was incubated in the shaker bath with the reactor and placed in the hot water bath when the next sample was drawn. By comparing the glucose concentrations in the two portions of each sample, enzyme leakage into the lumen could be detected and quantified. Since no significant differences between the two portions of any sample were detected, this method confirmed the absence of leakage.

At the end of each experiment, the fluid remaining in the system was drained into a graduated cylinder. Residual liquid was forced out and into the graduated cylinder by blowing air through the system. The total volume in the graduated cylinder was then recorded as the residual volume. Total initial volume in the system and volumes during sampling intervals were determined by adding sample volumes to the residual volume.

Flow Rate

Product concentration and reaction rate were compared at flow rates of 2.7, 6.5, and 19.2 ml/min in a reactor loaded with 3.05 mg/ml enzyme (Table 11, Experiment 7). Samples were collected at 0, 15, 30, 60, 105, and 120 minutes.

Enzyme Loading

To determine the effect of varying the amount of enzyme loaded, product concentration and reaction rates with enzyme loadings between 0.4 and 7.35 mg/ml (Table 11, Experiments 1-6) were measured. Samples were collected at 0, 15, 30, 60, 90, 120, 150, and 240 minutes.

Reactor Life

A. oryzae β-galactosidase stability in contact with PA ultrafiltration fibers and when exposed to sodium hypochlorite was assessed using the SFR prepared for the flow rate experiment. To check stability in contact with the fiber, the SFR was stored 8 days in a refrigerator at 4°C and then set up in the system and operated. Product concentration was compared with the results from the first day's operation.

Following operation at 8 days, the reactor was washed by pumping 100 ml of distilled water, then 125 ml of 200 ppm NaOCl, and finally 125 ml of buffer through the lumen and out the sample port at 6 ml/min. The SFR was then operated by the procedure described above. Conversion was compared with operation before treatment with NaOCl.

The reactor was then refrigerated another 8 days, operated, sanitized, and then operated again.

Data Treatment

Data were initially acquired in the form of glucose concentration versus time. Since the volume of solution in the system varied among experiments and changed with each sample collection during an experiment, comparisons among experiments required translating sampling time into an average residence time in the SFR. Mean residence time (τ_i) for each sampling interval was determined from the following:

$$\tau_i = \frac{V_L}{q} + V_L \Sigma \left(\frac{t_i - t_{i-1}}{V_{si}} \right) \qquad (31)$$

where V_L = the lumen volume (0.185 cm3), q = volumetric flow rate, V_{si} = volume of fluid in the system during the time interval $t_i - t_{i-1}$. Product concentration was plotted versus τ_i.

With competitive inhibition, the rate of reaction was a function of both product and substrate concentrations. Reaction rates, as the first derivatives of the product (glucose or galactose) concentration versus time curves, were calculated by a method described by Burden et al. (1981). Apparent specific activity of the enzyme was plotted versus the product concentration for the various loadings. The predicted rate curve was generated from the kinetic parameters determined in Chapter 2.

To enable the comparison of catalytic performance with effectiveness factors described in the literature, a FORTRAN program (see Appendix) was written to calculate the effectiveness factor from experimental data and the generalized modulus (Moo-Young and Kobayashi 1972) from diffusivity and kinetic constants. The effectiveness factor was calculated by dividing the observed rate of reaction by the predicted rate of reaction using free-solution kinetic parameters at the substrate and product concentrations in the lumen. Moo-Young and

Kobayashi (1972) expanded the form of a generalized modulus (Bischoff 1965) for use with competitively inhibitted reactions:

$$m = \frac{h}{\beta_1+\beta_2} \cdot \frac{1}{\sqrt{I_2(1,0)}} \tag{32}$$

where,

$$I_2(1,0) = \frac{1}{\beta_2^2}\left(\beta_2-\beta_1 \ln \frac{\beta_1+\beta_2}{\beta_1}\right) \tag{33}$$

$$h = \sqrt{V_m/2D_s S} \cdot L \tag{34}$$

$$V_m = \frac{Ke_o}{V_c} \tag{35}$$

and

$$\beta_1 = \alpha_1(a - \omega\alpha_2)$$

$$\beta_2 = 1 - \zeta\alpha_1\alpha_2$$

$$\alpha_1 = \frac{K_m}{S} \qquad \omega = \frac{P}{s} + S$$

$$\alpha_2 = \frac{S}{K_c} \qquad S = \frac{D_s}{D_P}$$

and V_c = volume of catalyst. Values for the kinetic parameters V_m, K_m and K_c were described in Chapter 2. Lumen substrate concentration was

assumed to be determined directly from the stoichiometry of the reaction, i.e. $S = S_o - P$. Substrate diffusivity ($D_s = 2.9 \times 10^{-6}$ cm^2/s) was calculated in parallel experiments conducted by another student (Knob, R. unpublished data). The ratio (ζ) of lactose and galactose diffusivities was assumed to be 3.7 based on free solution diffusivities (Perry and Chilton 1972).

Equation 35 yields a value of the modulus for a flat sheet geometry; therefore, modulus values are adjusted by defining the characteristic length (L) as the ratio of catalyst volume to lumen surface (Froment and Bischoff 1979):

$$L = \frac{V_c}{S_L} \qquad (36)$$

This simple adjustment is most accurate for first order reactions. Since the minimum K_{mapp} for the reaction was expected to consistently exceed substrate concentration, the approximation for first order reaction was regarded as acceptable.

Results and Discussion

Flow Rate

Varying flow rates yield no apparent differences in conversion with time (Figure 29) or the rate of reaction at various substrate concentrations (Figure 30). Lack of variation with flow rate indicates that lumen-side diffusion does not constitute a significant mass transfer resistance in the SFR over the range of flows examined. Several of the models described in Chapter 1 consider lumen-side resistance (Waterland et al. 1974, Kim and Cooney 1976), but these results suggest that

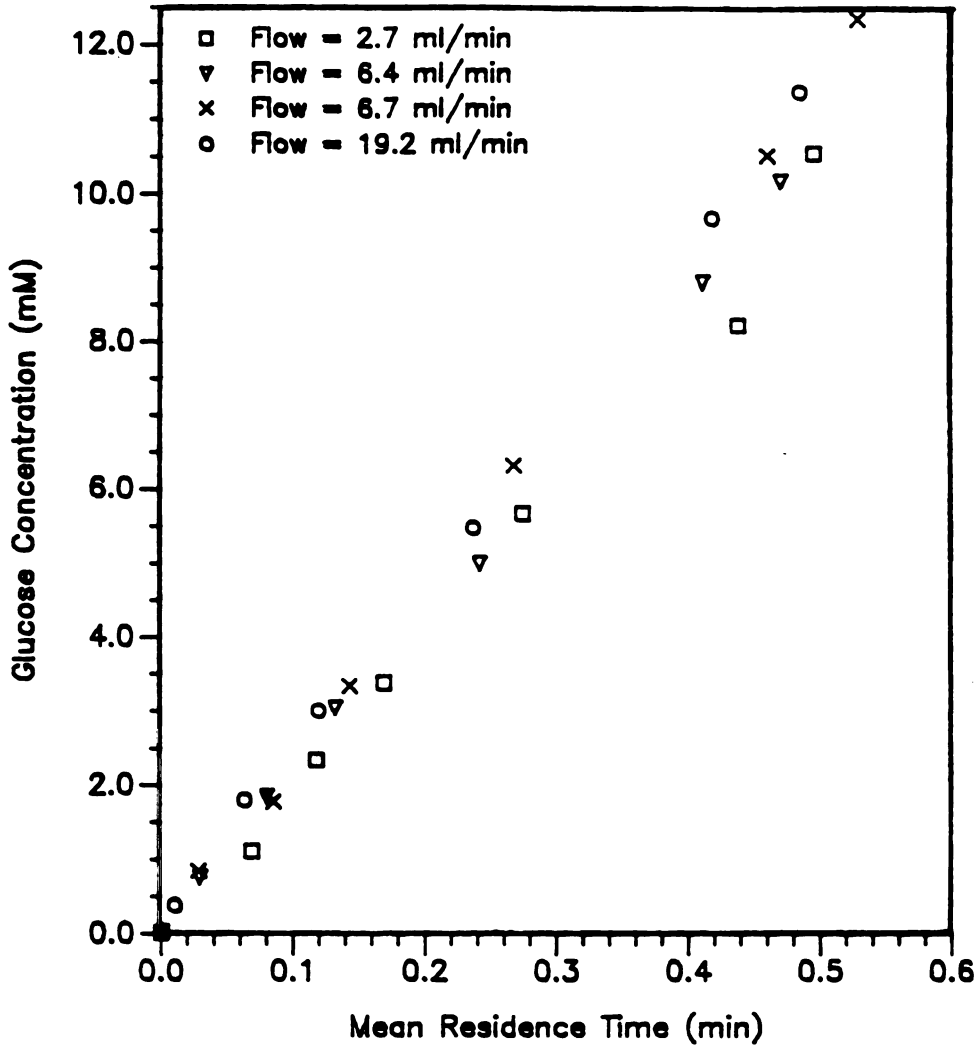

Figure 29. Effect of varying flow rate on conversion in a SFR.

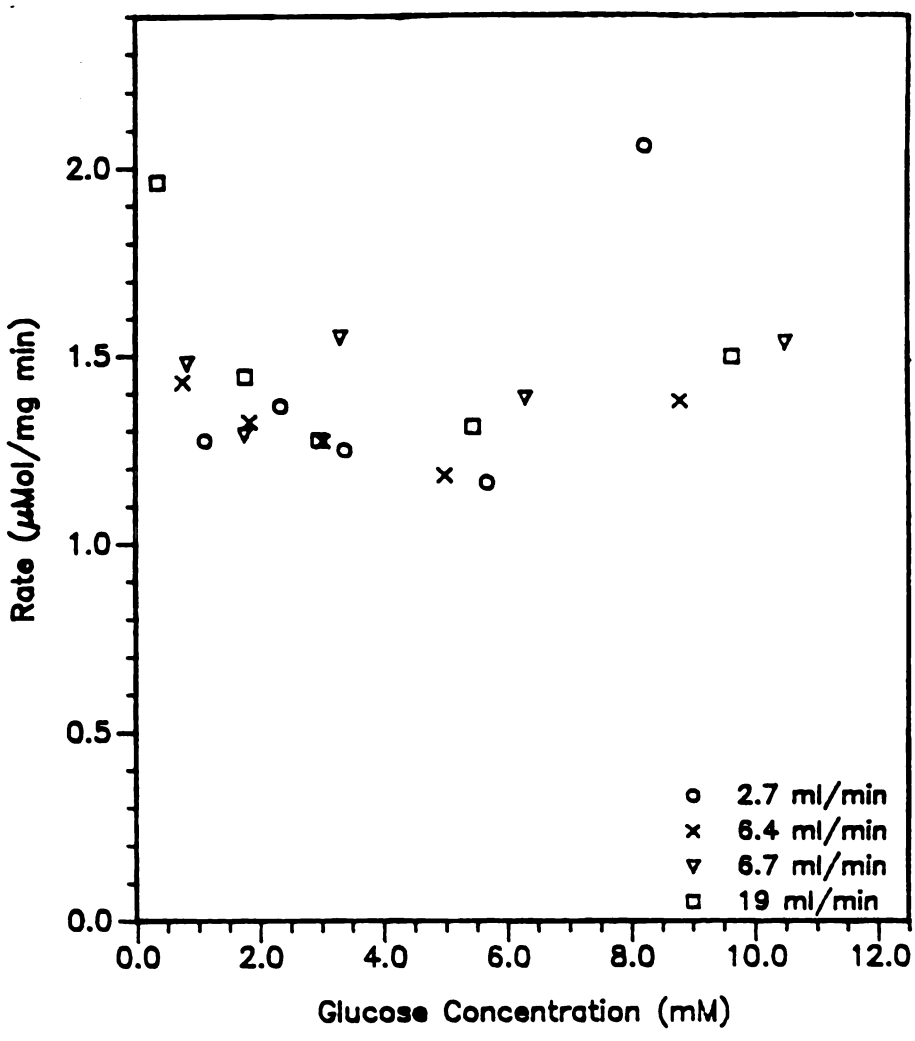

Figure 30. Rate of reaction versus product concentration at three different flow rates in a SFR.

consideration of radial concentration gradients on the lumen-side may not always be necessary.

The results obtained from operating an SFR at different flow rates may not easily be scaled up to longer systems. The relatively short residence time in the SFR may not have permitted the development of concentration gradients possible in a longer reactor. To illustrate this point, the Sherwood number may be used to estimate mass transfer coefficients in laminar flow (Bennett and Myers 1982):

$$Sh = \frac{k_c d}{D_{AB}} \qquad (37)$$

where d = tube diameter, k_c = mass transfer coefficient, and D_{ab}= diffusivity. The Sherwood number is a function of the Reynolds number, Schmidt number, tube diameter, and length. Using the operational parameters of the experiments in this section, the Sherwood numbers with lactose for the SFR and a reactor 15 times longer are 10.5 and 3.2, respectively, indicating a lower average mass transfer coefficient in longer tubes. While the above figures are based on uniform wall concentration and are, therefore, not entirely accurate for the reactor, they do point to an impediment to assuming negligible tube side resistance when scaling up the length of an SFR.

Loading

The rate of glucose production increases at greater enzyme loading in the SFR (Figure 31). However, the enzyme's apparent specific activity (Figure 32) declines at higher loadings. Specific activity of immobilized enzyme is consistently much lower than the free solution activity.

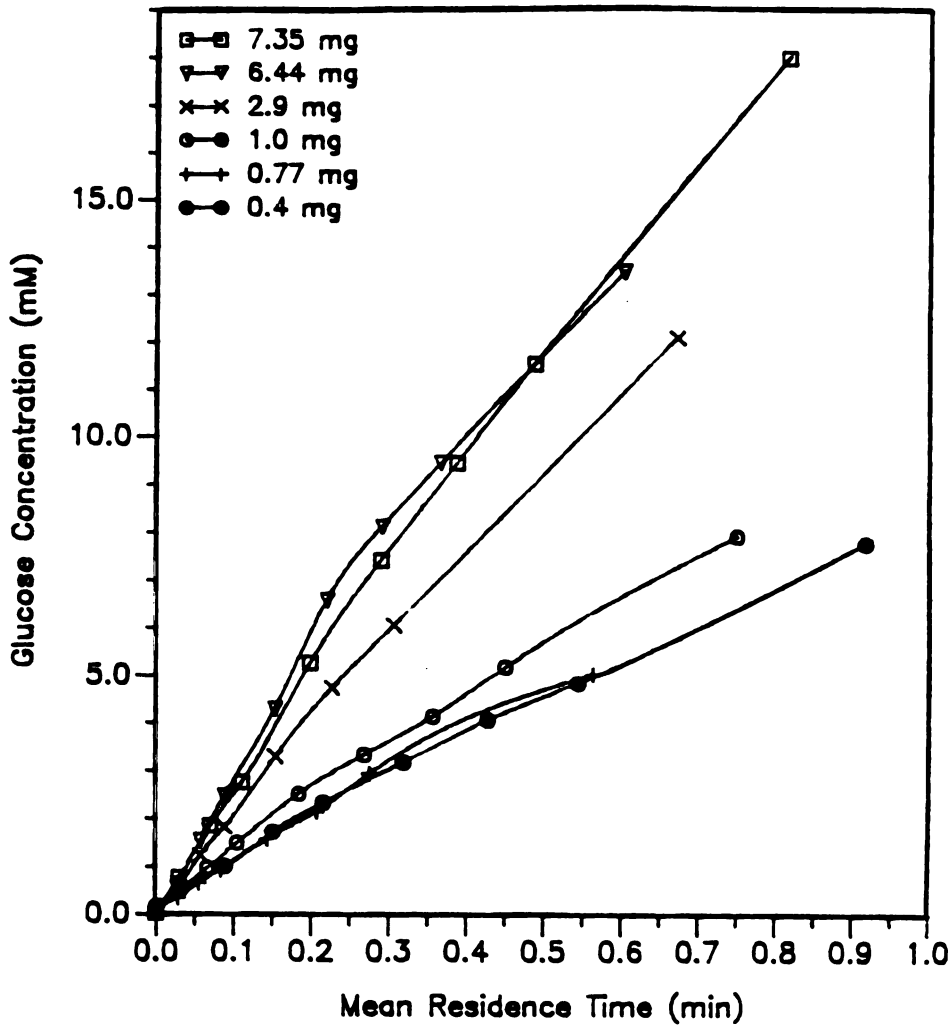

Figure 31. Conversion versus time at various enzyme loadings in a SFR.

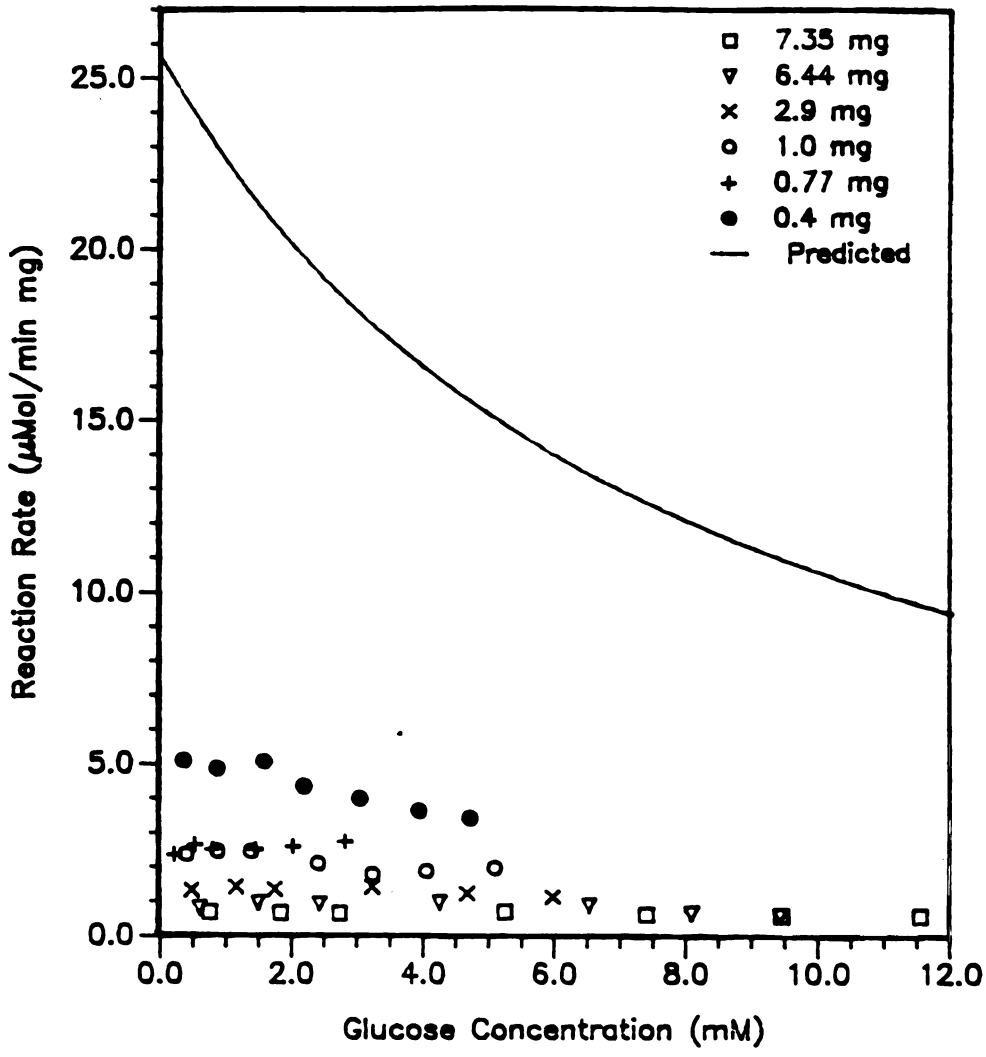

Figure 32. Reaction rate (apparent enzyme specific activity) versus product concentration at various enzyme loadings in a SFR.

Lower apparent specific activity in immobilized enzymes may arise from reduced reactivity or mass transfer resistances. Calculation of the generalized modulus, described in the preceding section, yields values between 80 and 500 (Figure 33) for the enzyme loadings and substrate concentrations in these experiments. This indicates approach to a diffusion limited regime. Thus diffusion resistance may account for the low apparent specific activity and consequent low effectivenss factors observed in these experiments.

The observed effectiveness factors are, in fact, somewhat higher than predicted by Moo-Young and Kobayashi (1972). While they predicted an effectiveness factor of 0.24 at a modulus value of 4, the modulus value observed in this work is 80. The higher modulus may arise from lower than predicted mass transfer resistances, changes in enzyme kinetics on immobilization, or uneven enzyme distribution.

Since the permeation experiments, from which diffusivity was calculated, were conducted under conditions that simulated reactor operation, the diffusivity value used to calculate the modulus is probably fairly accurate.

Undetected increases in the rate of reaction also would yield higher than predicted effectiveness factors. The kinetic parameters of the immobilized enzyme used in this work are assumed the same as the free solution parameters, and any changes in enzyme kinetics due to immobilization were not investigated. Immobilization may, however, increase, decrease, or not affect enzyme reactivity (Bailey and Ollis 1986). While one of the advantages cited for the physical entrapment method used in this study is that enzyme kinetics are generally not altered (Chambers et al. 1976), the data are not sufficient to determine immobilization effects on kinetics. Also the incomplete recovery of enzyme on ultrafiltration cleaning of the fibers (Table 11) indicates

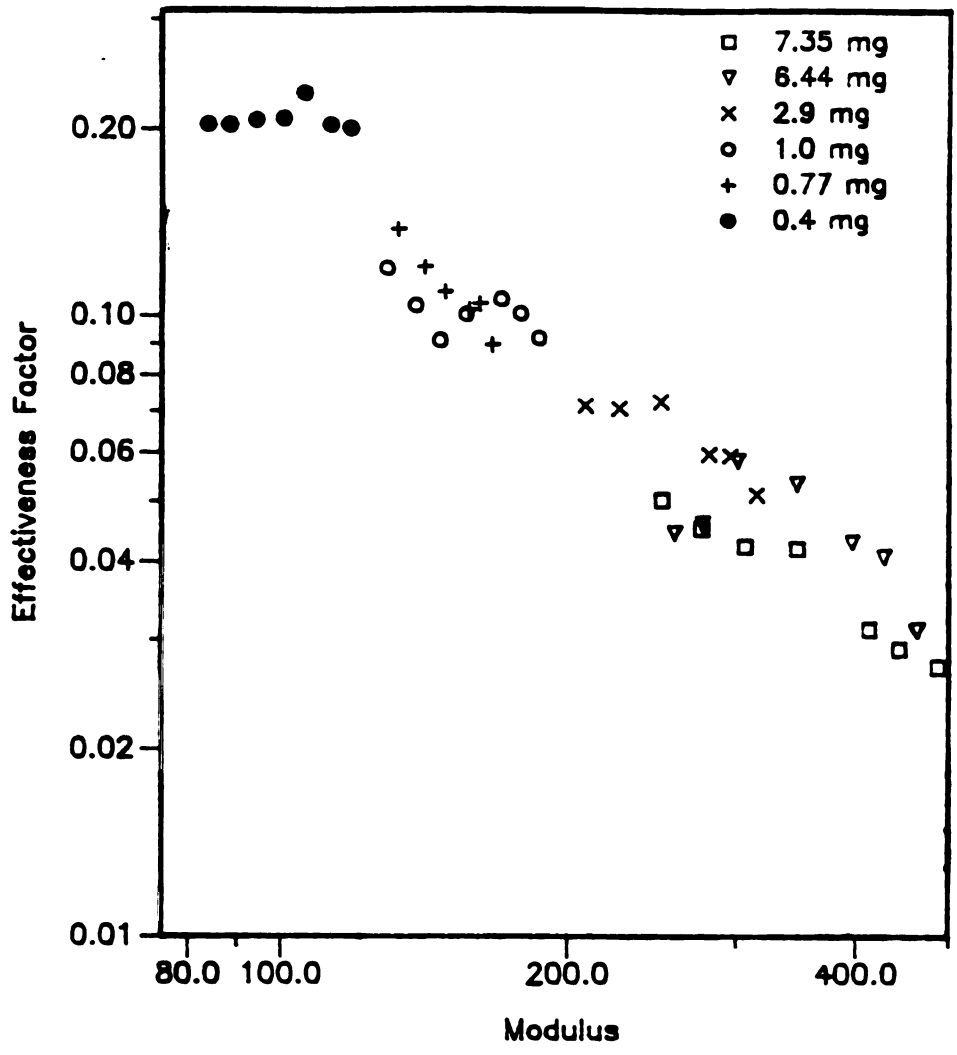

Figure 33. Generalized modulus versus effectiveness factor at different enzyme loadings.

that some β-galactosidase may have adsorbed to the support. Adsorption may also affect the rate of enzyme reaction.

Calculation of the modulus also assumes even distribution of enzyme in the spongy layer of the fiber. Backflush loaded lactase may, however, form a concentrated layer outside the lumen (Breslau and Kilcullen 1978, Chambers et al. 1976). Examination of Equations 32, 34, 35, and 36 reveals that the modulus varies as the square root of V_c, and thus is reduced with the effective radius of the catalytic layer. If the enzyme adsorbs around the inner membrane, the modulus again might be reduced. A concentrated layer of enzyme around the lumen, therefore, may yield lower values for the modulus than calculated above assuming evenly distributed activity.

Reactor Life

A. oryzae β-galactosidase remained stable in contact with PA10 fibers over at least eight days at 4°C. Glucose production in the SFR did not change significantly between the first and eighth day of the experiment (Figure 34). Similarly, enzyme activity on day 20 approximated that on day 12. These results demonstrate that stability of enzyme in contact with the fibers probably will not limit the useful life of the system.

Since sodium hypochlorite is commonly used to sanitize ultrafiltration fibers, its effect when used to sanitize the system with the enzyme in situ was evaluated. After each treatment with sodium hypochlorite, conversion over time dropped sharply (Figure 34). While cleaning with milder agents may be feasible, the standard method recommended by Romicon for food industry use includes sodium hypochlorite. To preserve activity, the enzyme should be flushed from the fibers before cleaning.

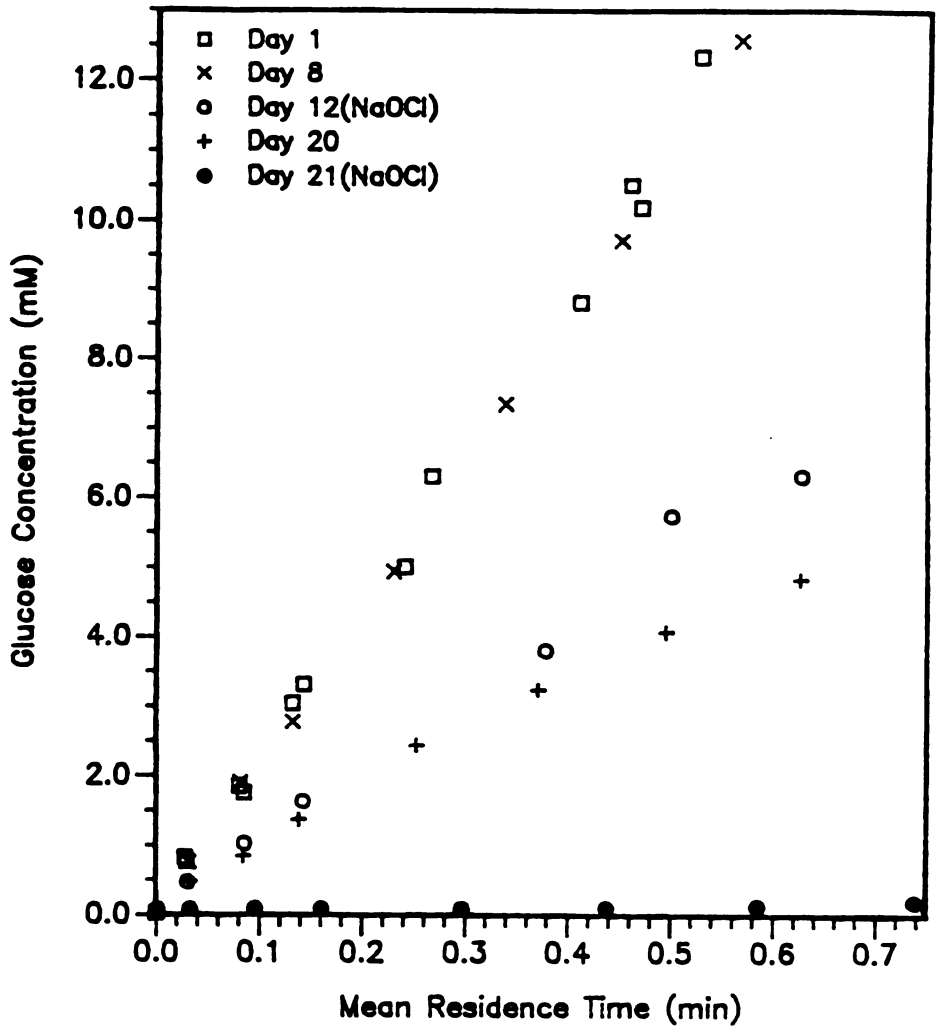

Figure 34. Product concentration in hollow fiber reactor system following storage and treatment with sodium hypochlorite (200 ppm).

CHAPTER 6

CONCLUSIONS AND RECOMMENDATIONS

Enzyme

Enzyme Selection

The advantages of using A. oryzae β-galactosidase, as described in the introductory chapter, include its activity over a range of pH values, stability at high temperature, commercial availability, and GRAS status. The experiments in this study support the manufacturer's claim and literature reports that the enzyme is relatively insensitive to divalent cations. Consequently, this lactase may be the most suitable commercially available enzyme for milk and sweet whey applications in an immobilized enzyme system.

The results of this study, however, suggest that alternatives to A. oryzae lactase should be considered. The lactose hydrolysis product, galactose, strongly inhibits the enzyme. Consequently, this enzyme's rate of reaction declines far more rapidly with conversion than other lactases with a lower degree of product inhibition.

Also, backflush loading yields relatively low retention of the enzyme in polyamide fibers despite nominal molecular weight cutoffs much lower than the enzymes molecular weight. Since Huffman-Reichenbach and Harper (1982) reported that the enzyme was poorly retained in two other common UF fiber types, changing fiber materials to improve retention does not appear to be a viable option.

Alternative thermostable lactases, which are not yet commercially available, are produced by Bacillus stearothermophilus and Streptococcus thermophilus, an organism used in yogurt production. Both organisms are nonpathogenic. Of the two organisms, the B. stearothermophilus enzyme may show the most promise for dairy application. Its optimum temperature is 60°C, and it is quite stable at that temperature (Griffiths and Muir

1978). The enzyme retains at least 75% of its maximum activity between pHs 5.6 and 7.0. It has not yet been assayed for activity in dairy products; however, its activity is not much reduced by several of the divalent cations present in milk.

Lactases have been isolated from a number of S. thermophilus strains (Greenberg and Mahoney 1982, Hemme et al. 1980, Ramana Rao and Dutta 1977). These enzymes are generally stable to at least 55°C and show optimum activity between 49 and 56°C. Their pH optima are near 7. Activity of the S. thermophilus β-galactosidase in milk and sweet whey is at least 90% of its rate with lactose in buffer solutions (Ramana Rao and Dutta 1981, Greenberg et al. 1985). While the S. thermophilus enzyme is reported to be difficult to produce (R. R. Mahoney, correspondence 1986), screening a variety of strains and selecting optimally producing cultures may yield a commercially viable enzyme.

The competitive inhibition constants reported for both the S. thermophilus and B. stearothermophilus enzymes (Table 5, Chapter 2) are far higher than reported for A. oryzae lactase. Product inhibition of the fungal lactase (Figure 35) yields sharply decreasing values of the generalized modulus described in Chapter 5. The modulus for the bacterial lactases is far more stable with conversion. Thus a bacterial lactase loading optimized to approach a diffusion controlled regime initially will remain in that regime throughout the operational cycle.

Enzyme retention and stability in HFRs are wholly unknown for both bacterial β-galactosidases. Thus both enzymes require extensive evaluation as described in this thesis for A. oryzae lactase before application.

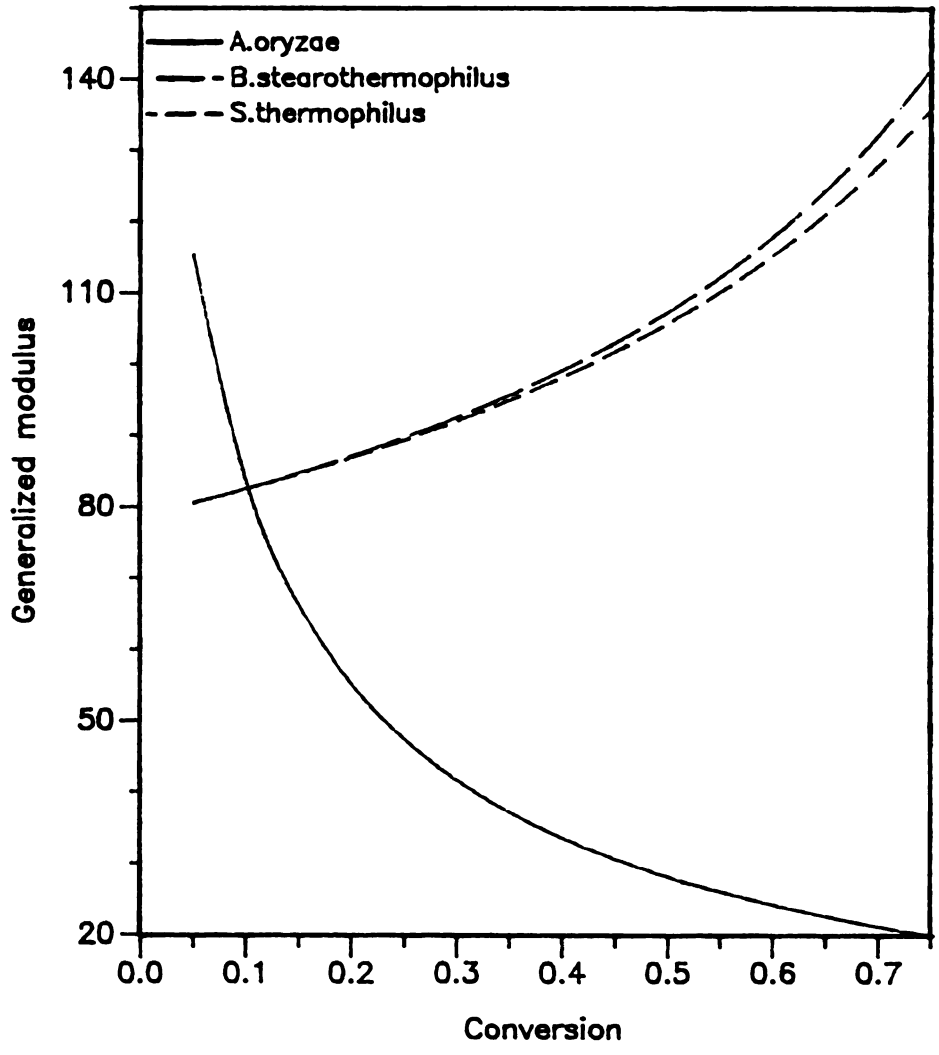

Figure 35. Generalized modulus versus conversion for A. oryzae and bacterial lactases.

Enzyme Retention

If A. oryzae β-galactosidase is selected for further evaluation, the use of whey proteins to improve retention and the phenomenon of increased enzyme retention with repeated use of the HFR should be investigated. While BSA is quite expensive for use in stabilizing enzyme retention, the cost of whey proteins is very low especially in dairy applications. One reason for initially using BSA in the SFR is that albumin also comprises a portion of the whey proteins that might eventually be substituted for BSA. Thus BSA may reasonably simulate the behavior of that fraction of whey protein in improving retention. Retention and enzyme stability in the system should be evaluated in the presence of whey proteins.

Enzyme retention in the SFR that endured repeated use increased after the first loading (Table 11, Chapter 4). One hypothesis explaining that observation is that enzyme retention improves after the fiber has been conditioned by the first loading. This hypothesis, however, requires testing under a regime of controlled pressure and constant backflush volume to eliminate the other variables that appear in the experiments reported in Chapter 4. If average retention does improve from 45% on the first application of enzyme to 80% on subsequent loadings, utilization of the A. oryzae enzyme may become more feasible.

Reactor

Single Fiber Reactor

The SFR is a useful tool for testing enzyme and substrate behavior in hollow ultrafiltration fibers. As a far smaller system, it costs less than a hollow fiber cartridge and requires the expenditure of less enzyme, substrate, and cleaning solution. Thus the SFR provides an easily prepared and inexpensive means of selecting enzyme loadings for

further evaluation and obtaining initial operational data that may be refined for scaling-up.

Data obtained from the single fiber reactor indicates that conversion is relatively insensitive to flow rate in flow regimes that may be required to prevent fouling. As described in Chapter 5, that observation may not be as valid for longer reactors if a radial concentration profile develops. When longer reactors are utilized, experiments should be conducted to determine whether the lack of correlation between conversion and flow rate continues to hold. If mass transfer across the lumen does become a limiting factor, evaluation of smaller diameter fibers may be desirable.

Full-scale systems also will require greater pressure drops to maintain a given flow rate through the fibers. In longer reactors, therefore, the possibility of toroidal flow across the inner ultrafiltration membrane and down the shell side of the fiber arises. Such flow may redistribute the enzyme (Waterland et al. 1975) and increase the bulk flow across the membrane sufficiently to reduce the applicability of mass transfer coefficients derived from single fiber data.

Immobilization Method

Backflush loading, despite A. oryzae enzyme leakage, is a satisfactory method for applying enzyme to the reactor. Compared to the static loading method used by Waterland et al. 1975, the method is relatively quick and permits attaining high enzyme concentrations in the outer layer of the fiber. Unlike methods that chemically cross-link the enzyme, lactase may be partially recovered (35-95%) from the fibers after backflush loading. Consideration of cross linking may, however, be indicated if toroidal flow results in substantial translocation of the enzyme.

Enzyme Distribution

The higher than expected effectiveness factors observed at high modulus values indicate that the enzyme may be concentrated near the lumen. Development of a model for the backflush loaded HFR requires the measurement of radial enzyme distribution in the fiber. Protein distribution may be visualized by permeating the fiber with fluorescein, sectioning it, and examining the sections microscopically (Dennis et al. 1984). If greater resolution and quantification are required, electron microscope autoradiography with radio-isotope labelled enzymes may be employed.

To avoid sacrificing the reactor, it may be desirable to employ an alternative method that permits determining the enzyme distribution by inactivation kinetics (Do and Hossain 1986). The method was developed for catalase, an enzyme that is slowly inactivated by its substrate. The technique might be applied to β-galactosidase by observing the rate of lactose hydrolysis, while slowly poisoning the enzyme with an inactivator.

Enzyme Loading

The experiments conducted in this study demonstrated increases in conversion with enzyme loading. Since the operational regime was approaching diffusion control, increases in production with enzyme loading were not directly proportional. Assuming the enzyme is mostly recoverable and relatively inexpensive, operation approaching a diffusion controlled regime may, in fact, be desirable to obtain maximum conversion in a given fiber configuration. Optimization of enzyme loadings to minimize equipment and operational costs will eventually be required.

Fiber Material

Of the fibers evaluated, the polyamide (PA) fibers performed better than the polysulfone (PM) fibers with respect to the recoverability of enzyme and enzyme inactivation. Since polysulfone fibers appear to inactivate other enzymes (Korus and Olson 1975 and 1977, Kohlwey and Cheryan 1981), utilizing PA fibers should be examined first for immobilizing other lactases.

Cleaning

While polyamide fibers easily withstand a cleaning regime acceptable to the dairy industry, enzyme immobilized in the fibers is susceptible to using sodium hypochlorite as a sanitizer. Since flushing enzyme from the fibers and subsequent reloading are time consuming and result in some loss of enzyme activity, it is desirable to increase, as much as possible, the time interval between cleaning cycles. That time interval is, in part, dictated by the necessity of preventing microbial contamination. Thus it is desirable to find a noninactivating sanitizing agent to reduce of frequency of cleaning cycles. Alternatives include quaternary ammonium compounds that have successfully been used to sanitize cellulose acetate fibers containing β-galactosidase (Pastore and Morisi 1976)

Operation

Method of Operation

Both the small difference between lactose and blue dextran residence times in the HFR and the low apparent specific activity of enzyme in the reactor indicate substantial diffusion limitation to the rate of conversion in the reactor. It may, therefore, be worthwhile to consider

alternative schemes of reactor operation. Alternatively imposing positive cross-membrane pressure on the lumen- and shell-sides of the reactor yields pulsatile convective mass transfer across the membrane (Furusaki et al. 1977, Kim and Chang 1983, Park et al. 1985). This scheme greatly reduces the effect of diffusional mass transfer resistance. In addition to increasing the rate of exchange between the lumen- and shell-sides, bidirectional cross-membrane flow may reduce fouling. Determination of whether such a scheme is more economical than a recycle reactor without a pulsatile cross-flow scheme requires evaluating whether increased conversion justifies equipment, operating, and maintenance costs.

Since the pulsatile scheme may involve filling the shell side with enzyme solution during operation, the potential for shell-side contamination may also increase. That is, the larger hold-up volume may be less easily sanitized by passing biocidal agents through the lumen than the current configuration.

Substrate Solutions

Since the ultimate objective of this project is to evaluate hollow fiber enzyme reactors for dairy use, the behavior of whey permeate, sweet whey, and milk in the system must be evaluated. Operation with lactose solutions provides baseline data, absent from the interfering ions, peptides, and fouling expected with dairy products.

Fouling, in particular, is a concern with dairy products. The accumulation of a relatively thin fouling layer may present a substantial barrier to diffusion across the membrane. Since fouling is a complex interaction of milk components, the membrane, and pressure (Delaney and Donnelly 1977, Horton 1982), low pressure operation cannot

be assumed to eliminate the problem. The effect of fouling on conversion may be observed by repeated operation of the reactor with whey or milk. To demonstrate that fouling and not enzyme inactivation decreases yields, the enzyme's activity in dairy products should be compared with activity in buffer solutions. Also, comparing hydrolysis of milk and sweet whey lactose with similar reactors operated with lactose solution (or possibly whey permeate) is desirable.

Changes in permeation of lactose from dairy products during repetitions of the permeation experiments used to determine diffusivity may be used to directly measure the effect of fouling on mass transfer. The fouling layer may also be visualized by methods similar to those suggested above for the visualization of enzyme distribution.

Several of the recommendations given in the above paragraphs are being studied by other investigators.

Summary

In summary, this thesis presents initial data for application β-galactosidase in a HFR to hydrolyze milk and whey lactose. The important results include the following:

1) The kinetic parameters for A. oryzae β-galactosidase (K_m = 153 mM, V_m = 55 μMol mg^{-1} min^{-1} and K_c = 4.4 mM) predict conversion in batch reactions at 55°C and pH 6.5.
2) Comparisons of the nondiffusing species, blue dextran, and the substrate, lactose, show that lactose mean residence time in the catalytic layer of the reactor is very short compared with its mean residence time in the lumen.
3) While polysulfone fibers apparently retain more enzyme with backflush loading, the enzyme may be more easily recovered from and is not inactivated in contact with polyamide fibers.

4) Enzyme retention following backflush loading in polyamide fibers is enhanced by the addition of BSA to the enzyme stock solution.

5) While flow rate does not change the rate of conversion in the reactor, conversion increases with enzyme loading, but not in direct proportion. Thus the apparent specific activity of the enzyme decreases as loading increases.

6) Although the reactor's operation approaches a diffusion controlled regime, the effectiveness factor is higher than expected for the calculated modulus values. This may be a result of the enzyme forming a tightly packed layer around the lumen during backflush loading.

7) The sanitizer, sodium hypochlorite, inactivates A. oryzae β-galactosidase.

APPENDIX

COMPUTER PROGRAMS

COMPUTER PROGRAMS

The WILKIN program (Figures 36a,b,c) determines K_m and ke_o (Equation 15) by the method of Wilkinson 1961. The equations in the program are found in Tables 1 and 2 of Wilkinson's paper. WILKIN is written in BASIC.

KINDET (Figures 37 a,b,c) predicts conversion as a function of time by iteratively solving Equation 23 to obtain conversion as a function of time. The program also compares the experimental data and predicted values using Chi-squared values (Equation 24). KINDET 1 is written in BASIC.

ERRPROP (Figure 38) predicts likely variance of Kinetics results from Equation 25. The effect of predicted errors was determined by inputting small finite variations to the following system of equations: that describe Kinetics experiments - without inhibition:

$$Papp = \frac{m}{\epsilon_b} OD \tag{38}$$

where Papp is apparent product (glucose) concentration and

$$m = \left(\frac{\nu_{as} + \nu_{gs}}{\nu_{gs}} \right) \frac{OD}{Cg} \tag{39}$$

and

$$OD = \epsilon_b \frac{\nu_s + \nu_{er}}{\nu_s + \nu_{er} + \nu_a} P \tag{40}$$

where P is product concentration.

For Equation 39:

$$Cg = \frac{5.56\ \nu_{gs}}{\nu_g + \nu_{bs}} \tag{41}$$

The quantity P in Equation 40 is obtained by integrating Equation 15:

$$P = -(1 + K_m/So) + [(1 + K_m/s_o)^2 + 2\,(K_m/s_o)^2\,(ke_o t)]^{\frac{1}{2}} \qquad (42)$$

where

$$s_o = \frac{L}{V_L\left[1 + \frac{\nu_B}{\nu_L}\right]\left[1 + \frac{\nu_{eR}}{\nu_s}\right]} \qquad (43)$$

and

$$e_o = \frac{e}{V_e\left[1 + \frac{\nu_s}{\nu_{er}}\right]} \qquad (44)$$

Table 12 lists variables and estimated errors. Errors were converted to fractional errors for operation of program. Temperature and pH effects were estimated from slopes of curves in enzyme data sheets. ERRPROP is written in the BASIC language.

THIELE (Figure 39) determines an effectiveness factor for inhibited enzyme reactions using equations 32 - 35 in Chapter 5. THIELE is written in the FORTRAN language.

```
10 REM statistical estimates in enzyme kinetics per wilkinson, biochem j. 80
   (1961):324
20 PRINT "Statistical estimates for enzyme kinetics (ref: Wilkinson, Biochem J."
      ,"  80(1961):324. Estimates Km and Vmax from substrate concentration,"
30 PRINT "  product concentration, enzyme concentration, and time of reaction."
40 PRINT "  Initial velocity may also be used."
50 DIM S(105),V(100),IDT(100),P(100),IDTS(10)
60 N=0
70 K=0
80 L=1
90 INPUT "Input temperature and inhibitor concentration (mM)    ";TEMP,INH
100 PRINT "If using velocity instead of product concentration,use 1,1 for enzyme
           concentration and reaction time. Additional enz conc and time may be
           input later.
110 L= N+1
120 CLOSE
130 K1=N+1
140 INPUT "enzyme concentration (mg/ml) and reaction time (min)";E,T
150 PRINT "Read from data file (1) or input data manually (2)? If both do manual
 input     first. "
160 INPUT YQ
170 IF YQ = 1 THEN 1310
180 PRINT " input date, substrate concentration (mm) and product concentration
(mm)"
190 PRINT " date = 0 ends input, date = 1 repeats previous date"
200 N = N+1
210 INPUT IDT(N),S(N),P(N)
220 IF IDT(N) = 1 THEN 1560
230 IF IDT(N) () 0 THEN 200
240 N = N-1
250 FOR I = L TO N
260 PRINT I,IDT(I),S(I),P(I)
270 NEXT I
280 INPUT "edit (y=1 or n=2)";YQ
290 IF YQ = 1 THEN 1420
300 INPUT "create a new data file? (yes = 1,no = 2)    ",YQ
310 IF YQ = 1 THEN 1480
320 L = N+1
330 INPUT "Input additional data? (yes =1,  no=2)     ",YQ
340 IF YQ = 1 THEN 180
350 INPUT "input data from data files? (yes=1 no=2)    ",YQ
360 IF YQ = 1 THEN 1300
370 OPEN "a:results" FOR APPEND AS #2
380 PRINT  "Where T=  ";TEMP;"C  and [inhibitor] = ";INH;"mM"
390 PRINT #2, "Where T=  ";TEMP;"C  and [inhibitor] = ";INH;"mM"
400 PRINT #2, "Enzyme concentration = ";E;" mg/ml"
410 PRINT #2, "Reaction time = ";T;" min"
420 PRINT "  date       substrate (mm)  product (mm)  vel(mm/mg min)     1/vel"
430 PRINT #2, "   date       substrate (mm)  product (mm)  vel(mm/mg min)     1/ve
l"
440 FOR I= K1 TO N
450 V(I)= P(I)/(T*E)
460 V(I)= P(I)/(T*E)
470 VI = 1/V(I)
480 PRINT USING " ###.#####    ";IDT(I),S(I),P(I),V(I),VI
490 PRINT #2,USING "  ###.#####    ";IDT(I),S(I),P(I),V(I),VI
500 NEXT I
510 INPUT "Additional enzyme concentrations or times? (yes=1,no=2)  ",YQ
520 IF YQ = 1 THEN 110
530 PRINT " input dates for processing (0 stops selection)"
540 K =0
550 K= 1+K
560 INPUT IDTS(K)
570 IF IDTS(K) = 0 THEN 580 ELSE 550
580 K=K-1
```

Figure 36a. The WILKIN computer program.

```
590 PRINT #2,"results for dates"
600 FOR J = 1 TO K
610 PRINT #2, IDTS(J)
620 NEXT J
630 SX=0
640 A = 0
650 B = 0!
660 G = 0!
670 D = 0!
680 EP = 0!
690 FOR J = 1 TO K
700 FOR I = 1 TO N
710 IF IDT(I) = IDTS(J) THEN 720 ELSE 800
720 X = V(I)^2
730 Y = X/ S(I)
740 SX= SX + X
750 A= A + (V(I)*X)
760 B= B + (X^2)
770 G = G + (V(I)*Y)
780 D = D + (X*Y)
790 EP = EP + (Y^2)
800 NEXT I
810 NEXT J
820 DEL = (A*EP) - (G*D)
830 KMP = ((B*G) - (A*D)) / DEL
840 VMAXP = ((B*EP) - (D^2)) / DEL
850 PRINT #2,"vmax provisional = ";VMAXP,"   km provisional = ";KMP
860 PRINT "vmax provisional = ";VMAXP,"   km provisional = ";KMP
870 F= 0!
880 FP= 0!
890 AL= 0!
900 GA= 0!
910 DE= 0!
920 BE= 0!
930 EP= 0!
940 FOR J = 1 TO K
950 FOR I = 1 TO N
960 IF IDT(I) = IDTS(J) THEN 970 ELSE 1050
970 SK = S(I) + KMP
980 F = (VMAXP * S(I)) / SK
990 FP = -(VMAXP * S(I)) / SK^2
1000 AL = AL + (F^2)
1010 GA = GA + (F*FP)
1020 DE = DE + (V(I) * F)
1030 BE = BE + (FP^2)
1040 EP = EP + (V(I) * FP)
1050 NEXT I
1060 NEXT J
1070 DEL = (AL*BE) - (GA^2)
1080 B1 = ((BE*DE) - (GA*EP)) / DEL
1090 B2 = ((AL*EP) - (GA*DE)) / DEL
1100 VMAX = VMAXP * B1
1110 KM = KMP + (B1*B2)
1120 VAR = (SX - (B1*DE) - (B2*EP)) / (N - 2!)
1130 SD = SQR(VAR)
1140 SEKM = (SD/B1) * SQR(AL/DEL)
1150 SEVM = (VMAXP * SD) * SQR(BE/DEL)
1160 PRINT "km = ";KM;"+/-";SEKM
1170 PRINT #2,"km = ";KM;"+/-";SEKM
1180 PRINT "vmax = ";VMAX;"+/-";SEVM
1190 PRINT #2,"vmax = ";VMAX;"+/-";SEVM
1195 PRINT #2,"     "
1200 INPUT "other date combinations (y=1 or n=2)?"; YQ
1210 IF YQ=1 THEN 530
1220 PRINT "This is your last chance to stay in the program. Do you want to :"
1230 PRINT "  Input more data (type 1)"
```

Figure 36b. The WILKIN computer program.
Page 2

```
1240 PRINT "  Try more date combinations (type 2)"
1250 PRINT "  Get out of this infernal mess (type 3 or any other number)"
1260 INPUT "  Your choice ? "; YQ
1270 IF YQ = 1 THEN 110
1280 IF YQ = 2 THEN 530
1281 FOR I= 1 TO 4
1282 PRINT #2,"  "
1283 NEXT I
1290 CLOSE
1300 END
1310 REM enter data from data file on disc
1320 INPUT "name of data file ? (enter as a:filename if on floppy)    ",N$
1330 OPEN "i",1,N$
1340 N = N+1
1350 INPUT #1, IDT(N), S(N), P(N)
1360 IF EOF(1) THEN 1380
1370 GOTO 1340
1380 CLOSE #1
1390 INPUT "input additional files?(yes = 1, no =2)    ",YQ
1400 IF YQ = 1 THEN 1320
1410 GOTO 370
1420 REM edit input
1430 INPUT "number of line in error",M
1440 INPUT "input correct values for date, substrate and product";IDT(M),S(M),P(
M)
1450 INPUT "other changes (y=1 or n=2)"; YQ
1460 IF YQ=1 THEN 1430
1470 GOTO 300
1480 REM create a new file from input data or adds to existing file
1490 INPUT "enter name of file (enter as a:filename for floppy)  ", N$
1500 OPEN "a",1,N$
1510 FOR I = L TO N
1520 PRINT #1, IDT(I),S(I),P(I)
1530 NEXT I
1540 CLOSE #1
1550 GOTO 320
1560 M=N-1
1570 IDT(N) = IDT(M)
1580 GOTO 230
```

Figure 36c. The WILKIN computer program.
Page 3

```
5 REM  Kindet1 predicts conversion with time from kinetic parameters and
       compares predicted with mean observed conversions.
10 DIM T1(3),T2(3),S1(3),S2(3),CL(5),VL(5),CH(5),VH(5),TIME(5)
20 OPEN "a:data" FOR OUTPUT AS #1
30 KM= 157!
40 SEK = 4!
50 J1 = 2
60 VM  = 56!
70 SEV = 1!
80 J2 = 1
90 KM1 = 510!
100 SEK1 = 10!
110 J3 = 3
120 VM1 = 55.8
130 SEV1 = 1!
140 J4 = 1
150 G1 =10!
160 REM Observed mean values from experimental data
170 CL(1) = 2.08 : VL(1) = .21:TIME(1)=10!
180 CL(2) = 5.58  : VL(2)=.38 : TIME(2) =30!
190 CL(3) = 8.58       : VL(3) =.55: TIME(3) = 60
200 CL(4) = 13.2  : VL(4) =1.51 : TIME(4) = 120!
210 CL(5) = 18.23 : VL(5) =1.67:TIME(5) = 240
220 CH(1) = 10.33 : VH(1) = .98
230 CH(2) = 20.22 : VH(2) = 1.28
240 CH(3) = 30.73 : VH(3) = 1.74
250 CH(4) = 40.38 : VH(4) = 2.53
260 CH(5) = 51.55 : VH(5) = 4.99
270 PRINT "Conversions with time from experimental data."
280 PRINT #1, "Conversions with time from experimental data."
290 PRINT "    Time          (.0125 mg/ml)          (.1 mg/ml)"
300 PRINT "                 Conv.      Var          Conv.        Var"
310 PRINT #1,"    Time          (.0125 mg/ml)          (.1 mg/ml)"
320 PRINT #1,"                Conv.     Var          Conv.        Var"
330 FOR I= 1 TO 5
340 PRINT USING "   ####.## ";TIME(I),CL(I),VL(I),CH(I),VH(I)
350 PRINT #1, USING "   ####.## ";TIME(I),CL(I),VL(I),CH(I),VH(I)
360 NEXT I
370 IF J1>1 THEN  Q1 = 2 * SEK / (J1 - 1) ELSE Q1 = 0
380 IF J2>1 THEN Q2 = 2 * SEV / (J2 - 1)  ELSE Q2=0
390 IF J3>1 THEN Q3 = 2 * SEK1/ (J3 - 1)  ELSE Q3 = 0
400 IF J4>1 THEN Q4 = 2 * SEV1/ (J4 - 1)  ELSE Q4 = 0
410 FOR I= 1 TO J1
420 KS = KM - SEK * (Q1) *(I-1)
430 FOR J = 1 TO J2
440 V = VM - SEV * (Q2*(J - 1))
450 FOR M = 1 TO 3
460 PRINT "  "
470 NEXT M
480 PRINT " WHERE Km = ";KS;"  and Vmax = ";V
490 PRINT #1, " WHERE Km = ";KS;"  and Vmax = ";V
500 FOR K = 1 TO J3
510 KI1 = KM1 - SEK1 * (Q3*(K-1))
520 FOR L = 1 TO J4
530 VI1 = VM1 - SEV1 * (Q4 *(L-1))
540 KI = G1 / ((V/VI1) - 1)
550 IF KI< 0! THEN KI = 10^6
560 KCU = G1 / (V * KI1 / (KS * VI1) -1)
570 PRINT " "
580 PRINT #1, " "
590 PRINT "   Apparent values for 10mM galactose: Km = ";KI1;"  Vmax = ";VI1
600 PRINT #1, "   Apparent values for 10mM galactose: Km = ";KI1;"  Vmax = ";VI1
610 PRINT "            Calculated ku = ";KI;"    kc = ";KCU
620 PRINT #1, "            Calculated ku = ";KI;"    kc = ";KCU
630 GOSUB 700
640 NEXT L
650 NEXT K
```

Figure 37a. The KINDET1 computer program.
Page 1

```
660 NEXT J
670 NEXT I
680 CLOSE
690 END
700 REM Integrate using derived kinetic parameters comp/uncomp model.
710 S0 = 138.9
720 GAL1 = .5556
730 GAL2 = .5556
740 S1(3) = 0!
750 S2(3) = 0!
760 AL1 = 1 / (V * .0125)
770 AL2 = 1 / (V * .1)
780 BE = 1 + (S0 / KI) - (KS / KCU)
790 GA = KS * ( 1 + (S0 / KCU))
800 DE = 1 / (2 * KI)
810 PRINT "    Predicted conversions for competitive/uncomp inhibition"
820 PRINT #1, "    Predicted conversions for competitive/uncomp inhibition"
830 PRINT "                          Vmax = ";AL1;"      Vmax =";AL2
840 PRINT #1, "                             Vmax = ";AL1;"      Vmax =";AL2
850 PRINT "    Time          (.0125 mg/ml)          (.1 mg/ml)"
860 PRINT "                 Conv.      Chi^2        Conv.        Chi^2"
870 PRINT #1,"    Time          (.0125 mg/ml)          (.1 mg/ml)"
880 PRINT #1,"                  Conv.     Chi^2        Conv.        Chi^2"
890 IND =0
900 TI = 10!
910 TIN = 15!
920 VA1T = 0!
930 VA2T = 0!
940 FOR M1 = 1 TO 5
950 IF IND = 1 GOTO 1030
960 S1(2) = S1(1)
970 S1(1)= S0 - GAL1
980 T1(2) = T1(1)
990 T1(1) = AL1 * ((BE * (S0-S1(1))) + (GA * LOG(S0/S1(1))) + (DE * ((S1(1)^2)-(
S0^2))))
1000 IF (T1(2)(TI ) AND (T1(1)) TI ) THEN  GOSUB 1270
1010 GAL1 = GAL1 + .556
1020 CONV1 =  (1 - (S1(3)  / S0)) * 100
1030 S2(2) = S2(1)
1040 S2(1) = S0 - GAL2
1050 T2(2) = T2(1)
1060 T2(1) = AL2 * ((BE * (S0-S2(1))) + (GA * LOG(S0/S2(1))) + (DE * ((S2(1)^2)-
(S0^2))))
1070 IF (T2(2)(TI) AND (T2(1)) TI)  THEN GOSUB 1350
1080 GAL2 = GAL2 + .556
1090 CONV2 =  (1- (S2(3)  / S0)) * 100
1100 IF (ABS(T1(3) - TI) ) .04) OR  (ABS(T2(3) - TI) ).01 ) GOTO 950
1110 VA1 = (CL(M1) - CONV1)^2/ CL(M1)
1120 VA2 = (CH(M1) - CONV2)^2/ CH(M1)
1130 PRINT USING "   ####.## ";TI,CONV1,VA1,CONV2,VA2
1140 PRINT #1, USING "   ####.## ";TI,CONV1,VA1,CONV2,VA2
1150 VA1T = VA1T + VA1
1160 VA2T = VA2T + VA2
1170 TI = TIN
1180 TI = 2* TI
1190 TIN = TI
1200 IND = 0
1210 NEXT M1
1220 VA1T = VA1T/5
1230 VA2T = VA2T/5
1240 PRINT " Chi square for  low enzyme = ";VA1T;"for high enzyme = ";VA2T
1250 PRINT #1, " Chi square for low enzyme = ";VA1T;"for high enzyme = ";VA2T
1260 RETURN
1270 REM Solve for conversion at low enzyme concentration.
1280 S1(3) = S1(2) - ((S1(2) - S1(1)) * (TI - T1(2))/ (T1(1) - T1(2)))
```

Figure 37(b). The KINDET1 computer program (2).

```
1290 T1(3) = AL1 * ((BE * (SO-S1(3))) + (GA * LOG(SO/S1(3))) + (DE * ((S1(3)^2)-
(SO^2))))
1300 IF (ABS (T1(3) - TI)(.035) GOTO 1330
1310 IF  T1(3) - TI )0 THEN S1(1) = S1(3) ELSE S1(2) = S1(3)
1320 GOTO 1280
1330 IND = 1
1340 RETURN
1350 REM Solve for conversion at high enzyme concentration.
1360 S2(3) = S2(2) - ((S2(2) - S2(1)) * (TI - T2(2))/ (T2(1) - T2(2)))
1370 T2(3) = AL2 * ((BE * (SO-S2(3))) + (GA * LOG(SO/S2(3))) + (DE * ((S2(3)^2)-
(SO^2))))
1380 IF (ABS (T2(3) - TI)(.035) GOTO 1410
1390 IF  T2(3) - TI )0 THEN S2(1) = S2(3) ELSE S2(2) = S2(3)
1400 GOTO 1360
1410 RETURN
1420 INPUT " Uninhibited  Km and SE and interval# " , KM,SEK,J1
1430 INPUT " Uninhibited Vmax and SE and interval # ", VM,SEV,J2
1440 INPUT " 10 mM gal Km and SE and interval #  ", KM1,SEK1,J3
1450 INPUT " 10 mM gal Vmax and SE and interval #  ", VM1,SEV1,J4
```

Figure 37(c). The KINDET1 computer program (3).

```
5 REM Propagation of error analysis for determination of glucose appearance
      in enzyme reaction solution.
10 DIM V(18),E(18),N$(18),PA(2),M(2)
20 FOR I= 1 TO 18
30 READ N$(I),V(I),E(I)
40 NEXT I
41 KM=50
42 K = 30
43 EB = V(2) / (5.56 * V(6)/ (V(6) + V(8))) * (1 + V(3)/V(4))
44 VAR = 0
45 PRINT "  variance                magnitude     standard      df       variance"
46 PRINT "  source                                deviation     da"
50 FOR I = 1 TO 18
60 SA = V(I)
70 DIFF = V(I) * .0001
80 FOR J = 1 TO 2
90 EO = V(14) / (V(15) * (1 + V(5)/V(13)))
100 SO = V(11) / (( 1 + V(10)/V(9)) * (1 + V(13)/V(5))* V(12))
110 CG = 5.56 * V(6)/ (V(6) + V(8))
120 P = (-(1+(KM/SO)) + SQR (( 1 + (KM/SO))^2 + (2*KM*K*EO*V(16)/(SO^2)))) / (KM
      /SO^2)
130 M = V(2) / CG * (1 + V(3)/V(4))
135 V(I) = EB * P * (1 / (1 + V(7)/ (V(5) + V(13))))
140 IF (I = 1 ) AND (J = 2) THEN V(1) = V(1) + DIFF
150 PA(J) = V(1)* M/ EB
160 V(I) = V(I) + DIFF
170 NEXT J
171 IF (I()17) AND (J()2) THEN 174
172 DF = .0063
173 GOTO 190
174 IF (I()18) AND (J()2) THEN 180
175 DF = .82
176 GOTO 190
180 DF = (PA(2) - PA(1))/ DIFF
190 ER = (DF * E(I))^2
200 PRINT  N$(I);USING "  +#.###^^^^";V(I),E(I),DF,ER
210 VAR = VAR + ER
220 V(I) = SA
230 NEXT I
235 PRINT "Where substrate concentration = ";SO;" mM"
236 PRINT "  product concentration = ";P;" mM with "
240 PRINT "  variance = ";VAR
250 SD = SQR(VAR)
260 PRINT "  Standard deviation = ";SD
270 DATA " OD of sample          ",1.2,0.001
280 DATA " OD of standard        ",0.278, 0.00056
290 DATA " ml analytic sol std   ",5.0, 0.078
300 DATA " ml standard sol       ",0.5,0.022
310 DATA " ml substrate sol      ", 0.5, 0.022
320 DATA " ml glucose sol in std",0.5,0.022
330 DATA " ml analytic sol prod ",5.0, 0.078
340 DATA " ml buffer in std      ",9.5, 0.078
350 DATA " ml lactose stock sol ",16.0, 0.141
360 DATA " ml buffer in substrate",  4.0,.078
370 DATA " mMol lactose in stock" , 277.8,.02778
380 DATA "  l buffer in stock    " , 1.00,.0021
390 DATA " ml enzyme sol in react", .005,.0001
400 DATA " mg enzyme in sol      ", 50.,0.14
410 DATA " ml enzyme sol prep    ", 100.,0.237
420 DATA " time (min)            ", 10. ,0.25
430 DATA " normalized temperature", 0.0,0.5
440 DATA " normalized pH         ", 0.0,0.05
450 END
```

Figure 38. The ERRPROP computer program.

Table 12. ERRPROP program variables

Measured Variable	Value Ranges: Variable Magnitude	Value Ranges: Estimated Error (+/-)
OD = absorbance of product	0.2 - 1.0	0.0004 - 0.002
OD_s = absorbance of standard	0.3 - 1.0	0.0011
ν_{as} = volume of standard analyte	5 - 10 ml	0.078 ml
ν_{gs} = volume of diluted glucose solution in standard analyte	0.5 ml	0.022ml
ν_s = substrate solution reaction volume	0.5 ml	0.022ml
ν_g = standard glucose solution volume	0.5 - 1 ml	0.022ml
ν_a = volume of reaction solution analyte	5ml	0.078ml
ν_{bs} = buffer volume in standard	4 - 19ml	0.078 - 0.141ml
ν_L = lactose stock solution volume in reaction	2 - 16ml	0.078 - 0.141ml
ν_b = buffer volume in reaction	4 - 18ml	0.078 - 0.141ml
L = lactose weight in stock solution	100g	0.01g
V_L = lactose stock solution volume	1000ml	2.09ml
ν_{er} = enzyme solution volume in reaction	5μl	0.094μl
e = enzyme weight in stock solution	50mg	0.14mg
V_e = volume stock enzyme solution	100ml	0.297ml
t = time	10 min	0.25 min
T = temperature in reaction	59.5°C	0.3°C
pH = pH	6.5	$\pm$ 0.05

```
C       THIELE.for15
c       Determine inhibitted enzyme reaction Thiele modulus from
c       kinetic and diffusion parameters and determine effectiveness factor
c       from data
        DIMENSION UV(100),PI(100),TM(100),E(100)
C       INITIALIZE VALUES
        N=7
        E0=0.4
        eKM=153.
        EKENZ= 55.
        EKI= 4.4
        S0= 138.9
        VOL1 = 0.4473
        VOL = 0.4473
        SA = 0.3512
        DS = 2.9E-06
        ZETA = 3.7

C       READ DATA AND CALCULATE MODULUS AND EFFECTIVENESS
        DO 50 I=1,N
        READ (23,*)PI(I),UV(I)
 50     CONTINUE
        DO 100 I=1,N
C       CALCULATE EFFECIVENESS FACTOR
                VM = EKENZ * E0 / VOL1
                SI = S0 - PI(I)
                VP = EKENZ * SI / (SI + EKM*(1+PI(I)/EKI))
                E(I) = UV(I)/VP
C       CALCULATE THIELE MODULUS
                EL = VOL/ SA
                H = EL * SQRT(VM / (120*DS*SI))
                A1 = EKM / SI
                A2 = SI / EKI
                W = (PI(I)/SI) + ZETA
                B1 = A1 * (1 + (W*A2))
                B2 = 1 - (ZETA * A1 * A2)
                EI2 = 1 / (B2**2) * (B2 - (B1*LOG((B1+B2)/B1)))
                DV = SQRT(EI2)
                TM(I) = (H / (B1+B2)) * (1/ DV)
        WRITE (24,*)TM(I),E(I)
 100    CONTINUE
        STOP
        END
```

Figure 39. The THIELE computer program.

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BIBLIOGRAPHY

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