LACTOSE HYDROLYSIS BY IMMOBILIZED WHOLE CELLS OF *K. LACTIS* CBS 2357

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ABSTRACT

The application of immobilized yeast for lactose hydrolysis was investigated. The enzyme stability was tested as a function of pretreatment. The stability of *K. lactis* CBS 2357 cells after treatment with glutaraldehyde (GA) and the β-galactosidase activity of whole cells after immobilization in alginate bead and corn particles were studied.

Permeabilization using ethanol and chloroform (10% and 2%, respectively) at 37 °C and 120 rpm for 5 min, followed by stabilization with 10 mM glutaraldehyde at 30 °C for 1 hour with gently shaking deactivated 2.5% of the initial whole cells β-galactosidase activity, tested with the ONPG method. The glutaraldehyde treatment could significantly maintain β-galactosidase activity in phosphate buffer pH 6.5 containing 0.1 mM $MnCl_2$. Manganese and potassium ions in the Mn-Buffer were found to be essential to enhance the activity. The biomass activity of GA stabilized cells in Mn-Buffer can be maintained above 70% during 72 hours of incubation at 30 °C. An increase of incubation temperature from 30 to 37 °C deactivated 10% of biomass activity after 72 hours.

Direct stabilization of alginate biocatalyst with glutaraldehyde caused a significant reduction of β-galactosidase activity with the resulting deactivation depending on glutaraldehyde and alginate concentrations. When 40 g of biocatalyst containing $2x10^9$ cells/g alginate was stabilized in 100 ml of 0 to 4 mM glutaraldehyde, the optimum range of glutaraldehyde concentration was between 0.5 to 1.0 mM. When this concentration range was applied to stabilize 2%- to 3%-alginate biocatalyst, the average biocatalyst activity remained within 56-74% of the initial activity.

It was shown that the adsorption of *K. lactis* on corn particles through a "double liquid cultivation stage" followed by permeabilization of biocatalyst gave a higher activity. The activity obtained was 0.84 μmol lactose hydrolyzed /min/g biocatalyst under the conditions tested. This activity was about 5 times higher than the case without permeabilization and about 2 times higher than that of the permeabilized biocatalyst prepared with a "single liquid cultivation stage". When tested in the packed-bed reactor, during the initial stages the degree of hydrolysis (d.h.) was 45% within the operational conditions tested. Free enzyme was detected during the first 5 hours of operation, especially when non-stabilized corn biocatalyst was used. After 5 hours, free enzyme was no longer detected in the reactor outlet, suggesting that direct adsorption might have rendered good cell confinement inside the corn particles.

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(In the name of Allah, Most Gracious, Most Merciful)

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INTRODUCTION

β-Galactosidase is one of the most studied enzymes from a scientific and technological point of view (Richmond, et al., 1981; Gekas and Lopez-Leiva, 1985; Berger, et al., 1995). This enzyme is one of the best examples for studying enzyme kinetics and modeling. This enzyme has been applied to the hydrolysis of lactose into its isomolecular mixtures comprising glucose and galactose (Carrara and Rubiolo, 1994). More recently, it has gained much importance for its application in the synthesis of some promising oligosaccharide compounds via a galactosyl transfer reaction (Berger, et al., 1995; Sheu, et al., 1998).

Lactose is found abundantly in milk and whey. There are two main reasons for the intensive research on β-Galactosidase and its substrate lactose (Santos, 1988; Carrara and Rubiolo, 1996; Siso, 1996). Firstly, lactose is a sugar fastidiously digestible by non-Caucasian people. Secondly, because whey and other dairy wastes are by-products of the dairy industry with high BOD's, they create a major problem for their disposal. In addition, the physical characteristic of lactose to be easily crystallized is troublesome in some processes in the dairy industry. To overcome this, lactose must be hydrolyzed into its more soluble constituents that offers technological advantages, such as improvement of the sweetness and solubility thereby increasing its usability.

There are two hydrolysis methods used so far. The first is the use of acid at a high process temperature (150 °C), and the second is the use of enzyme operated at a temperature between 4-70 °C. The use of enzymes has several advantages over acid hydrolysis; i.e. no brown color formation, the protein present in the substrate is not denatured, and formation of undesirable by-products can be prevented. For these reasons, enzyme use is becoming the more popular method in the food industry (Santos, et al., 1988; Siso, 1996).

β-Galactosidase (EC 3.2.1.32) is an intracellular enzyme that exists in many sources. Animal and plant sources are not taken into account for commercial use due to their high cost and low productivity. β-Galactosidases from microorganisms are the first choice because they can be produced in a large quantity. However, not all β-

Galactosidases from microorganisms can be used in the food industry, but only those which are already considered safe (GRAS). The most acceptable β-Galactosidase enzymes are derived from the yeasts *Kluyveromyces* sp. (*K. lactis, K. marxianus* and *K. fragilis*) that can be applied in neutral pH (between pH 6.5–7.0). These enzymes are suitable for the hydrolysis of milk and sweet whey.

Recently, the use of whole cell to accomplish lactose hydrolysis has gained much interest in developing the process technology for lactose hydrolysis; therefore the cost of extracting the intracellular enzyme from yeast is reduced (Siso, 1996). However, the reported results so far have shown that the stability of whole cell biocatalyst is not comparable yet to that conferred by immobilized bulk β-Galactosidase, which can maintain the degree of hydrolysis required in the process range for long periods of time.

The objectives of this research are:

- To study the activity of whole cell β-galactosidase from yeast *Kluyveromyces* sp.;
- To test the stability of treated whole cell biocatalyst on storage at operational temperatures (30 and 37 °C);
- To find an effective immobilized whole cell β-Galactosidase for lactose hydrolysis in a packed-bed reactor.

Chapter 1
LITERATURE REVIEW

1.1 LACTOSE

1.1.1 General Properties

Structurally, lactose [4-(β-D-galactopyranosyl)-D-glucopyranose] is a disaccharide comprising two monomers glucose and galactose, joined by β-1.4-glycosidic linkage (Stryer, 1988). Lactose is a class of non-sweet sugar. It has a low solubility; therefore it can not be absorbed readily from the intestine. In contrast, a mixture of glucose and galactose as a lactose hydrolyzed product has a sweetening power of about 0.8 relative to sucrose. In addition, these monosaccharides are 3 to 4 times more soluble than lactose and well absorbed from the intestine (Nijpels, 1981).

1.1.2 Sources and Functions

Lactose, commonly known as milk sugar, is found in mammalian milk and separated from whey as a by-product of the cheese industry (Shukla, 1975). Increasing world cheese production generates more than 115 million tons of liquid whey per year, of which 6 million tons is lactose (Castillo, 1990). Lactose can be produced from cheese whey or permeate by crystallization

Lactose is commonly used as a supplement in baby milk and as an excipient for pharmaceutical products, such as capsules and tablets. However, world consumption of purified lactose can be realized from 5% of the whey available (Coton, 1980; Moulin and Galzy, 1984). Lactose is also used as a raw material to produce derivatives such as lactitol, lactulose, lactosyl urea, lactobionic acid and gluconic acid through chemical modification (Castillo, 1990). Figure 1.1 illustrates the possible utilization of cheese whey (Siso, 1996).

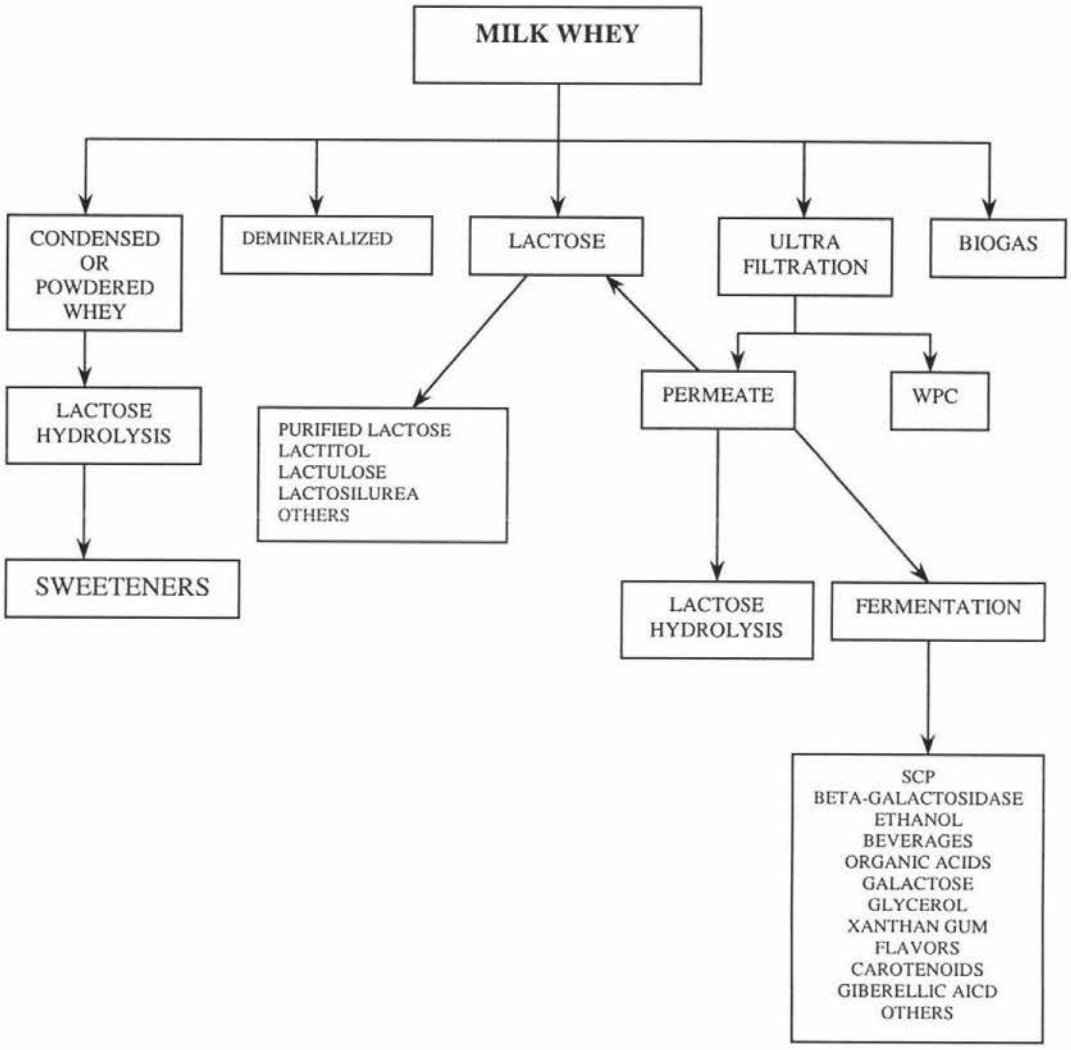


Figure 1.1 The established commercial and currently-under-study utilization of cheese whey (Siso, 1996).

1.1.3 The Lactose Problems

There are several problems due to the presence of lactose in food:

a. Intolerance problem

The deficiency of β-galactosidase in certain ethnic groups is a great concern since it causes some implications especially for those with a deficiency in intestinal β-galactosidase (Shukla, 1975). Excessive intake of lactose, when consumed by these groups cannot be hydrolyzed in the intestine and passed on to blood circulation, which is finally excreted in the urine. The path of lactose through the large intestine has been found to be the prominent cause for tissue dehydration due to osmotic effects; poor

calcium absorption because of acidity; fermentative diarrhoea with a bloated feeling, flatulence, belching, and cramps; and watery explosive diarrhoea. β-Galactosidase has been administered orally to treat β-galactosidase-deficient people in the past, but then it was rejected due to allergic reaction (Bodalo, et al., 1991).

Shukla (1975) concluded that based on the data collected in 1970 many more ethnic groups in the world are intolerant to lactose than are tolerant. These tolerant represent only a small number of people, restricted to northern european and nomadic pastoral tribes in Africa, where approximately 90% and 80%, respectively, are tolerant.

b. Waste disposal problem

Disposing of acid and sweet whey as waste in the dairy industry becomes major problem because of the high BOD (Carrara & Rubiolo, 1996; Siso, 1996). Cheese whey is the liquid portion that remains following the precipitation and removal of milk casein during cheese processing, which represents 85-95% of the milk volume. This byproduct contains milk nutrients with the major nutrient lactose (4.5-5% w/v), soluble proteins (0.6-0.8% w/v), lipids (0.4-0.5% w/v) and mineral salts (8-10% of dried extract) (Siso, 1996).

Although many possibilities of utilizing cheese whey have been explored over the last 50 years, approximately half of world cheese whey production is disposed of as effluent or dumped into the sewage system (Cheetam, 1994; Siso, 1996). Cheese whey is a major problem for disposa systems because of the large volume produced and the high organic content. It exhibits a BOD_5 of 30000-50000 ppm and COD of 60000-80000 ppm with lactose being largely responsible for the high BOD/COD (Decleire, et al., 1985; Cheetam, 1994; Siso, 1996).

c. Lactose crystallization problem

Technically lactose is crystallized easily. This sets limitations to certain process in the dairy industry and influences the quality of dairy products (Gekas and Lopez-Leiva, 1985). The crystallization of lactose in the dairy products can develop a mealy, sandy, or gritty texture. The crystallization of lactose in sweetened condensed milk, concentrated whey products, and preserved concentrated ice creams is generally not prevented and the problem is acute. However, the treatment with β-galactosidase prior to the condensing operation decreases the lactose content, which prevents lactose

crystallization (Shukla, 1975). The sweetening power of the hydrolyzed products favors the use of whey in food products (Kosaric and Asher, 1985).

1.1.4 Lactose Hydrolyzed Products

Enzymatic hydrolysis of lactose into glucose and galactose with simultaneous protein removal is the most reliable method to eliminate lactose content in milk and milk by-products (Bodalo, et al., 1991). Richmond et al. (1981) suggested that the use of β-galactosidase (either free or immobilized) could improve the quality as well as processing economics in many products. They mentioned application of hydrolyzed milk as a fluid product and a skim milk powder and also the use of hydrolyzed milk in cultured diary products. Gyuricsek and Thompson (1976) utilized enzymatic hydrolyzed lactose milk to make yogurt, buttermilk and cottage cheese. During the process time required to reach the desired pH was considerably reduced compared to that of conventional methods. In addition, hydrolyzed lactose cheese was favored over plain yogurt.

MacBean (1979) reviewed the literature on lactose crystallization and hydrolysis. He discussed the utilization hydrolyzed syrups derived from whey or ultrafiltrate, and listed many products that could be prepared using this process including flavored yogurt, imitation maple syrup, juices, canned fruits, wine and beer. Gekas and Lopez-Leiva (1985) reviewed the application of lactose hydrolyzed whey as a source of sugar, and in some cases, of protein in bakery products, in confectionery, in soft fruit drinks, in ice cream, in feedstuffs for cattle replacing molasses, in dairy desserts, or as a basis for further fermentation to alcohol. They also mentioned high quality syrup with 80% total solids could be prepared from cottage cheese whey through deproteination, hydrolysis, decolorisation and concentration. When given to vanilla ice cream, this syrup gave a quality corresponding to that of maize syrup.

The use of β-galactosidase for new product development as reviewed by Shukla (1975) and Richmond, et al. (1981) is listed in Table 1.1.

Table 1.1 The use of β-galactosidase

No	Products
1	Low lactose milk processing
2	Low lactose diary products
3	Low lactose yogurt
4	Sweetened yogurt
5	Low lactose concentrate for ice cream
6	Lactose processing of acid and sweet whey
7	Food syrups and sweetener manufacture
8	Lactose treatment during cheese making

1.2 THE ENZYME β-GALACTOSIDASE

1.2.1 General Properties of β-Galactosidase

β-Galactosidase is a common name for the enzyme β-D-Galactosidase or previously known as β-D-Galactosidase galactohydrolase (EC.3.2.1.23). This enzyme hydrolyzes lactose and other β-Galactosides such as *o*-nitrophenyl-β-D-Galactopyranoside (ONPG). The enzymes catalyze also transgalactosidation to form up to 11 different oligosaccharides corresponding to about 11% of the original lactose (Gekas and Lovez-Leiva, 1985; Castillo, 1990). Solid material of β-galactosidase can be obtained in crystalline form and is easy to prepare. This enzyme has been extensively studied by biochemists and geneticists because it offers the best example of induced enzyme synthesis (Shukla, 1975; Stryer, 1988). Shukla (1975) pointed out that the structure, size and optimum conditions for lactose hydrolysis of β-Galactosidase are not identical from one source to the other. All such enzyme systems, however, carry out the hydrolysis lactose into glucose and in some cases catalyze the formation of oligosaccharides through the transfer of sugar residues to another sugar or alcohol. The advantage over differences in optimum pH and process temperature is that a particular process can be adapted according to the requirement imposed by the nature of the product and substrate. For example, β-galactosidase from *Aspergillus* with the pH optimum 3.5-4.0 is suitable for lactose hydrolysis in acid whey, while that from yeast with the pH optimum close to 7.0 is preferred for saccharifying whole milk, skim milk or sweet whey. From an industry point of view, however, there is a disadvantage associated with

the high cost of enzyme extraction and low yield obtained from intracellular yeast β-galactosidase due to enzymatic instability (Fenton, 1982; Gekas and Lopez-Leiva, 1985; Gonzales and Monsan, 1991).

Molecular weight of β-galactosidases lie between 200000 to 700000 (Shukla, 1975; Castillo, 1990). The large molecular weight gives rise the assumption of the presence of more than one sub-unit as proposed for *K. fragilis* (Mahoney and Whitaker, 1978) and *K. lactis* (Dickson, et al., 1979). Temperature is considered dictating the number of active sites per molecule β-galactosidase, with the number of active sites at 4-6 °C = 1.0 and at 20-22 °C = 4.7, suggesting that some active sites are turned-off at low temperatures. The number of subunits in *E. coli* β-galactosidase was proposed to be 4, known as tetrameric, whilst those of the other sources may or may not have subunit structures. For instance, *Streptococcus lactis* β-galactosidase has two unidentical subunits, but *Thermus aquaticus* β-galactosidase, a class of extreme thermophile, is unlikely to have subunit structure (Bodalo et al., 1991).

1.2.2 The Sources of β-Galactosidase

β-Galactosidases can be found in a variety of sources consisting of plants, animal organs, bacteria, yeast, fungi or moulds. However, there are various industrially important microorganisms that are capable of metabolizing monosaccharides such as glucose and galactose than small number of microorganisms that are directly utilizing lactose as sole C-source and energy (Shukla, 1975; Richmond, et al., 1981; Gekas and Lovez-Leiva, 1985; Siso and Doval, 1994; Siso, 1996). There are at least 98 yeast species grouped into 19 genera capable of metabolizing lactose. Lactose utilization requires two sets of enzymes, i.e. β-galactosidase and permease. β-Galactosidase activity has been identified in *Saccharomyces cerevisiae* strains, but these strains can not utilize lactose as a sole carbon source because they lack lactose transport systems (Castillo, 1990). Among β-galactosidases from bacteria, *E. coli* β-galactosidase is the most studied enzyme; but this enzyme is not considered as safe in food industry (GRAS) because of the digestive problems caused by the bacteria (Gekas and Lopez-Leiva, 1985; Santos, et al., 1998).

The possible sources of β-galactosidase are shown in Table 1.2 (Shukla, 1975; Richmond, et al., 1981; Gekas and Lovez-Leiva, 1985; Castillo, 1990).

Table 1.2 The source of β-galactosidase

Plants :
Peach
Apricot
Almond
Kefir grains
Tips of wild roses
Alfalfa seeds
Coffe berries
Animal organs :
Intestine
Brain and skin tissue
Yeast :
Kluyveromyces lactis
Kluyveromyces fragilis
Kluyveromyces marxianus
Candida pseudotropicalis
Brettanomyces anomalus
Wingea roberstsii
Bacteria
Escherichia coli
Bacillus megaterium
Thermus aquaticus
Streptococcus lactis
Streptococcus thermophillus
Lactobacillus bulgaricus
Lactobacillus helveticus
Bacillus sp.
Bacillus circulans
Bacillus stearothermophillus
Lactobacillus sporogenes
Fungi
Neurospora crassa
Aspergillus foetidus
Aspergillus niger
Aspergillus flavus
Aspergillus oryzae
Aspergillus phoenicis

Mucor pucillus
Scopuloriopsis
Alternaria palmi
Curvularia inaegualis
Fusarium moniliforme
Alternaria alternara

Some β-galactosidase producing strains that are considered to be safe are *Aspergillus niger*, *A. oryzae* and *Kluyveromyces* sp. (*lactis* or *fragilis*). These sources have been shown to be safe for use and have been tested for their adverse effects (Pariza and Poster, 1983). Yeasts, especially *Kluyveromyces lactis* and *K. fragilis* are the most important source of β-galactosidase (Castillo, 1990; Siso and Doval, 1994).

There are several reasons why microorganisms have become the most appropriate source of enzymes for industrial purpose (Chibata, et al., 1986): the production cost is low, the conditions for production are not restricted by location and time, production time is short, and mass production is possible. In terms of productivity on commercial scale, the selection of suitable strains of microorganism is a limiting factor. The strain selected should be able to produce a high amount of the desired enzyme (Vojtisek, 1989).

1.2.3 Yeast β-Galactosidase Stability and Activity

1.2.3.1 Storage Stability

There are numerous publications that report the effect of environment on the stability and activity of β-galactosidase. The pure enzyme of *Kluyveromyces fragilis* β-galactosidase was relatively unstable compared to a crude preparation, e.g. cell free extract which was stored at –20 °C for one year without loss of activity (Mahoney and Whitaker, 1977). β-Mercaptoethanol retains the β-galactosidase stability because this compound can protect the sulfhydril groups of the enzyme from oxidation or other chemical inactivation during storage (Lencki, et al., 1992).

Siso and Doval (1994) on their study about whole cell *K. lactis* immobilized to corn grits through covalent linkage found that the immobilized biocatalyst lost 10% of its β-galactosidase activity during the first month of storage in phosphate buffer at pH 7.0, 4 °C. However, a complete disappearance of activity was observed within 1 week in free cells stored under the same conditions. Treating the free cells with glutaraldehyde could significantly improve their storage stability. When this treatment was applied to *K. lacits*, the activity decreased by 20% in phosphate buffer containing 0.1 mM manganese ions after 14 months storage at 4 °C (Champluvier, et al., 1988). This finding was tested by Flores, et al. (1995) to prove that the treatment with glutaraldehyde maintained the stability for at least 6 months storage at 5 °C without loss of activity.

1.2.3.2 The Effect of pH

Food-grade β-galactosidases isolated from *Kluyveromyces* sp. have a neutral pH optima, and hence are suitable for the hydrolysis of sweet whey and milk (Tomaska, et al., 1995b). The optimum pH for *K. lactis* β-galactosidase ranges from 6.5 to 6.8. Below pH 5.9 enzymatic activity declines rapidly (Guy and Bingham, 1978). *Kluyveromyces fragilis* β-galactosidase was found to be stable at pH 6.5 – 7.5 for at least 10 minutes at 37 °C (Mahoney and Whitaker, 1977). They also found pH below 6.5 and above 8.0 to be the range in which the enzyme was rapidly inactivated.

Siso and Doval (1994) found that pH 7.0 was optimum for whole cell *K. lactis* immobilized on corn grits, with marked drop of activity on both sides of the optimum. The optimum pH for whole cell preparation of *K. lactis* was 6.5-6.6 (Champluvier, et al. 1988; Flores, et. el., 1995). Champluvier, et al. (1988) also discovered the whole cell *K. lactis* biocatalyst exhibited the highest stability when stored in phosphate buffer pH 6.5 – 7.0 containing 0.1 mM manganese ion. In contrast, Flores et al. (1995) found the pH range displaying the highest stability for glutaraldehyde treated cells was at pH 7.0–7.5 and declined continuously as pH decreased.

1.2.3.3 The Effect of an Ionic Environment

The effect of several cations on the stability of soluble and whole cell *Kluyveromyces lactis* β-galactosidase have been tested (Guy and Bingmam, 1978; Voget et al., 1994).

Several divalent cations have a strong stabilizing effect on β-galactosidase when Mn^{2+}, and to a lesser extent Mg^{2+}, were included in the potassium phosphate buffer pH 6.6. The optimum concentrations of Mn^{2+} and Mg^{2+} were 0.1-0.2 mmol/L and 2.5-5.0 mmol/l, respectively. Higher concentrations of Mg^{2+} were found to be destructive for the enzyme. The presence of K^{+} increased the activity of the enzyme. In contrast, Na^{+} and Ca^{2+} significantly reduced the activity.

Of all the tests carried out by Voget et al. (1994), the whole cell preparation of *K. lactis* was proven to be more resistant toward deactivation by divalent cations than the corresponding cell free enzymes. This could be explained by the fact that whole cells have ligand groups in the cell structures that are capable of capturing the destructive cations available.

1.2.3.4 The Effect of Temperature

Generally, yeast β-galactosidases are only stable at a low reaction temperature, ranging from 4-35 °C. That temperature range may limit their application because low temperature is usually accompanied with lower efficiency of hydrolysis. Yet an appreciable activity of *K. lactis* was found at 4-6 °C, which is of practical importance since low temperature commonly suppresses the growth of spoilage bacteria (Nijpels, 1981). In fact, the operation at moderate temperature is more prone to contamination (Tomaska, et al., 1995b). The activity of *K. lactis* β-Galactosidase was no longer detected after it was heated in phosphate buffer at 70 °C for 1 min (Guy and Bingham, 1978).

Tomaska, et al. (1995b) attempted to improve the temperature stability of the whole cell *K. marxianus* β-galactosidase. To do this, the whole cell was permeabilized with a mixture of chloroform-ethanol (1:9), followed by stabilization with 0.4% glutaraldehyde. They found that both the cell-free enzyme and whole-cell preparations exhibited the maximum activity at 50 °C and 52 °C respectively, for a 5 hour testing period in batch mode. In contrast to that optimum, the most stable temperature for whole cell *K. marxianus* β-galactosidase was 45 °C at which about 60-70% of the original activity was retained after 5 hours. On the other hand, soluble β-galactosidase was nearly totally inactivated within 4 hours. Tomaska, et al. (1995a) proposed that an

intracellular protection mechanism might have prevented β-galactosidase from temperature destruction. On the effort of improving the stability of β-galactosidase, they immobilized the permebilized cells in calcium alginate and then hardened the beads with polyethyleneimine and glutaraldehyde. By carrying out the same procedures, they found the immobilized whole cell β-galactosidase system to be relatively stable to perform 25 hydrolysis cycles with the conversion of lactose varied between 80-88%.

The temperature ranging from 30 to 42 °C was suitable for milk whey saccharification using permeabilized *K. lactis* whole cells covalently attached to corn grits. In that temperature range, 90% hydrolysis of the lactose was achieved within 6 hours; but the hydrolysis of lactose using purified and immobilized β-galactosidase was less than 75% (Siso and Doval, 1994).

1.2.4 Immobilization of β-Galactosidase

1.2.4.1 Immobilization Method

Immobilized enzyme systems allow enzymes to be utilized more efficiently because they are made in such a way that resembles ordinary solid phase catalysts used for synthetic chemical reactions. The hydrolysis of lactose in one phase (aqueous phase) with soluble enzyme is uneconomical because β-galactosidase can perform only one batch reaction. This has encouraged much research into developing heterogeneous phase where enzymes are immobilized on solid support or polymerized using insoluble material (Siso, 1996). In immobilized enzyme systems, the catalyst can be re-utilized many times thereby reducing cost. Immobilization of pure β-galactosidase is already well established compared to whole cell immobilization. As an example, β-galactosidase entrapped in fibres of polymers has been successfully used on an commercial scale (Gekas and Lopez-Leiva, 1985).

There are several immobilization techniques and these are summarized below.

(a) Covalent attachment

This method involves the reaction between a nucleophilic group (usually amino group) of the enzyme or cells with an electrophilic group (such as hydroxylic, carboxylic, or amino groups) that is already available or previously formed on a solid carrier through a

proper activation process. Examples of activating agents include cyanogen bromide, ethyl chloroformate, and glutaraldehyde. This method offers the advantage of a strong attachment, therefore preventing leakage. However, the preparation procedure is rather complicated and therefore expensive. The most common method used is β-galactosidase co-polymerization with glutaraldehyde as a bifunctional cross-linking agent that is claimed to be safe in the food industry (Gekas and Lopez-Leiva, 1985; Chibata et al., 1986).

(b) Adsorption

In this method, enzyme or cells are simply attached to solid carriers through adsorption. Hydrogen bonds or Van der Waals forces are thought to be the binding forces. This is an inexpensive method. However, desorption and leakage of enzyme is hardly prevented due to weak forces, especially as the pH and temperature change (Wang, et al. 1979; Gekas and Lopez-Leiva, 1985).

(c) Adsorption and cross-linking

Since cross-linking alone is an inefficient method, the combination of adsorption and cross-linking can improve the stability of the adsorbed enzyme or cells. The common cross-linking agent is glutaraldehyde (Wang, et al., 1979; Gekas and Lopez-Leiva, 1985; Chibata et al., 1986).

(d) Entrapment

Enzyme or cells are entrapped within the internal structure of a solid material (usually polymers) in the form of gels, microcapsules, fibres, films or a membrane. In some cases, the polymer structure is cross-linked with bifunctional agents to strengthen the bound enzyme. In this method, enzyme can leak out through diffusion, even with small pore sizes in the matrix. By its simplicity, this method is preferred for whole cell immobilization (Gekas and Lopez-Leiva, 1985; Bucke, 1986; Chibata, et al., 1986).

(e) Ultrafiltration membrane retainment

In this method, a membrane unit is used to retain the soluble enzyme in a liquid. The low molecular weight substances cross through the membrane freely. This makes possible the re-use of the enzyme, which characterizes the bound enzymes (Gekas and Lopez-Leiva, 1985).

1.2.4.2 Immobilized soluble β-Galactosidase

β-Galactosidases derived from different sources have been immobilized by numerous techniques but with a varying degree of success (Shukla, 1975; Gekas and Lopez-Leiva, 1985).

Gekas and Lovez-Leiva (1985) have summarized the research on the immobilization of β-galactosidase in the period of 1978-1984. In their list, the β-galactosidase from several sources have been immobilized employing various methods and tested using different substrates (ONPG, lactose, acid whey, raw whey, whey permeate, skim milk, demineralized whey). The content of Table 1.3 accommodates only those using lactose or lactose-containing substrates as the test substrates.

Table 1.3 The important immobilized β-galactosidase systems developed during 1978-1984 (Gekas and Lopez-Leiva, 1985)

Source	Carrier	Method/Immobilization Agent	Substrate	Load and Activity Results
A. niger	Phenol-formaldehyde resin	Adsorption/cross-linking with Glutaraldehyde Fluidized bed reactor	Acid whey	20 mg IME/g, 70% hydrolysis, 0.6 kg lactose/kg IME/hour
A. niger	Porous alumina	Adsorption/cross-linking with GA. Pilot Plant. Fluidized bed reactor	5% lactose solution	50% binding
A. niger	Phenol-formaldehyde, resin, particle size: 0.25-0.35 mm	Adsorption/cross-linking. Jacketed glass column. Industrial experience 80.000 L whey/day	Whole whey, whey UF-permeate	25 mg/g of resin, 220U/g, 70% for UF-whey, 39% for whole whey
Bacillus circulans	Phenol formaldehyde, resin Duolite ES 762	Adsorption/cross-linking with Glutaraldehyde	Skim milk	225 U/g wet resin
A. niger	Phenol formaldehyde, resin Duolite ES 762	Adsorption/cross-linking with Glutaraldehyde	Lactose solution, whey	250 mg/g dry resin, 4000 U/g dry resin. Half-life for whey: 120 days.
E. coli	Egg white powder	Adsorption/cross-linking with Glutaraldehyde	Lactose solution, whey	50% conversion in 8 hours
A.niger	Feather protein	Adsorption/cross-linking with Glutaraldehyde	Lactose solution	100 mg/g protein, 300 U/g protein
A. niger	Phenol formaldehyde, resin Duolite ES 761	Adsorption/cross-linking with Glutaraldehyde	Whey permeate	Flow: 75.5 mL/min, 70 -75% convers., 30 ° C
S. lactis	Polyacrylamide beads containing benzenemercaptane	Adsorption/hydrophobic bonds formation	Whey permeate	Load: 70 U/g for polypylated enzyme, 67% binding efficiency

A. oryzae	PVC-silica sheet	Covalent coupling on the contained silica particles	Whey permeate	2000 IU/g, 90% hydrolysis in 4 min
S. lactis	Aminoethyl sepharose	Covalent coupling, diazo method	Milk	Not available
A. niger	Porous silica	Covalent coupling (corning method). Semi-industrial plants. Fixed-bed continuous reactor	Demineralized whey	250 mg/g, 500 U/g at 50 °C, 360 L/h, 80% hydrolysis
A. niger	SiO_2 beads, 0.8-1.2 mm, porosity 1050 A	Covalent coupling, silanization method use of glutaraldehyde	Lactose solution	17.4 mg/g
A. niger (Rapidase)	Chitosan	Covalent coupling with glutaraldehyde	Lactose solution, whey, whey permeate	195 U/g dry chitosan
A. oryzae	Sepharose derivative (gel)	Covalent coupling. Packed-bed reactor (2.6x40 xm and 1.6 x40 cm)	Lactose solution, whey permeate	1 mg/ml of gel, 64 U/mg, pH 4.52, 50 °C
S. fragilis	Polyethyleneimine coated with gelatine	Covalent coupling, glutaraldehyde	Lactose	500 U/g, 25.5 U/g
A. niger (Hydrolact)	Mn-Zn ferrite non porous particles	Covalent coupling, silanization and use of glutaraldehyde	Whole milk	1 U/g, 16 U/g
A. niger	Porous silica	Covalent coupling, silanization and use of glutaraldehyde	5% lactose whey, UF whey permeate, whole whey	initial activity: 540U/g, 310-430 U/g
A. niger	Silica beads	Covalent coupling. Pilot plant 450 L/day, jacketed column. Diam.=100 mm x 915 mm height	UF-permeate, deionized whey	Average: 87.5% hydrolysis over 29 days
A. oryzae	Macroporous bead plexigels like material. Pores: 0.1-0.3 μm	Covalent coupling. Fixed-bed reactor. Pilot plant containing 2 lb of IME	Whole whey	80% hydrolysis remained for 100 days, 240 Imp. gal per day
A. oryzae	Ion exchange resin	Covalent bonding by glutaraldehyde	Skim milk acid whey, whey permeate	1000 U/g IME, 80% conversion
S. lactis	Cellulose triacetate	Entrapment in fiber, wet spinning method, industrial use. Packed-bed column	Skimmed sterile milk	30 mg/g, 22 U/g dry fiber. Conversion: 71-81% within 20 h
S. lactis	Polyvinyl alcohol gel	Entrapment	Whole milk	100% hydrolysis in 200 min with 80 mL IME
K. fragilis	Fibrous collagen	Whole cell entrapment	Lactose, whey, skim milk, whole milk	30% binding yield, 1680 U/g collagen, 54%, 32-37 °C

Chen and Wang (1991) have studied lactose hydrolysis in an aqueous two-phase system with β-galactosidase. They tested three different enzymes namely *E. coli* β-galactosidase, *Saccharomyces fragilis* β-galactosidase, and *A. oryzae* β-galactosidase. Through using continuous hydrolysis of 50 g/l lactose with a mixer-settler enzyme reactor using the fungal enzyme system, the hydrolysis could be operated for 84 h with only 10% loss of enzyme activity. During that period, maximum glucose concentration attained in the effluent was 36 g/l at 60 hours and then gradually decreased beyond 60 hours.

β-Galactosidase has also been covalently immobilized on uncoated porous glass, entrapped on both alginate and κ-carrageenan beads and physically adsorbed on chromosorb-W (Bodalo, et al., 1991; Mammarella and Rubiolo, 1991, 1996; Brady, 1998). However, the activity of β-galactosidase entrapped in calcium alginate and carrageenan gels showed very low activity, suggesting that the enzyme activity has been restrained by an unfavorable microenvironment inside the gel matrix (Bodalo et al., 1991). The highest amount of protein immobilized onto a support with the best activity was obtained for β-galactosidase covalently linked to porous glass after activation with γ-aminopropil-triethoxylane and glutaraldehyde. Later, Bodalo, et al. (1995) covalently immobilized β-galactosidase on chromosorb-W showing an excellent percentage of protein loading and activity yield. Since chromosorb-W is a cheap material, they suggest it as a suitable material for industrial scale application.

It has been proven that agarose or gellan gum beads were not suitable for the immobilization of a thermostable β-galactosidase isolated from *Thermus aquaticus* YT-1 due to enzyme leakage (Bodalo, et al., 1991; Berger et al. 1995). Instead of lactose hydrolysis, they have used this enzyme for synthesis of oligosaccharides. An attempt to use high gel concentrations to avoid enzyme leakage has led to a very low activity. To increase agarose gel stability, a better technique was developed; co-cross-linking of β-galactosidase with bovine serum albumin (BSA) and glutaraldehyde followed by entrapment in agarose gel. BSA was embodied as the "bridge" of polymeric network to improve mechanical and enzymatic stability.

β-Galactosidase has also been immobilized on chitosan beads (Martino, et al., 1996a & 1996b; Portaccio et al., 1998). Before immobilization, chitosan was neutralized with 5%

(v/v) acetic acid aqueous solution, followed by the addition of an excess 0.5 M NaOH aqueous solution. After washing with water until neutral, the dry chitosan was obtained by a successive procedure of precipitation, freezing and lyophilization. This pre-treated support was then mixed with enzyme solution in phosphate buffer and stirred thoroughly for 1 hour. Coupling and stabilization was accomplished by adding 2.5% glutaraldehyde for a period of 2 hours. Finally, the solid phase of immobilized β-galactosidase on chitosan beads was recovered by centrifugation. Sheu, et al. (1998) has employed a slightly different approach of immobilizing β-galactosidase on chitosan to be used for galactooligosaccharides (GOS) synthesis in a plug flow reactor. Before enzyme attachment, chitosan was firstly activated with 10% (w/v) glutaraldehyde for 1 hour at 30 °C. The cross-linking of activated chitosan with enzyme solution was allowed to proceed under mild shaking at 4 °C for 4hour.

On finding a more stable immobilized system for long term application, *K. lactis* β-galactosidase has been reversibly immobilized onto agarose and acrylic beads activated with thiol reactive structures (thiosulfonate/thiosulfinate moieties) (Ovsejevi et al., 1998). The immobilization involves two steps of pretreatment (activation) and cells attachment. The first step was the formation of thiol groups on the enzyme structure. This step was carried out to provide SH (thiol) group on the enzyme molecule. This group is needed to react with thiol reactive group on the gel. In this step, β-galactosidase was reduced with DDT (2,2'-dipyridyl disulfide). Ovsejevi et al. (1998) found that the optimal conditions for reduction were 30 min incubation with 100 mM DDT in 20 mM potassium phosphate buffer pH 7 or 8. The second step was the synthesis of thiol reactive structures on the support gel (agarose and acrylic). In the final step (attachment), the enzyme was immobilized by incubating the reduced enzyme solution in phosphate buffer with the thiol-reactive adsorbents (gels) for 22 h at room temperature. Alternatively, the enzyme can be bound by re-circulating it through a column containing adsorbents. The enzyme systems developed above were able to hydrolyze 85-90% of the 5% lactose in saline solution, whey, whey permeates, and skimmed milk, either in batches or in packed beds. The activity of the immobilized enzyme was fully conserved upon storage at 4 °C in buffer for 10 months.

It is widely accepted that the immobilization treatment is usually followed by changes in the structure of the immobilized macromolecule. The structure modification may alter

the active site behavior leading to changes in kinetic parameters. This phenomenon has been observed by Portaccio et al. (1998) who found different values of inhibition constants (k_i) of *A. oryzae* β-galactosidase immobilized in immunodyne and in chitosan. The difference in k_i value for the two immobilized systems indicated that each support might have introduced a different modification to the enzyme molecule.

1.2.5 The Whole Cell β-Galactosidase Systems

Enzymes that are intracellular can be applied in a pure or crude extract form by firstly extracting them from the cells. However, enzymes in that form may be unstable in certain cases and not applicable for use as immobilized enzymes. Isolation and purification of intracellular enzymes is also costly and often inefficient process. Therefore, immobilization of whole cell for enzymatic conversion is one field in enzyme technology that is being extensively developed because it offers a more comfortable way for enzyme system preparation. By using whole cells as catalysts, there is no need to extract the enzyme, and the enzyme stability can be maintained. In addition, most cell immobilization methods are cheap and effective (Bucke, 1986; Chibata, et al., 1986; Siso and Doval, 1994; Flores et al., 1995)

There are several requirement to use the whole microbial cell as a biocatalyst (Chibata, et al., 1986): the enzymes are intracellular, the enzymes are unstable during and after immobilization, the whole cell system performs only the desired reaction without being interfered with by other enzymes, and the substrates and products are low-molecular-weight compounds.

The use of *Kluyveromyces bulgaricus* whole cells to hydrolyze lactose solution and whey has also been studied (Decleire, et al., 1985). All the tests were carried out in phosphate buffer media at pH 7 containing 1 mM $MgCl_2$. There was no report on the effort to increase the permeability of cells. For hydrolysis, the optimum temperature was found to be 48 °C. At this temperature, 15 mg/ml dried yeast cell hydrolyzed 80% of lactose in 3.5 and 9.0 min for of 5% lactose and whey, respectively. The reusability of the biocatalyst was also tested. It was found that the degree lactose conversion in phosphate buffer solution markedly decreased after 3 repeats. In contrast, 80%

conversion of lactose in whey was retained even after 8 repeats. The authors suggested that some compounds in whey might have stabilized β-galactosidase.

Lactose uptake in living cells of *Kluyveromyces lactis* is mediated by an inducible transport system (Dickson and Barr, 1983). Induction is evoked by the presence of lactose or galactose and requires protein synthesis. The protein, known as permease, facilitates active transport of lactose into the cell. This carrier is required for transportation when lactose concentration within the cell boundary is significantly greater than that of extracellular concentration. Since lactose transport becomes a rate limiting step in the hydrolysis of lactose by whole cells (Joshi, et al., 1987 & 1989), the permeability of cellulose membranes to lactose may be increased by treating with organic solvent, surfactant and freezing-thawing cycles. Permeabilization means the treatment to change membrane permeability without the destruction of cell integrity (Decleire et al. 1987; Flores, et al., 1994).

Decleire et al. (1987) compared various permeabilization treatments of *Kluyveromyces bulgaricus* cells and measures the whole cell β-galactosidase activity using synthetic substrate, ONPG. They found the minimum solvent concentrations required for a good permeabilization were either: 10% n-butanol; 20% propanol; 30% isopropanol or tert-butanol; 40% ethanol or acetone; or 70% dimethylsulphoxide. Physical treatment through five cycles of freezing and thawing gave enzymatic activity comparable to those treated with organic solvents. It is worth noting that the quantity of cells employed in each treatment was not reported.

Champluvier et al. (1988) studied permeabilization of β-galactosidase-containing cells from *Kluyveromyces* sp. using various solvents or polycations. The species in this genus tested were *Kluyveromyces marxianus, K. fragilis*, and *K. lactis*. Among the permeabilizing agents tested (chloroform, ethanol, protamine, chitosan, polybrene, butanol, and the combinations), the combination of chloroform-ethanol rendered the best permeabilizing effect. The procedure is as follows: 1 volume of chloroform-ethanol (1:9) was mixed with 1 volume of cell suspension with 0.8 mg dry weight (DW) per mL phosphate buffer pH 6.5 (containing Mn) and shaken at 30 °C for 30 min. However, it was observed that the total activity of permeabilized cells decreased significantly when stored for 24 hours at 30 °C, with shaking at 100 rpm. To stabilize the confinement of β-

galactosidase within the cells, cells were treated with glutaraldehyde. Glutaraldehyde could form a network in the cell wall thereby allowing physical retention of the enzyme, or connecting β-galactosidase molecule to other cell components. For a range of concentrations of glutaraldehyde tested, it was found that a concentration of 0.4% glutaraldehyde gave the maximum β-galactosidase activity to be retained in the cells. Owing to solvent permeabilization followed by the treatment of cells with glutaraldehyde (GA), Champluvier et al. (1988) concluded that, firstly, GA treatment retained about 90% the initial activity of permeabilized cells; secondly, the treated biocatalyst showed good stability upon storage; and thirdly, the activity of permeabilized cells not treated with GA remained at 25% after 22 h storage at 30 °C. The reusability of stabilized free cell was also tested in repeated batch hydrolysis of 5% lactose solution in Manganese-phosphate buffer at 30 °C. The results showed that about 80% conversion could be repeatedly achieved without loss of activity after 7 cycles. This implied a good operational stability and stable confinement of biocatalyst.

Flores et al. (1994) studied more details the permeabilizing effect of ethanol, chloroform and toluene to β-galactosidase activity of whole cell *K. lactis.* It was found that the effectiveness of these solvents was primarily dependent on the incubation time, the incubation temperature, and the concentration of both cells and solvents. The intact cells of *K. lactis* did not show β-galactosidase activity toward ONPG since this synthetic substrate could not be transported via the permease system. They concluded ethanol played a synergistic role toward chloroform or toluene on enhancing the membrane permeability. Ethanol could increase membrane fluidity, allowing more contact of other solvents with membrane structures. These effects were further augmented when permeabilization temperature was increased up to 37 °C. At this temperature, permeabilization occurred within 5 min. It was also proven that the solvent treatment fully retained β-galactosidase activity within the cells and did not alter the cell wall structure. When chloroform or toluene was used alone, the effective concentration lies between 1.5-3.0%. However, when using ethanol these values can be reduced to 0.75-2.5%. Ethanol at the concentration up to 40% either alone or in the mixture was effective for permeabilization at 30 °C for 15 min; but ethanol concentration above 40% or further incubation time gradually inactivated β-galactosidase. The optimum conditions suggested for permeabilization of whole cell *K. lactis* were: $8x10^9$ cells/ml in

potassium phosphate buffer (50mM) at pH 6.6 containing 0.1 mM manganese, carried out at 37 °C for 5 min with 2% chloroform and 10% ethanol. In this condition, no extracellular β-galactosidase activity was observed and the activity was fully conserved. The permeabilized cells were found to be non-viable as a result of essential compound leakage from the cell during treatment.

Siso and Doval (1994) have developed an immobilization method that was acclaimed as a new low-cost type of insolubilized whole cell β-galactosidase. They grew *K. lactis* cells on milk whey, covalently linked the cells to corn grits and permeabilized the immobilized cell with ethanol. Immobilization on corn grits involved four steps:

(1) The oxidation of corn grits with sodium metaperiodate (40 g/l) at 25 °C for 24 h in the dark;
(2) The amination of corn grits with 1 M ethylene diamine at 25 °C for 72 h;
(3) The activation of corn grits with 2% glutaraldehyde in 0.05 M pyrophosphate buffer (pH 8.6) at 25 °C for 6 h;
(4) The linkage of *K. lactis* cells on to corn grits in 15 mM phosphate buffer (pH 7.0) at 4 °C for 15 h.

All above steps were done with a gentle rotary shaking. After immobilization, permeabilization with 70% ethanol was carried out to increase substrate accessibility. It was found that ratio of volume of 70% ethanol to mg of immobilized cell that gave the greatest permeabilization to be 0.4. The β-galactosidase activity increased 240 fold after permeabilization when tested with ONPG method. Permeabilization also hindered further monosaccharide fermentation, whereas the test using unpermeabilized biocatalyst showed that ethanol production rate was considerably higher (0.22 mg ml^{-1} min^{-1}). Corn grits was chosen because they are cheap, very porous, and exhibit a good mechanical strength. This support consists of 25-35% cellulose, 47% hemicellulose and lignin. The extensive macropore structure in the support enables yeast cells to enter freely. This means a direct adsorption of cells is actually possible. Immobilization by adsorption can only occur when the pore diameters of the supports are bigger than the cell size to allow the cells to penetrate and to be retained (Siso and Doval, 1994). However, Siso and Doval (1994) favored covalent linkage since the bond formed is more resistant to the changes in conditions (temperature, pH and ionic strength) encountered when treating raw materials. During the covalent attachment, the aldehyde

groups of activated corn grits reacted with amino group of the lysine in yeast cell walls. They found that more cells were fixated to activated support through covalent linkage (70 mg biomass g^{-1} support) than direct adsorption (66 mg biomass g^{-1} support) when using 0.8 mm diameter of corn particles. The corn grits biocatalyst, when tested using a packed-bed reactor, converted more than 90% lactose in the feed substrate, which has never been achieved with immobilized pure enzyme (Siso and Doval, 1994).

Whole cells usually exhibit non-viable following physical or chemical treatments, which obstruct metabolization of the substrate (Flores, et al., 1995). This is an advantage when the unwanted metabolic reaction is to be prevented. Unfortunately, non-viable cells represent an unstable system because solubilization of cell components and enzyme inactivation both occurs simultaneously with time (Breddam and Beenfeldt, 1991; Flores, et al., 1995).

It has been reported that a proper permeabilization procedure applied to yeast cells could retain the enzyme within cell boundaries during the treatment, but as incubation proceeds the enzyme is slowly released (Flores at al., 1994 & 1995). Flores et al. (1995) suggested that solubilization was the major factor for the disappearance of β-galactosidase activity from permeabilized cells of *K. lactis*. At 30 °C incubation temperature, the activity almost completely ceased after 48 h. With the aim to stabilize and retain the enzymes within the cell boundaries, they treated the cells with glutaraldehyde (GA). In this treatment, soluble enzymes inside the cells were cross-linked to form water insoluble enzyme, thereby preventing them from solubilization and inactivation. After GA treatment, extracellular activity was not detected in any case. However, the rate of β-galactosidase deactivation increased with the increase of GA concentration. A good confinement and stability of the cell biocatalyst could be achieved by selecting a good combination of time, temperature and GA concentration. A reliable combination obtained was the use of 50 mM GA containing permeabilized *K. lactis* cells ranging from 1 to 2 $x10^{10}$ cells/cm^3, incubated for 60 min at 30 °C. Such treatment has reduced the activity of the enzyme down to 75 % of the initial activity. Stabilized cell biocatalyst obtained by such treatment was reported be able to perform 25 cycles of lactose hydrolysis with 70-80% conversion within 1 month, operated at 5 °C without loss of activity. At a higher temperature (45 °C), the biocatalyst activity was also conserved after 12 cycles of 1 hour reaction time.

Tomaska, et al. (1995a) tested the performance of calcium pectate gel (CPG) and calcium alginate gel (CAG) as the carrier for permeabilized whole cells of *Kluyveromyces marxianus* CCY eSY2 as the source of β-galactosidase. For permeabilization, 100 g/l DW of harvested and washed biomass was mixed with 13% (v/v) chloroform/ethanol (1:9, v/v) for 15 min, at 25 °C, under agitation. For entrapment, either sodium alginate (3% w/w) or potassium pectate (5% w/w) containing permeabilized biomass 40 g/l DW was extruded into $CaCl_2$ solution to form beads. For long-term hydrolysis of lactose in the presence of phosphate ions, it was shown that stabilization (hardening) of beads with polyethyleneimine (PEI) and glutaraldehyde (GA) is essential. For this purpose, three volumes of 2% (v/v) PEI in 0.05 M $CaCl_2$ was added to one volume of CAG or CPG particles and stirred for 6 h. After washing, particles were thoroughly mixed with two volumes of 1% GA for 1 min. The hydrolysis of 5% lactose in 50 mM phosphate buffer at pH 6.5 containing 0.1 mM $MnCl_2$, carried out in stirred batch reactor (35 °C) showed that the conversion of more than 80% of the initial lactose could be sustained for as many as 55 cycles and 25 cycles for hardened CPG and CAG beads, respectively. For a continuous process using a packed-bed reactor, more than 80% conversion could be maintained within 11 days and 6 days, respectively, for CPG and CAG beads. It could be seen that CPG beads exhibit a better performance than CAG beads, since calcium ion is a strong β-galactosidase inhibitor (Dickson et al., 1979). A lower concentration of free calcium ions in calcium pectate beads provides an additional advantage over calcium alginate beads, despite the higher calcium pectate concentration (5% pectate against 3% alginate). In a stability test of both beads, no loss of activity was observed after 2 months storage at 4 °C (Tomaska, et al., 1995a).

1.3 INDUSTRIAL APPLICATION

1.3.1 The Choice of Bioreactor

Richmond et al. (1981) reported that for industrial applications, the suitability and durability of β-galactosidase reactors must take into account operational temperature, substrate flow, carrier/support material, immobilization method, and substrate

component. Monsan and Combes (1984) reported that packed-bed reactors are, in fact, preferable. The choice of packed-bed reactor for enzymatic conversion over other bioreactor systems is due to their efficiency to generate high conversion rates, and for the condition where products released exhibit inhibitory effects to enzyme reaction. As an illustration, the efficiency between the packed-bed reactor and the continuous stirred tank reactor with amyloglucosidase immobilized on DEAE cellulose showed that both reactors give similar results (O'Neill, et al., 1971). But the volume required to obtain the same productivity is 40 times bigger for the continuous stirred tank reactor compared to the packed-bed reactor (Monsan and Combes, 1984). In the case the substrate is insoluble or if it inhibits the reaction, continuous stirred tank reactors are preferable. Plug-flow reactors have been applied to many industrial applications, such as the glucose isomerization into fructose and lactose hydrolysis by acidic β-galactosidase (Richmond et al., 1981)

1.3.2 The Commercial Process

Richmond et al. (1981) reviewed a typical commercial process of lactose hydrolysis in whey, whey permeate and whey milk, as depicted in Figure 1.2. The substrate is pumped into the feed tank where temperature and pH is adjusted to optimum values (35 to 50 °C at optimum pH), and then pumped into an immobilized enzyme column. Lactose in the feed solution is hydrolyzed as the substrate moves down through the column. Residual lactose in the effluent is then removed, followed by the concentration of glucose-galactose syrup to 60% solids. The column is sanitized once a day by back washing with dilute acetic acid. It was reported that 360 to 500 liters per day of feed could be hydrolyzed with an average 80% conversion.

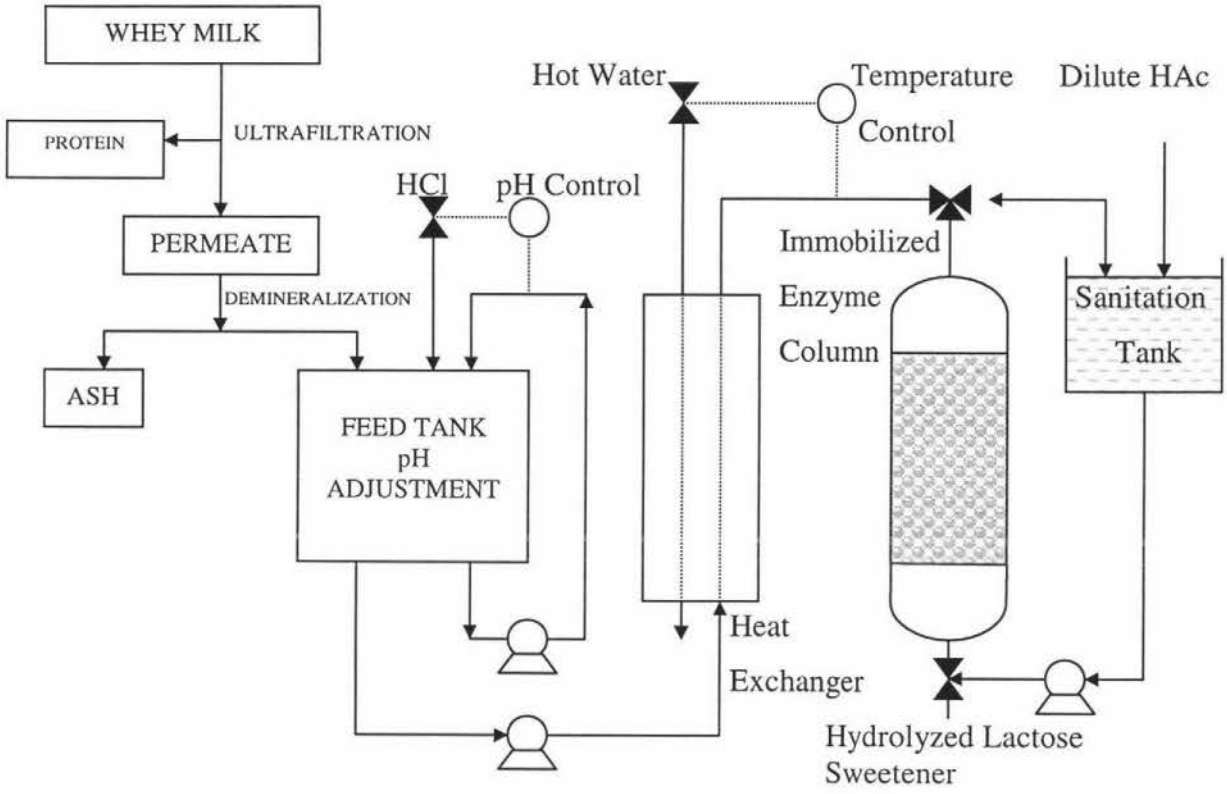


Figure 1.2 Schematic diagram for commercial process of the hydrolysis of lactose (Richmond et al., 1981).

1.4 THE KINETICS OF LACTOSE HYDROLYSIS

1.4.1 The Enzyme Kinetic Models

The Lineweaver-Burk linear transformation of the Michaelis-Menten equation is the more common method used to determine the maximum reaction rate (V_{max}) and Michaelis-Menten constant (K_M) and the Dixon plot is used for assaying the product inhibition constant (K_P). These methods use the initial rate data to determine kinetic parameters and neglect the high conversion effect. Other methods use integrated rate equations to fit the model with a set of experimental values obtained from low to high conversion of substrate (Halwachs, 1978; Yang and Okos, 1989).

On studying the kinetic parameters β-galactosidase, three widely accepted models of lactose hydrolysis by β-galactosidase have been proposed (Wondolowski and Woychik,

1974; Mahoney and Whitaker, 1977; Flaschel et al., 1982; Halwachs, 1978;Yang and Okos, 1989; Carrara and Rubiolo, 1996; Santos, et al., 1988):

1. *Model A*: Michaelis-Menten kinetics without product inhibition are derived from the following enzyme reaction :

$$S + E \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} (ES) \xrightarrow{k_2} E + P + Gl$$

where S, P, GI, and E are lactose, galactose, glucose and enzyme concentrations, respectively. Assuming a steady-state for the enzyme-lactose complex (ES), the reaction rate of substrate conversion (v_s) is described by :

$$-v_s = -\frac{dS}{dt} = \frac{E^o k_2 S}{K_m + S} \tag{1.1}$$

where E^o is the total enzyme and $E^o k_2 = V_{max}$.

2. *Model B*: Michaelis-Menten kinetics model with competitive product inhibition by galactose, derived from the following enzyme reaction :

$$S + E \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} (ES) \xrightarrow{k_2} E + P + Gl$$

$$EP \underset{k_{-3}}{\overset{k_3}{\rightleftharpoons}} (E + P)$$

If k_I is the inhibition constant and $P \approx GI$, the equation is :

$$-v_s = -\frac{dS}{dt} = \frac{E^o k_2 S}{K_m\left(1 + \frac{P}{k_i}\right) + S} \tag{1.2}$$

3. *Model C*: as postulated by Yang and Okos (1989) with the assumption that molecule glucose is the first product released from the active site leaving a chemical intermediate in the form of enzyme-galactosyl complex from which the galactose is released. The following equations describe this mechanism :

$$S + E \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} (ES) \xrightarrow{k_2} (E\,P) + Gl$$

$$(EP) \underset{k_{-3}}{\overset{k_3}{\rightleftharpoons}} (E + P)$$

Following a simplification similar to the previous models, the expression of reaction rate is:

$$-v_s = -\frac{dS}{dt} = \frac{E^o k_2 S}{K_m\left(1 + \frac{P}{k_i}\right) + (1 + K_p)S} \tag{1.3}$$

where the reaction rate constant is $K_P = k_2 / k_3$.

1.4.2 The Kinetic Parameters

Table 1.4 shows the kinetic parameters values of soluble β-galactosidase isolated from different sources. Table 1.5 presents the kinetic parameters values of immobilized lactase (immobilized enzyme and immobilized whole cell). All the tests used lactose as the substrates and were carried out at different temperatures.

Table 1.4 The Kinetic parameters of soluble β-galactosidase from different sources

Source	pH	Temp (°C)	Kinetic Parameters				Reference
			k_2 (μmol mg^{-1} min^{-1})	K_m mM	k_I mM	K_p mM	
E. coli	7.0	25		0.54	1.5	0.57	Wadiak & Carbonell, 1975
A. niger	3.5	50	12.20	53.9	0.92		Flaschel, et al., 1982
A. oryzae		37	10[a]	96	40		Shukla & Chaplin, 1983
K. fragilis	6.5	5	54	0.4	0.41		Santos, et al., 1988
	6.5	25	225	4.6	3.6		
	6.5	40	580	23	15.3		
A. niger	4.0	8	0.0040[b]	83.3	0.47		Yang & Okos, 1989
	4.0	30	0.0157[b]	80.9	0.53		
	4.0	40	0.0274[b]	79.9	0.55		
	4.0	60	0.0758[b]	78.2	0.60		
A. niger	4.0	40	0.040[c]	18.9	0.47		Papayannakos, et al., 1993
	4.0	60	0.085[c]	38.7	2.36		
K. lactis	6.6	5		29.7			Flores, et al., 1995
	6.6	37		28.2			
	6.6	45		27.9			
K. fragilis	6.86	43	2.0	43.6	51.9		Carrara & Rubiolo, 1996
K. marxianus	6.6	28		21.0		29.2	Illanes, et al., 1998
	6.6	35		27.8		31.6	

[a] IU/mg
[b] mg^{-1} mL min^{-1} M
[c] μmol U^{-1} min^{-1}

Table 1.5 Kinetic parameters of immobilized β-galactosidase (pure/crude and whole cell) from different yeast sources

Source	Enzyme systems	pH	Temp (°C)	Kinetic Parameters					References
				k_2 (μmol mg^{-1} min^{-1})	K_M mM	k_I mM	K_P mM	V_{max}	
K. bulgaricus	Untreated free cells	7.0	48		59				Decleire, et al., 1985
K .lactis	GA-stabilized free cells	6.6	5		35.5			86.74[a]	Flores et al., 1995
		6.6	37		33.1			86.74[a]	
		6.6	45		32.4			86.74[a]	
K. fragilis	Cells cross-linked to GA-activated chitosan	6.86	43	0.78	137.0	234.0			Carrara & Rubiolo, 1996
K. marxianus	β-Galactosidase cross-linked to GA-activated chitin	6.6	28		54.4		86.9	275[b]	Illanes, et al., 1998
		6.6	35		36.5		114.0	303[b]	
A. oryzae	β-Galactosidase cross-linked to chitosan using GA	6.5	30		150			4.5	Portaccio, et al., 1998

[a] μmol min^{-1} (10^9 cells)$^{-1}$

[b] μmol min^{-1} g^{-1} catalyst

1.4.3 Thermal Deactivation of enzyme

The first order process has been used as a general scheme to study thermal deactivation (Peterson, et al., 1989)

$$E \xrightarrow{k_d} E_d$$

where E is the active enzyme;

E_d is the deactivated enzyme;

k_d is the deactivation rate constant;

Deactivation of enzyme activity may be assumed to follow an exponential decay

$$A / A_o = e^{-k_d t}$$

where A is the total activity at time t;

A_o is the total activity at time t = 0;

Gianfreda et al. (1985) confirmed that the deactivation for β-galactosidase obeyed first order kinetics. These authors found the value of k_d at 45 °C for immobilized species to be 0.363 h^{-1}. For immobilized whole cells of *K. lactis,* the value of activation energy E_a found by Siso and Doval (1994) was found to be 7.78 Kcal/mol. The k_d values at other operational temperatures can be calculated based on the Arrhenius equation:

$$k_d = A_d \cdot e^{-E_a / RT}$$

where A_d is the pre-exponential factor, E_a is the activation energy, R is the gas constant, and T is the absolute temperature.

1.5 CONCLUSIONS

The conclusions than can be drawn from this review are:

- The operational conditions (pH, temperature, substrate and product concentrations, and ionic strength) play an important role in governing the activity of the enzyme β-galactosidases for lactose hydrolysis.
- β-Galactosidase enzymes from yeast species are suitable only for the hydrolysis of neutral substrates, such as sweet whey or milk.
- Immobilization of pure β-galactosidase is better established compared to whole cell immobilization, since whole cell, which is usually operated in non-viable form, represents an unstable system. Recently, stabilization is becoming a major concern in the utilization of whole cell for lactose hydrolysis.
- The common cross-linking and stabilizing agent for β-galactosidase is glutaraldehyde
- Permeabilization of yeast cell by organic solvent treatment has been proven conserving β-galactosidase activity inside the cells which does not alter the cell wall structure.

Chapter 2
MATERIALS AND METHODS

2.1 MATERIALS

2.1.1 Microorganisms

Kluyveromyces lactis CBS 2357 and *K. marxianus* CBS 712 were obtained from the Culture Collection of Microbiology Laboratory, Institute of Technology and Engineering, Massey University (Palmerston North, New Zealand). They were chosen from a number of β-galactosidase-producing strains based on the growth profile with lactose as previously reported by Russel (1993). It was reported that these strains showed fast growth on lactose, an indication of high β-galactosidase activity. *Kluyveromyces lactis* exhibited better β-Galactosidase activity and was used for nearly the entire experiment.

2.1.2 Media

The media composition:

(a) Maintenance and selection (Flores, et al., 1994):

Table 2.1 The composition of solidified media (SM medium)

Component	Concentration (g/l)
Lactose	5.0
Peptone	2.0
Yeast extract	1.0
Malt extract	0.01
Agar	2.0
pH 4.5	

(b) Pre-culture medium:

Table 2.2 The media composition for yeast cell germination (LM-1 medium)

Component	Concentration (g/l)
Lactose	22.5
Peptone	5.0
Yeast extract	10.0
pH 6.0	2.0

(c) Production media (Flores, et al., 1994):

Table 2.3 The composition of media for cell production (LM-2 medium)

Component	Concentration (g/l)
Lactose	25
Yeast extract	2.5
Urea	2.5
KH_2PO_4	2.5
$MgSO_4\ 7H_2O$	0.6
$CaCl_2\ 2H_2O$	0.1
$FeSO_4\ 7H_2O$	0.025
$ZnSO_4\ 7H_2O$	0.001
$MnSO_4\ H_2O$	0.0005
$CuSO_4\ 5H_2O$	0.00025
pH 5.0	

(d) The agar plate medium: PDA (Potato Dextrose Agar, Merck).

2.1.3 Chemical Specifications

Chemicals used for the experiment are listed in Table 2.4.

Table 2.4 Chemical specifications and Manufacturer

Name	Chemical Formula	Grade	Manufacturer
Agar		Microbiological	Gibco
Bone charcoal	Carbon	-	BDH Chemicals, Ltd., England
Calcium chloride	$CaCl_2$	AR	BDH Chemicals, Ltd., England
$CaNa_2$ EDTA	$(CH_2O)_n$	AR	Sigma Chemical Co., USA
Chloroform	$CHCl_3$	AR	BDH Chemicals, Ltd., England
Cooper sulphate	$CuSO_47H_2O$	AR	BDH Chemicals, Ltd., England
Corn of coarse particles			Healthries, New Zealand
D(+)-Dextrose anhydrate	$C_6H_{12}O_6$	AR	BDH Chemicals, Ltd., England
Ethanol 95%	C_2H_5OH	Technical	New Zealand
Ethanol 99-100%	C_2H_5OH	AR	Merck, Germany
Ferrous sulphate	$FeSO_47H_2O$	AR	BDH Chemicals, Ltd., England
Glutaraldehyde 25% solution	$C_5H_8O_2$	AR	BDH Chemicals, Ltd., England
Lactose monohydrate	$C_{12}H_{22}O_{11}H_2O$	Bulk Technical	BDH Chemicals, Ltd., England
Lactose monohydrate	$C_{12}H_{22}O_{11}H_2O$	AR	BDH Chemicals, Ltd., England
Magnesium sulphate	$MgSO_47H_2O$	AR	BDH Chemicals, Ltd., England
Malt extract		Microbiological	Oxoid, England
Manganese chloride	$MnCl_2 6H_2O$	AR	BDH Chemicals, Ltd., England
Manganese sulphate	$MnSO_47H_2O$	AR	Oxoid, England
o-Nitrophenyl-β-D-galactopyranoside	$C_{12}H_{14}O_8$	AR	Sigma, Chemical Co USA
Peptone		Microbiological	Oxoid, England
Potassiun dihydrogen phosphate	KH_2PO_4	AR	Scharlau, Spain
Sodium alginate		AR	BDH Chemicals, Ltd., England
Sodium carbonate	$Na_2 CO_3$	AR	BDH Chemicals, Ltd., England
Sodium chloride	NaCl	AR	BDH Chemicals, Ltd., England
Sodium hydroxide	NaOH	AR	BDH Chemicals, Ltd., England
Sulphuric acid	H_2SO_4	AR	Merck, Germany
Urea		AR	BDH Chemicals, Ltd., England
Yeast extract		Microbiological	Oxoid, England
Zinc sulphate	$ZnSO_47H_2O$	AR	BDH Chemicals, Ltd., England

2.1.4 Phosphate-Mn Buffer Solution

The buffer consisted of 50 mM K_2HPO_4/KH_2PO_4 supplemented with 0.1 mM $MnCl_2$ with pH ranging from 6.0 to 7.2. To make 1 liter of phosphate buffer solution, 6.8 g KH_2PO_4 was dissolved in about 900 ml distilled water. While stirring, drops 10% KOH solution was added to reach the desired pH value; then 1 ml of 100 mM $MnCl_2$ solution

was added and the solution was made up to 1 liter. The phosphate-Mn buffer is referred to as "Mn-Buffer" in this thesis.

2.1.5 The Substrate Lactose and ONPG

Unless otherwise stated, lactose or ONPG as substrate solutions were prepared by diluting in Mn-Buffer (lactose used was in the form of monohydrate - technical bulk material, BDH England). Since both lactose and ONPG have low solubility, vigorous stirring for sufficient time was required to dissolve completely.

2.1.6 Sterilization

Culture media and other solutions or materials were sterilized by autoclaving at 121 °C for 15 minutes. If a sterile lactose solution in Mn-Buffer was to be used as substrate for a long-term process, i.e. beyond a 24 hours period, sterilization was carried out by membrane filtration (Millipore) with a pore size of 0.45 μm.

2.2 ANALYTICAL METHODS

2.2.1 pH Measurement

The pH was measured using a benchtop pH meter (PHM82, Radiometer Copenhagen) or an Orion Research Digital Ion Analyzer (Model 701A; Watson Victor Ltd, New Zealand) which had been calibrated prior to use with standard buffer solution of pH 4.0 or 7.0.

2.2.2 Cell Enumeration and Microscopic Identification

Yeast cells were enumerated and identified microscopically using a standard haemocytometer (Assistant, Germany) at 400x magnification under a Microscope.

2.2.3 Cell Dry Weight (DW) Measurement

For cell dry weight measurement, 0.5 ml of yeast stock cell suspension was placed in a pre-weighed dish made of aluminum foil. The dish and its contents were dried in the oven at 105 °C overnight. After drying, the dishes were placed in a dessicator to prevent vapor absorption from air before being weighed. The difference in weight between the dish containing dried cell and empty dish measured net cell dry weight.

2.2.4 Enzymatic Glucose Determination

The enzymatic determination of glucose concentration was performed using a YSI-fixed glucose analyzer (Model 27, Yellow Springs Instrument Co., Inc., Yellow Springs, Ohio USA). Prior to measurement the instrument was calibrated using glucose standard of 2.0 g/l. The linearity of the analyzer was confirmed by measuring the standard glucose of 5.0 g/l.

2.2.5 Activity Determination of free cells

The activity based on the initial rate of *o*-Nitrophenyl-β-D-galactopyranoside (ONPG) hydrolysis was done by mixing 0.1 ml of cell suspension in a test tube with 0.9 ml of 5 mM ONPG solution in Mn-Buffer. The tube was quickly vortexed and incubated with agitation at 37 °C for exactly 5 min (longer incubation time was required if the activity was too low). The reaction was stopped by adding 2 ml of 0.2 M Na_2CO_3, followed by quick vortexing. 1.8 ml was centrifuged in a microfuge and 1.5 ml clear supernatant was taken for a direct reading of absorbance at 416 nm using a Spectrophotometer (Hitachi U-2000, Japan). Activity was expressed in μmoles ONPG hydrolyzed per minute per 10^9 cells. The molar extinction coefficient of *o*-Nitrophenol of 4662 M^{-1} cm^{-1} (Champluvier, et al., 1988) was used to calculate the activity. The value obtained represented the total β-galactosidase activity. To measure extracellular activity, the above procedure was repeated but the cell suspension was firstly centrifuged for 5 min at 12 000 rpm in an Eppendorf microfuge and then the supernatant activity was measured. The difference between total and extracellular activity is referred to as whole cell activity. Since the exact amount of β-Galactosidase confined within cell boundaries

is difficult to be quantified, the method used to express the enzyme activity is based on the cell number.

2.2.6 Sugars Determination

β-Galactosidase catalyzes the split of lactose into glucose and galactose. The glucose liberated was mainly assayed using the Glucose Analyzer (Section 2.2.4). Lactose, glucose and galactose were detected by an HPLC method (glucose was included to serve as comparative data to those obtained by enzymatic method). The mobile phase was deionized water containing 50 mg/l $CaNa_2$ EDTA, which was filtered through a 0.45μm membrane filter (Millipore) before use. A **Sugar Pak™ 1** Column (Waters, 6.5 x 300 mm; Millipore Corporation, Milford, MA) was conditioned to 90-92 °C by a column heater and was equilibrated with the mobile phase. The flow rate was adjusted to 0.5 ml/min. A 50 μl sample was injected manually. The sugars were detected by a refractive index detector (Waters R410 differential refractometer; Millipore Corporation). Peaks were recorded and integrated simultaneously by a Waters 740 data module (Millipore Corporation). The HPLC was calibrated using two-point calibration method employing two different concentrations of standard sugars. The standard solutions contained lactose monohydrate (0.1 to 10 g/l), glucose anhydrous (0.1 to 5 g/l) and galactose anhydrous (0.1 to 5 g/l). The samples, before being injected, were appropriately diluted to bring the lactose concentration within the concentration range of standard solutions to minimize the deviation.

The degree of hydrolysis (d.h.) is calculated as follows:

$$\text{d.h.} = \frac{\text{actual concentration of glucose (M)}}{\text{initial concentration of lactose (M)}} x\ 100\%$$

2.3 CULTURE CONDITIONS

2.3.1 Isolation and Maintenance of Microorganisms

The original yeast cultures were firstly purified to assure strain homogeneity. For yeast colony isolation, the original cultures were suspended and serially diluted with sterile physiological saline, then spread out onto SM agar plate medium. After incubation for 72 h at 30 °C, plates with well-separated colonies were picked up for colony isolations. The colonies with circular form and smoothly convex elevation of the surface with the diameter between 2-3 mm were chosen to be transferred to slope agar media in a bottle (2.5 x 7 cm) containing 5-7 ml SM medium (See Table 2.1), then incubated for 48h at 30 °C.

For maintenance, yeast cultures were regularly sub-cultured at least monthly and kept in cold room of 4 °C if not promptly used.

2.3.2 Biomass Production

(a) Cell germination stage (Pre-culture)

Yeast culture on the agar slope was suspended with 5 ml sterile water; 1 ml of which was transferred into 50 ml LM-1 medium (see Table 2.2) in 250 ml baffled Erlenmeyer flask. The flask was shaken 250 rpm at 30 °C for 24 h.

(b) Biomass production

For excessive biomass accumulation, 5 ml of pre-culture was inoculated in 50 ml LM-2 medium (see Table 2.3) in a 250 ml baffled Erlenmeyer flask. The flask was shaken at 250 rpm at 30 °C for the next 24 h. Following this, cells were centrifuged at 6500 rpm at a cold temperature 4-8 °C for 15 min in 500 ml centrifuge tube. The supernatant was discarded and the biomass was washed twice with cold Mn-Buffer pH 6.5. Biomass was re-suspended in Mn-Buffer pH 6.5 such that the final cell number lied in the range $2x10^9$ to $8x10^9$ cells/ml.

(c) Free cell permeabilization (Champluvier et al., 1988; Flores et al., 1995)

The cell suspension previously prepared was mixed with ethanol and chloroform with the final concentration 10% and 2%, respectively, and then incubated with shaking at

120 rpm at 37 °C for exactly 5 min. To reduce the permeabilizing effect of these solvents after incubation, the mixture was immediately diluted 10-15 times with cold Mn-Buffer pH 6.5, followed by centrifugation 6500 rpm at cold temperature (4-8 °C) for 15 min. The supernatant was discarded and the permeabilized cells were washed twice with cold Mn-Buffer pH 6.5. The cells were re-suspended in Mn-Buffer pH 6.5 and kept frozen at –20 °C if not used immediately.

2.3.3 Viability Test

Viability test of the prepared biocatalyst was tested by agar plate method. The cell suspension was diluted 100x and 1000x, then 0.2 ml was spread onto agar plate containing PDA (potato dextrose agar) and incubated at 30 °C for 48h.

Chapter 3
THE EFFECT OF PRETREATMENT ON WHOLE CELL β-GALACTOSIDASE ACTIVITY

3.1 YEAST SELECTION

The cultures of *Kluyveromyces lactis* and *K. marxianus* from the Culture Collection within the Institute of Technology and Engineering were purified by isolating the single colonies formed on agar plate media. This step was required to get cell population with more identical physiology and genetic characteristics. These new isolates were used throughout the project.

It has been reported that *K. lactis* and *K. marxianus* reached the end of growth phase after 24 h of incubation at 30 °C in the medium containing lactose and yeast extract (Champluvier, et el., 1988). In order to compare the biomass production ability of *Kluyveromyces lactis* CBS 2357 and *K. marxianus* CBS 712 both yeast were grown on LM-2 medium for 24 h and the cell number was enumerated. Ten replicates were made for each of the yeast strains examined. The results are shown in Table 3.1.

Table 3.1 shows that media composition and methods used gave a high biomass in the production stage for both strains, comparable to those utilizing glucose as carbon source as previously reported by Russel (1993). In addition, the biomass production after 24 h of incubation for both strains was quite similar as shown by the cell number data. Indeed, lactose was incorporated as the sole carbon source to induce high enzyme expression in the cell. However, the growth characteristic is not an indicative of the extent of β-galactosidase activity, therefore a further test was required to quantify the intrinsic activity.

Next, the activity test of β-galactosidase confined in the free cells was carried out to select only one of the yeast's for further study. The source of cells was from the above test, which were firstly permeabilized to increase the substrate accessibility. Because the exact amount of enzyme within a cell is unknown, a common way to quantify the extent of enzyme is based on the biomass (or cell number). Therefore, a new terminology of

activity is introduced, defined as μmol ONPG hydrolyzed/min/10^9 cells. Table 3.2 lists the test results that compare the whole cell β-galactosidase activity of *K. lactis* and *K. marxianus*. It can be seen that by applying the same treatment during biocatalyst preparation, the permeabilized whole cell of *K. lactis* CBS 2357 exhibited the average activity of 39.74 μmol/min/10^9 cells, or approximately twelve times greater than *K. marxianus* CBS 712 activity. As a consequence, *K. lactis* CBS 2357 was chosen for this investigation.

Table 3.1 Cell number of the cultures after 24 h of incubation

# Flask	Cell number/ml
Kluyveromyces lactis CBS 2357	
1	5.0×10^8
2	6.5×10^8
3	6.5×10^8
4	7.0×10^8
5	5.3×10^8
6	5.8×10^8
7	2.5×10^8
8	5.3×10^8
9	5.8×10^8
10	5.8×10^8
	Mean = 5.6×10^8
Kluyveromyces marxianus CBS 712	
1	5.5×10^8
2	5.5×10^8
3	5.5×10^8
4	5.8×10^8
5	5.5×10^8
6	5.5×10^8
7	7.0×10^8
8	5.8×10^8
9	6.0×10^8
10	5.5×10^8
	Mean = 5.8×10^8

Champluvier, et al. (1988) studied the activity of whole cells for the yeast strains *K. fragilis* (two strains), *K. lactis* (three strains) and *K. marxianus* (two strains). The best permeabilizing agent composition (ethanol 10% and chloroform 2%) reported by these authors was applied to this study. Among these strains, *K. lactis* CBS 683 exhibited the highest β-galactosidase activity, i.e. 2.38 U/mg DW (One unit was defined by Champluvier, et al. (1988) as the release of 1 μmol *o*-nitrophenol per minute). By

applying the same analytical procedures, the average activity obtained in this experiment for *K. lactis* CBS 2357 is equivalent to 2.83 U/mg DW. This corresponding value is obtained by converting activity expressed in the cell number into dry weight (DW). (Note: the correlation value used for conversion: 10^9 permeabilized cells equivalent to 14 mg DW; this value was averaged from six measurements as shown in Appendix 1). It is clear that the whole cell activity obtained is significantly higher (18.9% more active) than that obtained by Champluvier, et al. (1988). The average activity then became the reference value. Every new stock of cells produced had to fulfill this activity requirement before being used in experiments.

Table 3.2 Activity of whole cell β-galactosidase of *Kluyveromyces lactis* and *K. marxianus*

# Flask	Cell number per ml in reaction mixture	Absorbance After 5 min (λ = 416 nm)	Initial Rate (µmol ONPG hydrolyzed/min)*	Activity (µmol ONPG hydrolyzed/ min/10^9 cells)
Kluyveromyces lactis CBS 2357				
1	3.3×10^6	1.145	0.147	44.66
2	3.0×10^6	0.925	0.119	39.67
3	3.2×10^6	0.879	0.113	35.31
4	2.8×10^6	0.898	0.116	41.43
5	3.6×10^6	1.097	0.141	39.17
6	3.9×10^6	1.069	0.138	35.38
7	5.1×10^6	1.447	0.186	36.47
8	3.5×10^6	1.147	0.148	42.29
9	3.3×10^6	1.164	0.150	45.45
10	3.7×10^6	1.081	0.139	37.57
				Mean = 39.74 ± 3.66
Kluyveromyces marxianus CBS 712				
1	1.5×10^7	0.426	0.054	3.60
2	1.8×10^7	0.393	0.051	2.83
3	1.8×10^7	0.424	0.055	3.06
4	1.8×10^7	0.437	0.056	3.11
5	1.7×10^7	0.473	0.061	3.59
6	1.6×10^7	0.432	0.056	3.50
7	1.4×10^7	0.350	0.045	3.21
8	1.3×10^7	0.409	0.053	4.08
9	2.0×10^7	0.394	0.051	2.55
10	1.2×10^7	0.329	0.042	3.50
				Mean = 3.30 ± 0.44

* Calculated using the molar o-nitrophenol extinction coefficient = 4662 M^{-1} cm^{-1} (Champluvier, et al., 1988)

3.2 DETERMINATION OF KINETIC PARAMETERS

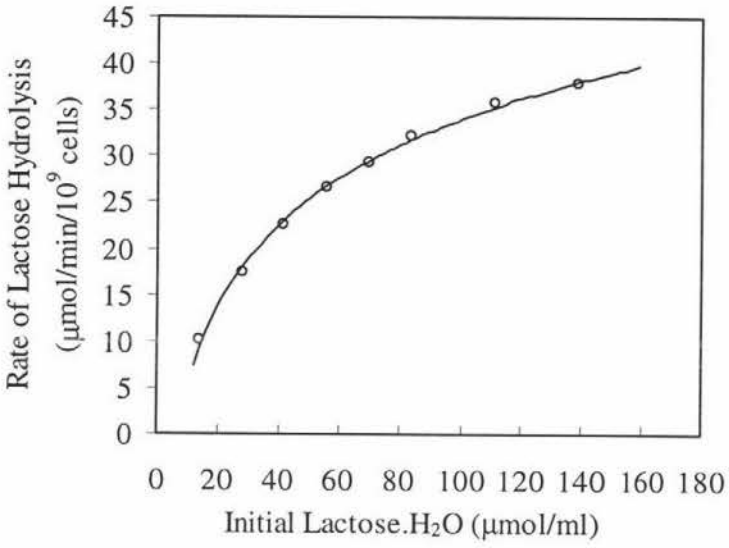
Instead of using ONPG, lactose was used as a substrate to determine the intrinsic kinetic parameters of β-galactosidase within free cells. For determination, 5 ml of permeabilized cell suspension (1.2 x10^8 cells/ml) in a large test tube (2.5x15 cm) was mixed with 5 ml of lactose solution of various concentrations (10 to 100 g/l). The final cell number in the reaction mixture was 6 x 10^7 cells/l. The mixture was incubated in the shaker water bath at 37 °C and 160 rpm for 15 minutes. To stop further hydrolysis of lactose, the tubes were immersed in a boiling water bath for 5 minutes. The supernatant after centrifugation in microfuge 12000 rpm for 5 min was used for glucose and lactose determination.

The hydrolysis processes were duplicated for each lactose concentration tested. From these duplicates, the amount of lactose hydrolyzed or glucose liberated was averaged for the calculations of initial rate of hydrolysis (calculations are presented in the Appendix 2). The data either derived from glucose analysis (enzymatic method) or lactose assay (HPLC) are analyzed and treated in an identical manner. The rate of hydrolysis was reported in μmol/min/10^9 cells. All data has been treated with the assumption that there was no product inhibition. Therefore, a linear transformation of the Michaelis-Menten equation is carried out. For a comparison study, three plot methods (Langmuir, Lineweaver-Burk and Eadie-Hofstee) were employed and evaluated.

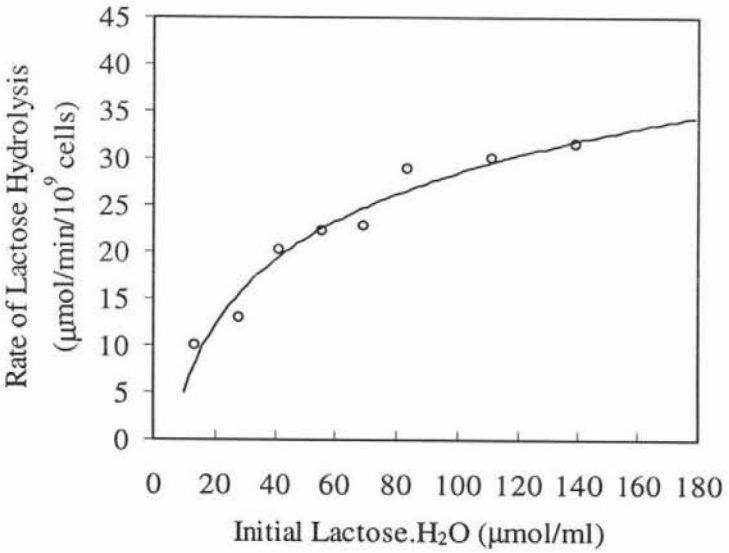
The effect of substrate concentration on the initial reaction rate is illustrated in Figure 3.1 (A) and (B) drawn based on the data derived from enzymatic assay of glucose and HPLC analysis of lactose, respectively. It could be seen that the enzymatic assay data gave a smoother curve than the HPLC assay data.

The graphs shown in Figure 3.2 and Figure 3.3 are those of Langmuir plot, because this method gave the best agreement between kinetic parameters obtained from enzymatic assay and HPLC analysis as shown in Table 3.3 (The other two plot methods are given in the Appendix 2). According to the R^2 value, the Lineweaver-Burk plot based on enzymatic assay of glucose gave the highest confidence, but these kinetic parameters showed greater deviations from their corresponding parameter values obtained based on HPLC analysis of lactose. Therefore the most acceptable kinetic parameters are those

obtained from Langmuir plot of based glucose assay with K_M and V_{max} found to be 59.34 μmol/ml and 54.64 μmol/min/10^9 cells, respectively.



(A)



(B)

Figure 3.1 The curves of the initial reaction rate of lactose hydrolysis at different lactose concentration based on: (A) enzymatic assay of glucose and (B) HPLC assay of lactose.

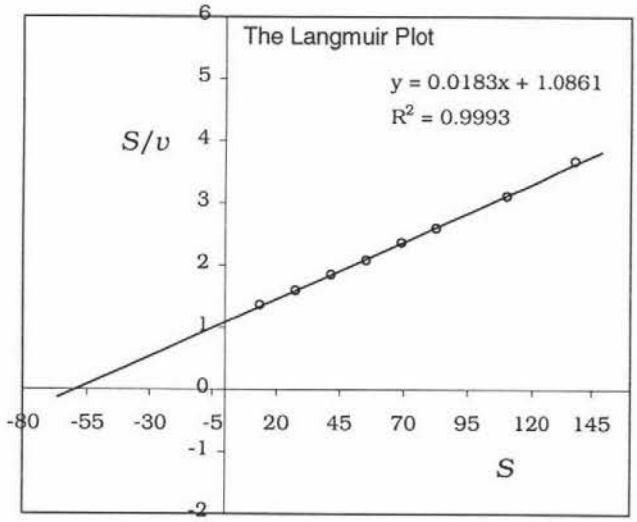


Figure 3.2 Langmuir plot of lactose hydrolysis based on enzymatic assay of glucose.

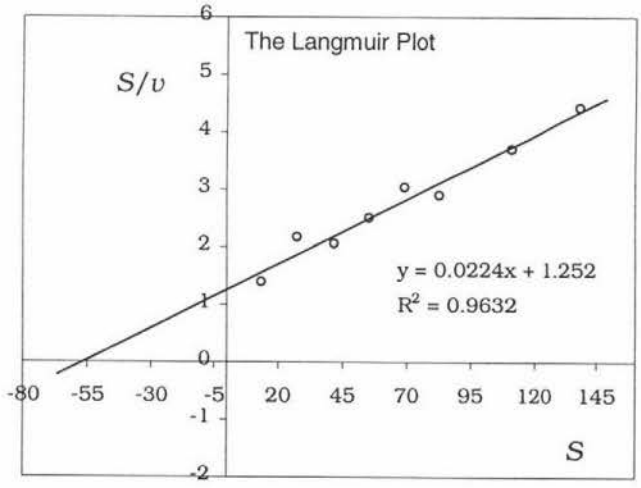


Figure 3.3 Langmuir plot of lactose hydrolysis based on HPLC assay of residual lactose.

Table 3.3 Summary of the kinetic parameters obtained from different techniques and determined based on enzymatic assay of glucose and HPLC assay of lactose

Plot techniques	R^2 (Glucose)	R^2 (Lactose)	Kinetic Parameters			
			K_M (μmol/ml)		V_{max} (μmol/min/10^9 cells)	
			Glucose	Lactose	Glucose	Lactose
Langmuir	0.9993	0.9632	59.34	55.89	54.64	44.64
Lineweaver-Burk	0.9998	0.9468	62.13	43.26	55.87	39.37
Eadie-Hofstee	0.9977	0.8126	60.63	45.42	55.17	40.74

3.3 STABILITY TEST OF WHOLE CELLS

3.3.1 Aims

The test aimed at testing the stability of untreated and Glutaraldehyde (GA)-treated cells of *K. lactis* after being stored at operational temperatures (30 and 37 °C) in different media compositions.

3.3.2 Glutaraldehyde (GA) Treatment of Free Cells

The glutaraldehyde treatment was carried out aseptically by adding one volume of GA solution (prepared by appropriate dilutions of 25% commercial GA in Mn-Buffer pH 6.5) to nine volumes of cell suspension of permeabilized cells, maintained with gently shaking in water at 30 °C for 1 hour. According to Florest, et al. (1995), the best concentration of GA for stabilization of yeast cell with cell number 10^{10} cells/ml is 15-20 mM. In this study, 9 ml of cell suspension containing 5.5 x 10^9 cells/ml was mixed with 1 ml of 99 mM GA. Therefore, the GA concentration in the reaction mixture was 9.9 mM. After incubation, the suspension was centrifuged at 6500 rpm for 20 min, washed twice with Mn-Buffer and re-suspended in 7.5 ml in Mn-Buffer (final volume obtained was 10 ml). The cell number in the final suspension enumerated using haemocytometer was 5.0 x 10^9 cells/ml. The stability of these GA-treated cells was then tested in different media (See Sub Section 3.3.3)

3.3.3 Stability Test

To test the efficacy of GA treatment on stabilizing the β-galactosidase confined within the cell, the GA-stabilized cell suspended in different media compositions were stored at operational temperatures (30 and 37 °C). The same conditions were also applied for untreated cells for a comparison study.

The suspension for stability test was prepared aseptically by mixing one volume of stock cell suspension (untreated stock cells containing 5.5x10^9 cells/ml or GA-treated stock cells containing 5.0x10^9 cells/ml) with nine volumes of liquid media. The media compositions tested were isotonic solution (0.89% saline), Mn-Buffer pH 6.5, and

0.89% saline containing 0.1 mM $MnCl_2$. These suspensions were then incubated at different temperatures (30 °C and 37 °C). Sample was taken every 24 h up to 72 h and tested for their total activity and biomass activity using ONPG method. Total activity was referred to as the combined activities of the β-galactosidase within and outside the cells. For the total activity assay, the activity in the sample was measured directly (see Sub-Section 2.2.5). Biomass activity was defined as the activity of β-galactosidase that was confined only within the cell boundaries (whole cell). For biomass activity assay, the samples were firstly treated according to the following procedure: 0.2 ml cell suspension was diluted with 1 ml Mn-Buffer at pH 6.5 in the microfuge tube and centrifuged at 12000 rpm for 15 min. The supernatant was discarded and the biomass was washed twice with Mn-Buffer. The resulting biomass was then re-suspended in 1 ml Mn-Buffer and transferred into a test tube. The microfuge tube was rinsed four times with 1 ml Mn-Buffer, and the washings were collected in the test tube (total volume was 5 ml). The activity was then assayed using ONPG method as previously described. The result is reported as μmol ONPG hydrolyzed/min/10^9 cells.

The stability tests of free cells were tested according to the following conditions:

- Untreated cells in 0.89% saline, stored at 30 °C (designated as CS-30) and 37 °C (designated as CS-37);
- Untreated cells in Mn-Buffer pH 6.5, stored at 30 °C (designated as CB-30) and 37 °C (designated as CB-37);
- Untreated cells in 0.89% saline containing 0.1 mM $MnCl_2$, stored at 30 °C (designated as CSM-30) and 37 °C (designated as CSM-37);
- GA-treated cells in 0.89% saline, stored at 30 °C (designated as GS-30) and 37 °C (designated as GS-37);
- GA-treated cells in Mn-Buffer pH 6.5, stored at 30 °C (designated as GB-30) and 37 °C (designated as GB-37);
- GA-treated cells in 0.89% saline containing 0.1 mM $MnCl_2$, stored at 30 °C (designated as GSM-30) and 37 °C (designated as GSM-37).

3.3.4 Results and Discussion

The initial activity (average) of the permeabilized stock cell suspension of untreated cells was 40.370 μmol/min/10^9 cells. This value was in the range required. After treatment with GA, the average activity decreased to 39.338 μmol/min/10^9 cells. Therefore, the treatment of permeabilized cells with GA did not cause a significant deactivation of β-galactosidase. Deactivation calculated after the GA treatment was 2.54%. Champluvier, et al. (1988) have previously reported that GA treatment induced an immediate deactivation of 10% enzyme activity. A modification of stabilization procedure has been performed by selecting the best conditions for permeabilization to minimize the loss of cell component as proposed by Champluvier et al. (1988), combined with the application of the optimal condition for GA treatment as described by Flores et al. (1995). Hence, the initial activity of untreated cells and GA-treated cells employed for stability tests were 40.370 and 39.338 μmol/min/10^9 cells, respectively. The activity expressed in percentage is then referred to as the ratio of activity obtained to the initial activity.

Figure 3.4 shows the plot total activity against incubation time up to 72 h for all conditions tested at 30 °C, whereas Figure 3.5 compares their percentage of total activity remained after storage. Likewise, for the corresponding samples, Figure 3.6 shows the plot of biomass activity against incubation time and Figure 3.7 compares the biomass activity remaining after storage.

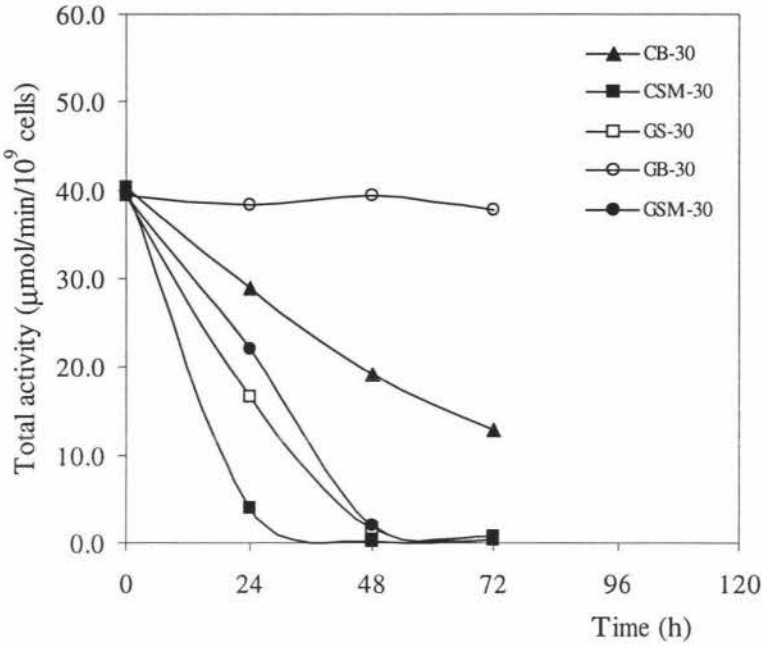


Figure 3.4 Comparisons of the total activity after storage at 30 °C. The activity expressed is μmol ONPG hydrolyzed/min/10^9 cells.

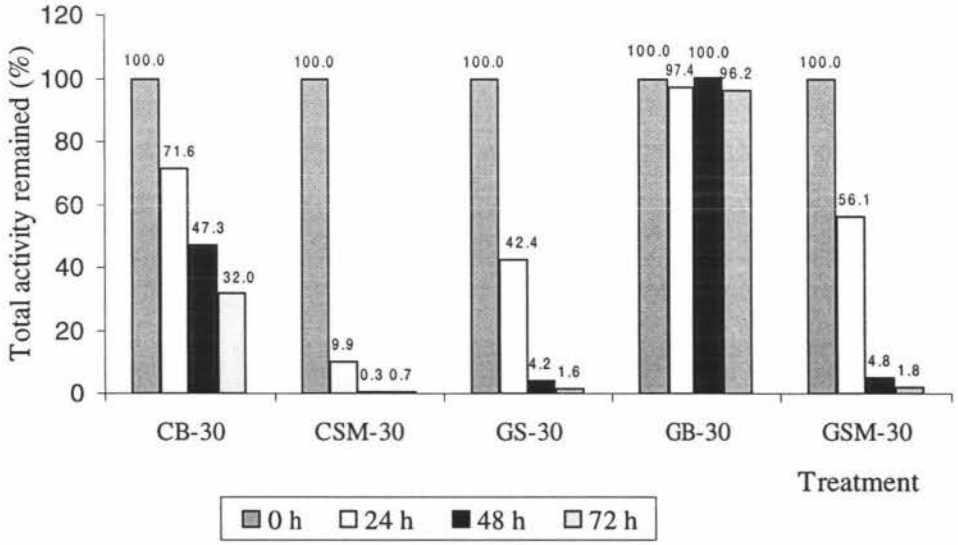


Figure 3.5 The percentage of total activity remained after storage at 30 °C

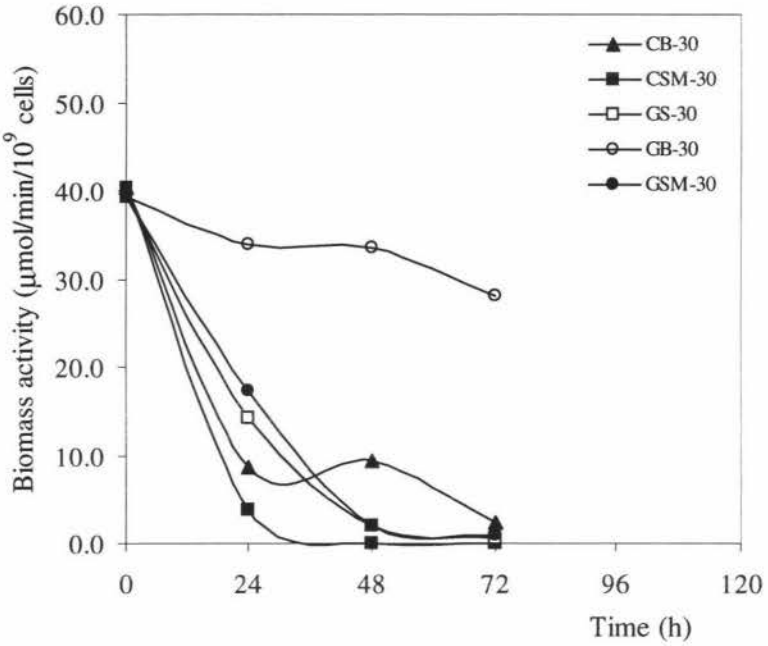


Figure 3.6 Comparisons of the biomass activity after storage at 30 °C. The activity expressed is μmol ONPG hydrolyzed/min/10^9 cells.

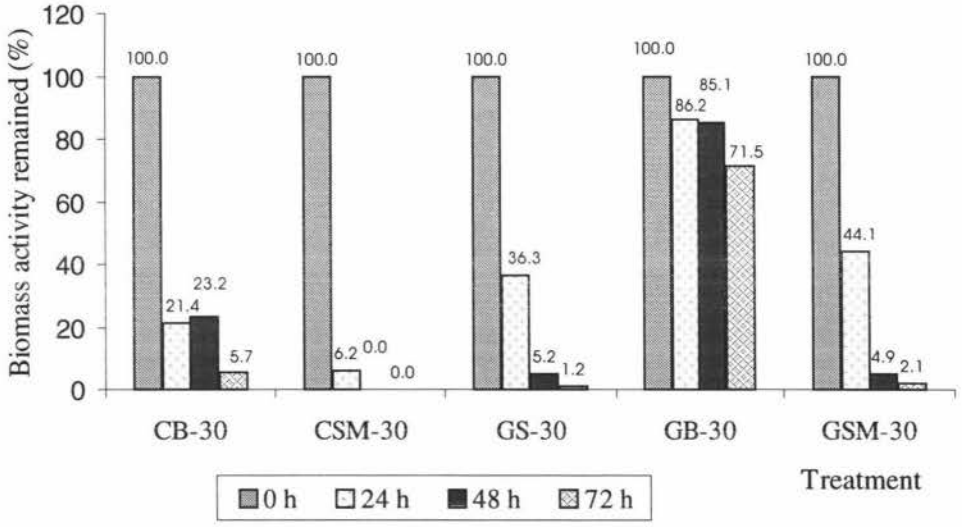


Figure 3.7 The percentage of biomass activity remained after storage at 30 °C

As can be seen from Figure 3.4, the GA-treated cells in Mn-Buffer stored at 30 °C (GB-30) maintained their total activity steadily during 72 h of incubation. Figure 3.5 confirms that the total activity of GB-30 after 72 h still remained reasonably high at 96.2%. In comparison, Figure 3.6 shows that the biomass activity of GB-30 declined slowly, reaching 71.5% at 72 h (Figure 3.7). In contrast to GB-30, the GA-treated cells

in 0.89% saline (GS-30) and in 0.89% saline containing 0.1 mM $MnCl_2$ (GSM-30) showed a sharp drop of total activity, remaining at only 4.2% and 4.8% (Figure 3.5), respectively, after 48 h. Meanwhile, the curves of biomass activity for GS-30 and GSM-30 (Figure 3.6) also displayed similar trends as for total activity where the biomass activity remained at 5.2% and 4.9% for GS-30 and GSM-30 (Figure 3.7), respectively; the values of which are comparable to the total activity. This implies that the GA treatment alone might not be the only factor that maintained the cell activity. In the absence of Mn-Buffer solution, a rapid deactivation also occurred for GA-treated cells either in 0.89% saline or in 0.89% saline supplemented with Mn.

For the untreated cells tested at 30 °C, the cells in Mn-Buffer (CB-30) showed a marked drop of total activity (Figure 3.4) and remained at 32% after 72 h (Figure 3.5). Based on total activity, during storage CB-30 showed better stability compared to GB-30 (Figure 3.4); but the biomass activity trend in Figure 3.6 confirmed that the total activity was exhibited mostly by the external β-galactosidase. The untreated cells kept in 0.89% saline containing 0.1 mM $MnCl_2$ (CSM-30) showed a dramatic fall in total activity (Figure 3.4) and remained at only 5% just after 24 h and then almost disappeared (Figure 3.5).

The results for identical stabilization tests, but carried out at a higher temperature (37 °C) are shown in Figure 3.8 to 3.11. Figure 3.8 shows the plot total activity against incubation time up to 72 h for all conditions tested, whereas Figure 3.9 compares their percentage of total activity remaining after storage. Similarly, for the corresponding samples, Figure 3.10 shows the plot of biomass activity against incubation time and Figure 3.11 compares their biomass activity remaining after storage.

The GA-treated cells in Mn-Buffer stored at 37 °C (GB-37) showed more rapid enzyme deactivation (Figure 3.8) compared to those stored at 30 °C. The total activity gradually decreased and remained at 67.3 % after 72 h (Figure 3.9). The biomass activity of GB-37 (Figure 3.10) showed a similar trend in activity, declining during incubation and remaining at 61.8% after 72 h (Figure 3.11). Therefore, the increase of temperature from 30 to 37 °C resulted in about 10% deactivation of biomass activity. On the other hand, the other the samples of other test conditions (CS-37, CB-37, CSM-37, GS-37, and GSM-37) mostly displayed a rapid drop of both total activity and biomass activity

during incubation. The total activity and biomass activity of those treatments nearly ceased (below 5%) at 72 h. Thus, the increase of incubation temperature has led enzyme deactivation to proceed more rapidly.

The phenomena found in above tests explain the efficacy of GA treatment combined with the availability of essential components in the media, especially the role of potassium that is available in the buffer solution for maintaining the biocatalyst stability. Studies conducted with *K. lactis* have confirmed the critical role of certain cations on enzyme activity (Voget, et al., 1994). β-Galactosidase is activated by monovalent cations such as K^+ and Na^+ (Mahoney & Whitaker, 1977; Dickson, et al., 1979). Eugene and Bingmam (1978) showed that Mn^{2+} has a strong stabilizing effect on β-galactosidase in potassium phosphate buffer at pH 6.6. There were the differences between the total activity and the biomass activity observed for GA-treated cells stored in Mn-Buffer (GB-30 and GB-37). These differences might be caused by:

- Enzyme dissolution into the liquid media;
- The internal destruction of the enzyme molecule had inevitably occurred due to the effect of temperature;
- The concentration of GA used was not sufficient to completely retain and stabilize the available enzyme within the cell boundaries, and therefore allowed the enzyme to leak out.

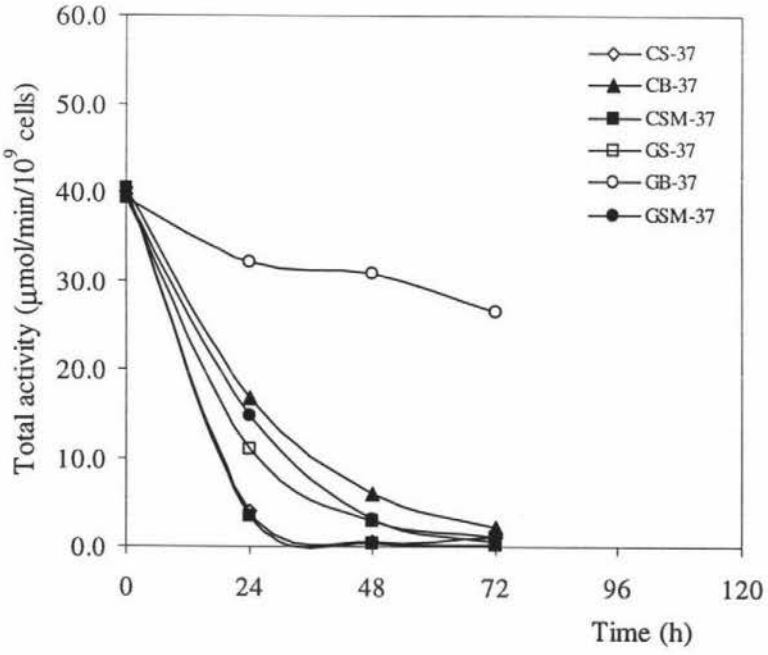


Figure 3.8 Comparisons of the total activity after storage at 37 °C. The activity expressed is µmol ONPG hydrolyzed/min/10^9 cells.

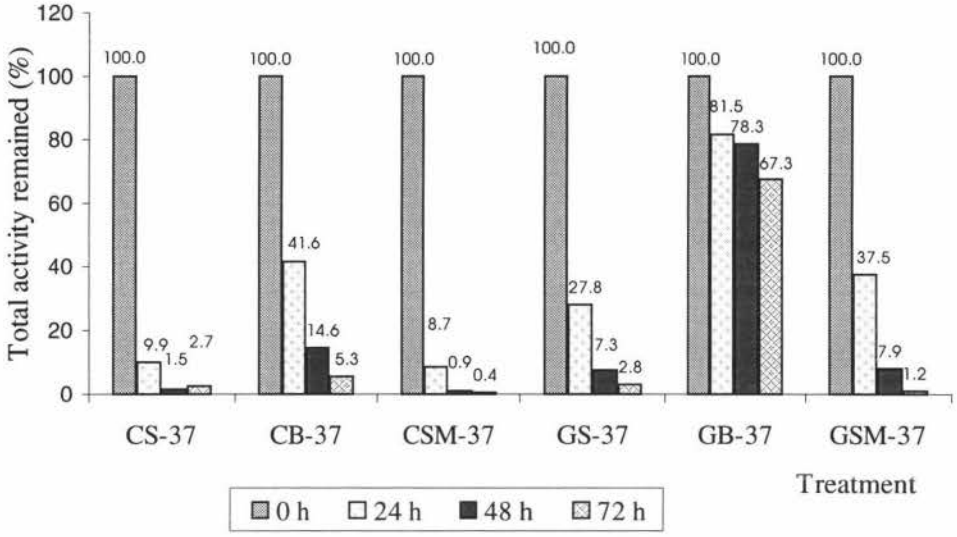


Figure 3.9 The percentage of total activity remained after storage at 37 °C

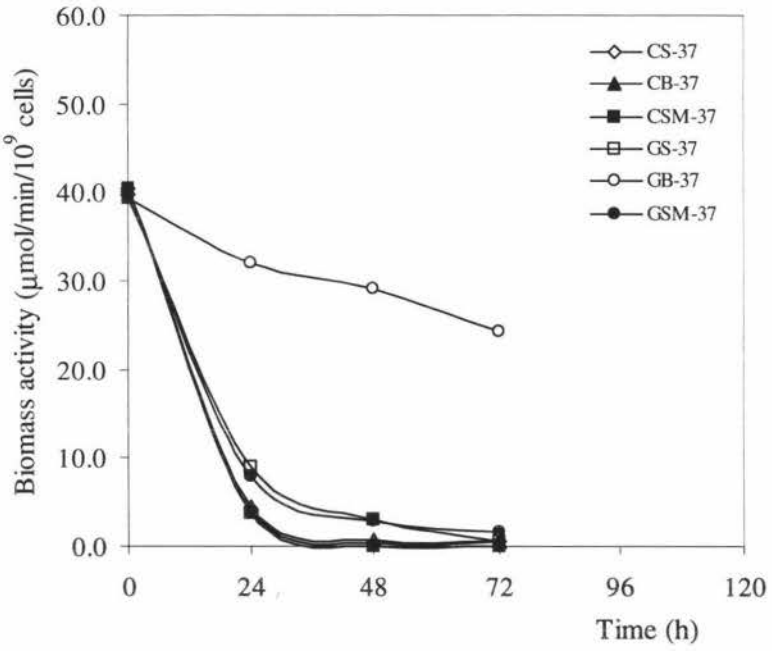


Figure 3.10 Comparisons of the biomass activity after storage at 37 °C. The activity expressed is μmol ONPG hydrolyzed/min/10^9 cells.

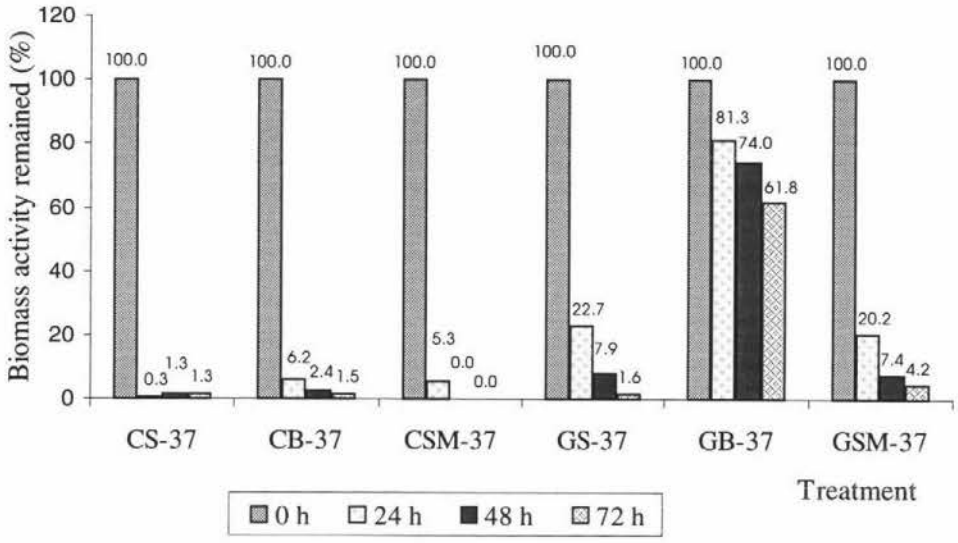


Figure 3.11 The percentage of biomass activity remained after storage at 37 °C

3.3.5 Conclusions

The conclusions from the stabilization tests of free cells are:

- GA treatment of permeabilized cells of *K. lactis* according to the procedure conducted in this study deactivated 2.54% initial β-galactosidase activity.
- The treatment of permeabilized cells with GA could significantly stabilize β-galactosidase activity in Mn-buffer media.
- Manganese and potassium ions in Mn-Buffer used are essentially required to retain the activity;
- The biomass activity of GA stabilized cells in Mn-Buffer can be maintained above 70% during 72 h of incubation at 30 °C. An increase of incubation temperature from 30 to 37 °C has led to the deactivation of about 10% biomass activity;

3.4 CELL IMMOBILIZATION IN ALGINATE

3.4.1 Biocatalyst Formation

Immobilization of cells was carried out under sterile conditions using sodium alginate. Sodium alginate powder was boiled in sterile water until completely dissolved then

allowed to cold at room temperature. Permeabilized cells, obtained from centrifugation of the stock cell, equivalent to 1.924 g dry weight (corresponds to 1.4×10^{11} cells) were mixed with 100 ml of alginate solution (2 to 3%) and gently stirred for 30 min to homogenize the suspension. The final cell concentration was 1.4×10^{9} cells/ml alginate solution. The temperature was kept at 0-5 °C with an ice bath. The mixture was then pumped through the 20G needle into a gently stirred 0.1 M $CaCl_2$ solution (300 ml) using a peristaltic pump (Masterflex^{R}) at a rate of about 100 ml/hour. The air was blown through the needle from two opposing directions (Figure 3.12) at a constant flow rate, which was adjusted using a set air flow regulator (consisting of an air-pressure regulator and an airflow meter). The drag force created by the concentric flow of gas contributed to shear the drop from the needle tip at a smaller size of alginate beads. The beads formed were kept gently stirred for 2 h to allow complete solidification before washing with sterile distilled water.

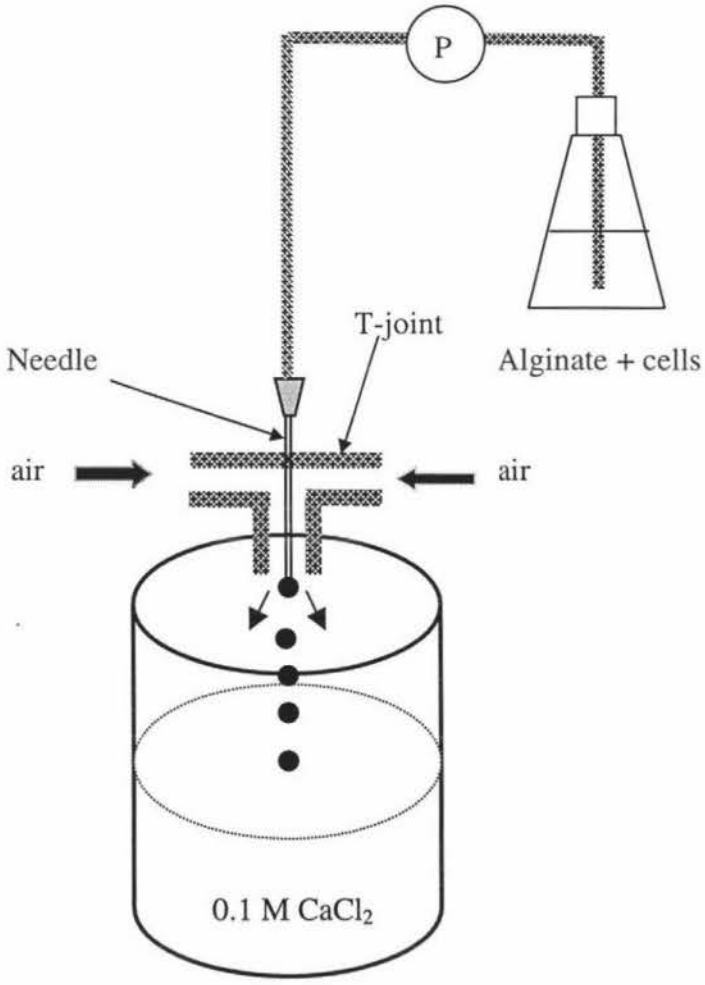


Figure 3.12 A schematic diagram of equipment used for the production of alginate biocatalyst.

3.4.2 Biocatalyst Stabilization

For stabilization, 40 grams of alginate biocatalyst were placed in 100 ml of water containing GA. The GA concentrations tested were 0.5, 1.0, 2.0 and 4.0 mM. The reaction was carried out at room temperature for 1 h employing mild stirring. The biocatalyst was then washed repeatedly with distilled water.

3.4.3 The Assay of Biocatalyst Activity

Since alginate beads are re-dissolved in phosphate solution, the use of Mn-Buffer as hydrolysis media was avoided. As a consequence, biocatalyst activity was always tested in water containing 0.1 mM $MnCl_2$. The distilled water used was at pH 6.6 – 6.8, which was close to the optimum pH for β-galactosidase (pH 6.6). The activity of alginate biocatalyst was assayed using the ONPG method according to the following procedure: 1 gram biocatalyst in 125 ml Erlenmeyer flask was mixed with 10 ml pre-warmed 0.1 M $MnCl_2$ solution (37 °C). Two ml of pre-warmed 5 mM ONPG solution (37 °C) was added and the flask was incubated in the water bath shaker (37 °C, 120 rpm). One ml of sample was taken at 2, 4 and 6 minutes. The sample was quickly mixed with 2 ml 0.2 M Na_2CO_3 to stop further reaction. The absorbance was measured at 416 nm. From the absorbance data, the total ONPG hydrolyzed was calculated and plotted against time. The slope obtained is the activity per gram of biocatalyst. The activity of immobilized cells is reported as μmol ONPG hydrolyzed per min per gram of alginate biocatalyst.

3.4.4 Results and Discussion

The gas shear method as previously described by Seifert and Phillips (1997) has been applied satisfactorily in this work to produce small size of alginate beads. The peristaltic pump used to deliver cells in alginate solution for gel formation was adjusted at a low flow rate of about 1-2 ml/min to get a more uniform size. It was noticed that fine particles were still formed concurrently by the action of gas shearing. These fine particles were removed by washing. The best adjustment of equipment yielded biocatalyst with diameter of 0.96 ± 0.09 mm.

For the stabilization of immobilized alginate biocatalyst, the choice of GA concentration used in the experiment was based on the experiment results obtained by Flores, et al. (1995) who treated permeabilized free cells of *K. lactis* with GA. The cells (10^{10} cells/ml were permeabilized at room temperature for 30 min. It was found that the effective stabilization at room temperature could be achieved with 15-20 mM GA where 20-25% β-galactosidase activity was lost. From this data, it is calculated that 10^{12} cells require 1.5 – 2.0 mmol GA which is equivalent to 0.12 - 0.16 mmol GA for 8 x 10^{10} cells.

In this experiment, the average cell number enumerated was 2 x 10^{9}cells/g of alginate biocatalyst. Therefore, if 40 g of biocatalyst was stabilized in 100 ml GA solution, the total cell number would be 8 x 10^{10} cells. The ranges of GA concentrations tested were between 0.5 mM to 4.0 mM which correspond to 0.05 to 0.4 mmol per 100 ml solution. These GA concentrations agreed with the range proposed by Flores and Ertola (1995).

The influence of GA concentration on the activity of whole cell β-galactosidase immobilized in different alginate concentration is shown in Table 3.4. The plot of data from Table 3.4 is shown in Figure 3.13. Figure 3.14 illustrates the percentage of relative activity remained after GA treatment. (Note: full collection of data for alginate biocatalyst activity measurement is shown in Appendix 4). As shown in Table 3.4 for control (biocatalyst without GA treatment), the average activity decreased as the alginate concentration increased. When the alginate concentration increased from 2% to 2.35%, the average activity decreased from 1.09 to 0.86 μmol ONPG/min/g biocatalyst. The average activity remained at 0.55 μmol ONPG/min/g biocatalyst when the alginate concentration was further increased to 3%. Those activity values were the initial activity for each alginate concentration tested before the biocatalyst was treated with different GA concentrations.

Table 3.4 The activity of biocatalyst at different alginate concentration treated with different GA concentration.

GA (mM)	Mean of Activity (μmol ONPG/min/g biocatalyst)		
	Alginate 2%	Alginate 2.35%	Alginate 3%
0 (control)	1.0871	0.8558	0.5539
0.5	0.6315	0.5729	0.4100
1	0.6131	0.4790	0.3143
2	0.4732	0.4044	0.3420
4	0.1248	0.2501	0.1022

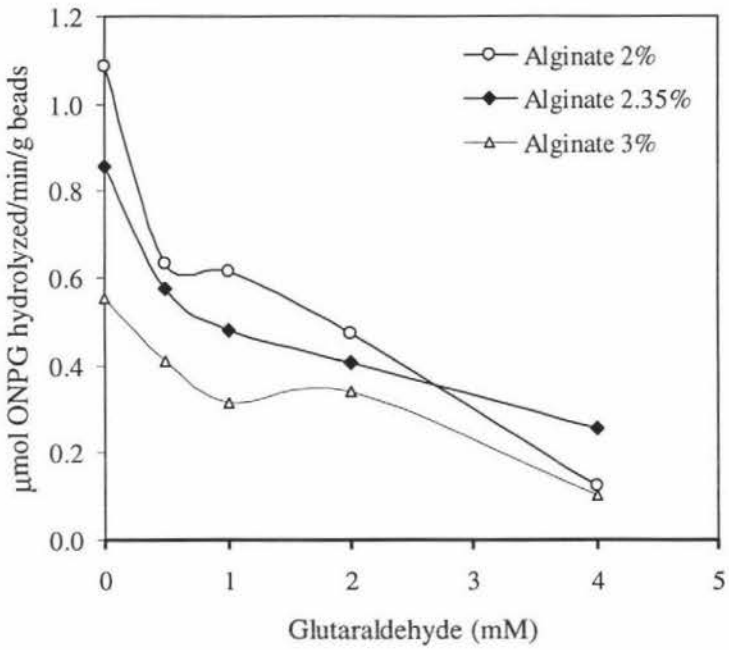


Figure 3.13 The influence of GA concentration on the deactivation of whole cell *K. lactis* β-galactosidase immobilized on alginate biocatalyst.

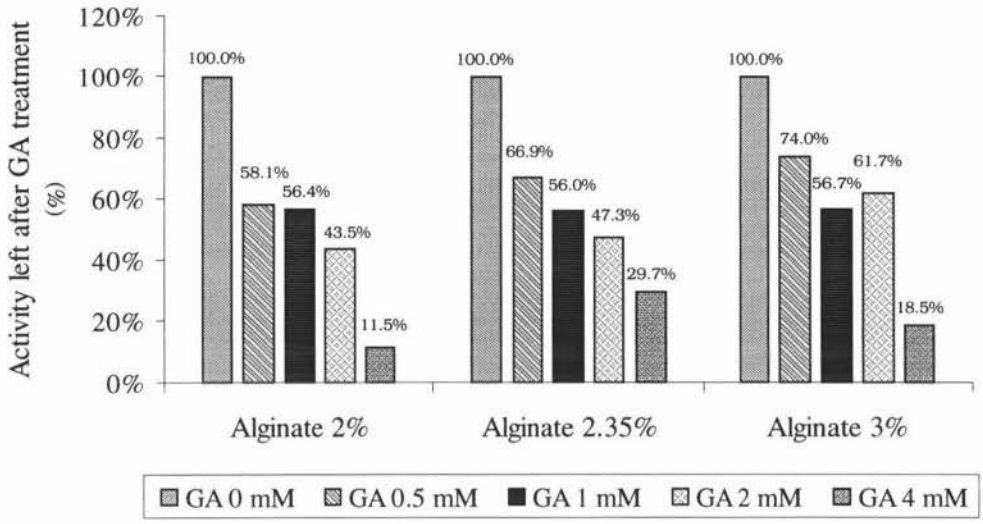


Figure 3.14 The percentage of enzyme deactivation for different alginate concentration after GA treatment.

Figure 3.13 shows that, in general, the treatment of alginate biocatalyst with GA caused a sudden drop of the activity for all alginate concentration tested, especially within 0 to 1 mM GA. The biocatalyst activity then steadily dropped at higher GA concentration. It is clear that the treatment of alginate biocatalyst with GA reduced the activity depending on alginate concentration. Figure 3.14 shows that stabilization with 0.5 mM GA contributed to the loss of 42% initial activity of 2%-alginate biocatalyst, whereas the same treatment reduced 33% and 26% of the activity of 2.35%- and 3%- alginate biocatalyst, respectively. Similarly, the treatment with 1 mM GA decreased 43.6%, 44% and 43.3% the activity of 2%-, 2.35%- and 3%- alginate biocatalyst, respectively. A further increment in GA concentration diminished the enzyme activity more rapidly. In general, even though the treatment of 2%-alginate biocatalyst with GA 0.5 – 2 mM showed significant fall in activity, the overall activity retained was still higher than that obtained from higher alginate concentrations. The decrease of biocatalyst activity as alginate concentration increased might be due to substrate diffusional resistance.

It can be seen from Figure 3.14 that the percentage of biocatalyst activity after stabilization with 0.5 to 1.0 mM GA remained within the range of 56-70% for all alginate concentrations tested. Accordingly, the GA concentration from 0.5 to 1.0 mM

is considered to be the best range for stabilization using the procedure applied. The actual activity value will then be dictated mainly by alginate concentration. Thus, the higher the alginate concentration used, the lower the activity obtained when treated with GA concentration between 0.5 to 1.0 mM.

After GA stabilization the biocatalyst in distilled water was kept at 37 °C without shaking. Upon storage, a strong decrease of biocatalyst activities was observed. The lactase activity was completely lost within 24 hours. This suggested that, firstly, there might be no stabilization effect upon direct treatment of alginate biocatalyst with GA. Birnbaum, et al., 1981 reported that alginate gel could be chemically stabilized by reacting with GA. This implies the GA molecule can react with alginate polymer thereby reducing the stabilization effect of GA toward the enzyme within the cell boundaries. Secondly, the absence of Mn and K ions that are usually available in Mn-Buffer might be a major cause of activity decline. This has been proven based on the previous experiment with free cells. The reason why the biocatalyst was kept in water instead of Mn-Buffer because calcium alginate structure is labile on contact with various complexing ions such as phosphate (Birnbaum, et al., 1981), an anion in the buffer used.

3.4.5 Conclusions

The conclusions drawn from the alginate biocatalyst study are:

- Direct stabilization of alginate biocatalyst with GA caused significant reduction of β-galactosidase activity depending on GA and alginate concentrations. When 40 g of biocatalyst containing $2x10^9$ cells/g beads was stabilized in 100 ml of 0 to 4 mM GA, the optimum range of GA concentration obtained lied between 0.5 to 1.0 mM. When that GA concentration range was applied to stabilize 2%- to 3%-alginate biocatalyst, the average biocatalyst activity remained within 56-74% of the initial activity.
- Since alginate biocatalyst is easily dissolved by phosphate ions, the GA-treated alginate biocatalyst kept in the absence of K (from phosphate buffer) and Mn ions showed poor activity after storage.

Chapter 4
PACKED-BED PROCESS

4.1 CELL IMMOBILIZATION

4.1.1 Immobilization to Bone Charcoal

Immobilization through direct adsorption of cells on bone charcoal was expected to occur. Bone charcoal comprising coarse particles was sieved through two different sieve sizes, 0.85 mm at the top and 0.60 mm as the second layer. The particles with sized between 0.60 – 0.85 mm were retained in the second sieve. They were collected and washed several times with distilled water until the washed water became clear. Wet bone charcoal (10 g) was autoclaved in a 250 ml Erlenmeyer flask, and then cooled at room temperature. 50 ml of propagation medium (LM-1) was added and 1 ml of *K. lactis* cell suspension, which was previously prepared by suspending the culture slope with 5 ml sterile water, was inoculated. The flask was shaken at 250 rpm for 24 h at 30 °C. After incubation, the liquid portion of the grown culture was discarded, and the solid was washed twice with sterile distilled water. Then 50 ml of production medium (LM-2) was added, and agitation was allowed to proceed for another 24 h. Finally, the biocatalyst produced was washed three times sterile with Mn-buffer and kept cold at –20 °C if not promptly used.

4.1.2 Immobilization to Corn Particles

Immobilization of cells through direct adsorption mechanisms was also applied to corn particles. The commercial corn particles (Healthries, New Zealand) were initially strained through the larger pore size sieve stacked on the top of the second smaller pore size sieve (two combinations of sieving used were 0.85/0.60 mm and 1.00/0.85 mm). The particles with a size range between the two mesh sizes were collected from the second sieve. Sieved corn grits (10g) in a 250 ml Erlenmeyer flask were autoclaved and cooled at room temperature. 5 ml of yeast cell suspension in LM-1 (slope culture was previously suspended with 20 m LM-1 medium) was distributed uniformly onto sterile

corn grits. The solid culture was then incubated at 30 °C. After incubation for 24 h, 5 ml of LM-1 medium was distributed onto particles in the flask to keep the solid moist. Further incubation was carried out for another 24 hours. After 48 h in solid state incubation, the modified production medium (LM-2 but with 5% lactose concentration) was poured into the flask to make a total volume (solid and liquid) of 100 ml. The liquid culture was shaken at 250 rpm at 30 °C for 24 h; this stage is referred to as a "single liquid cultivation stage". After incubation, the liquid portion was removed and the solid was washed thrice with Mn-buffer pH 6.5 followed by the addition of the second portion of modified production medium up to 100 ml of the total volume. The flask was shaken again for another 24 hours. This is referred to as a "double liquid cultivation stage". After the second incubation, the corn biocatalyst was washed three times with Mn-buffer and kept cold at -20 °C if not promptly used.

4.1.3 Permeabilization of Immobilized Cells

Biocatalyst and Mn-buffer in the ratio of 1:2 was placed in a rectangular box for permeabilization. Ethanol (10% v/v) and chloroform (2% v/v) were added and permeabilization was allowed to proceed at room temperature for 10 minutes with a gentle reciprocal shaking. The permeabilized biocatalyst was washed 3-5 times with sterile Mn-buffer pH 6.5 before used

4.1.4 Biocatalyst Activity Test on Lactose hydrolysis

The biocatalyst, before being applied to the packed-bed process, was tested for its activity towards lactose according to the following procedure: 8 ml of 2.5% lactose in Mn-buffer was mixed with 4 g biocatalyst in 50 ml Erlenmeyer flask. The flask was incubated in water a bath shaker at 120 rpm at 37 °C. Unless otherwise stated, 1 ml of samples was taken every 15 minutes for three times. To stop further lactase reaction, the samples were boiled for 5 min. A graph was plotted to allow measurement of the slope, which gives the lactase activity. The activity is reported in µmol lactose hydrolyzed/min/g biocatalyst.

4.2 PACKED-BED PROCESS

4.2.1 Reactor Configuration

A packed-bed process of lactose hydrolysis by immobilized whole cell of β-galactosidase was carried out in a jacketed tubular reactor with 1.5 cm of internal diameter and 25 cm height. The packed-bed reactor configuration was preferred because it creates less mechanical degradation of the support (Siso & Doval, 1994). Reactor temperature was maintained at a constant operational temperature by circulating the water between water incubator and reactor using a Masterflex^{R} peristaltic pump. A thermometer was inserted near the jacket water outlet to visually monitor the reactor temperature. The tip of the pH probe (Schott Gerate, Germany) was plugged in to a small chamber made from stainless steel, which was placed in the effluent line about 20 cm from the reactor outlet. The effluent pH was monitored using an Orion Research Digital Ion Analyzer (Model 701A; Watson Victor Ltd, New Zealand) and automatically recorded throughout the process by a chart recorder (Yokogawa Hokusin Electric, Model 3021 Pen Recorder; Japan). A peristaltic pump with Masterflex^{R} tubing was used to transfer the liquid substrate. The photograph of the packed-bed process is shown in Figure 4.1.

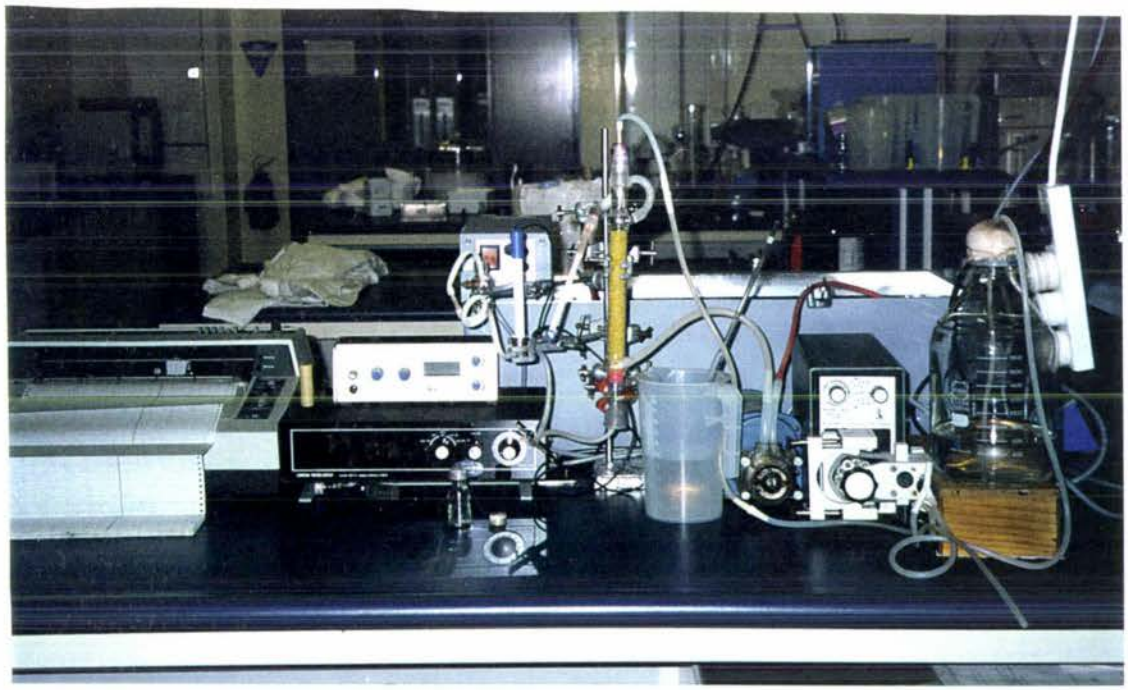


Figure 4.1 Photograph of the packed-bed process

4.2.2 Miscellaneous Assay

In order to check the availability of free enzyme in the reactor for certain packed-bed processes, 2 ml of sample in a test tube was shaken at 160 rpm in a water bath at 37 °C for 1 hour. The test tubes were immersed in the boiling water for 5 minutes to stop further reaction. The total glucose concentration after incubation was measured using the enzymatic method described in Chapter 2. If the total glucose concentration after incubation is higher than that before incubation, free enzyme in the liquid phase exists.

4.3 RESULTS AND DISCUSSION

4.3.1 Packed-bed Process Using Bone Charcoal Biocatalyst

Figure 4.2 plots the amount of lactose hydrolysis (μmol/g biocatalyst) versus time from bone charcoal biocatalyst in shake flasks to test the activity. The biocatalyst was not subjected to permeabilization. The slope, which represents the initial rate of lactose hydrolysis, was 0.07 μmol lactose hydrolyzed /min/g biocatalyst. The conditions operated for PBR-1 and PBR-2, which utilize the bone charcoal biocatalyst, are shown in Table 4.1.

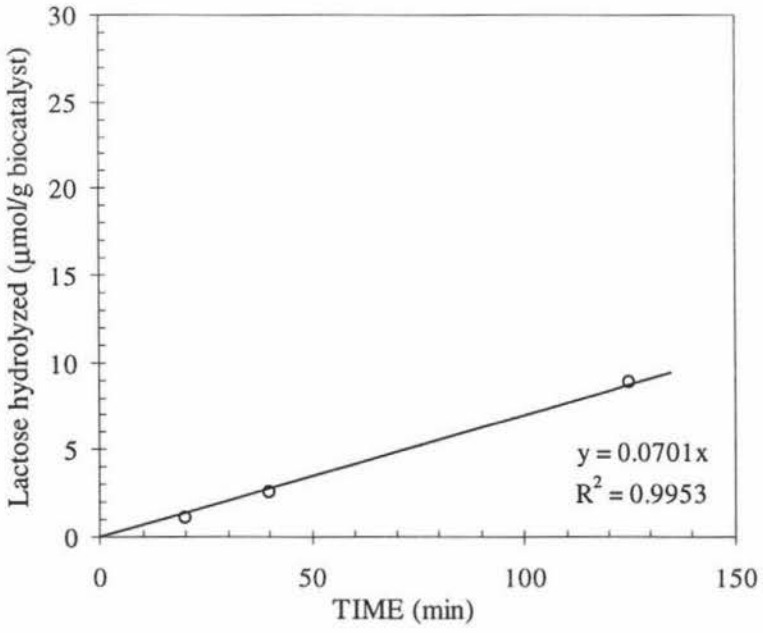


Figure 4.2 Activity test of bone charcoal biocatalyst used for PBR-1 and PBR-2. The activity (slope) = 0.07 μmol/min/g biocatalyst.

Table 4.1 Operating conditions for PBR-1 and PBR-2

Operating conditions	PBR-1	PBR-2
Feed Lactose	50 g/l (pH 6.5)	20 g/l (pH 6.5)
Temp	37 °C	37 °C
Biocatalyst	bone charcoal	bone charcoal
Permeabilization	No	No
GA stabilization	No	No
Biocatalyst diameter	0.60 – 0.85 mm	0.60 – 0.85 mm
Column height	24 cm	18 cm
Column diameter	2.4 cm	2.4 cm
Bed height	13 cm	13 cm
Feed rate	0.46 ml/min	0.57 ml/min
Biocatalyst activity (μmol/ min/g biocatalyst)	0.07	0.07

With a lactose concentration of 50 g/l in the reactor inlet, the initial trial of the packed-bed process (PBR-1) was run for a short period (below 4 hours). The results are shown in Figure 4.3. As shown in Figure 4.3, during the first half an hour the pH tended to decrease and then returned to a steady level afterwards. In the meantime, glucose concentration in the reactor outlet steadily increased, but remained below the level of 1 g/l (below d.h. 4%) during the course. By applying a lower inlet substrate concentration (20 g/l lactose) with an extended operation time in PBR-2 as shown in Figure 4.4, it was found that the outlet glucose concentration was still maintained at the same level as in PBR-1. The outlet glucose concentration of PBR-2 reached a plateau at about 0.7 g/l (d.h. 7%) after 20 h of operation, suggesting that the adsorption of glucose liberated from lactose hydrolysis to bone charcoal might have initially occurred. This suggestion was confirmed by a small decline of the pH in the first hour (Figure 4.3) that explained the adsorption of buffer components, which then altered the buffer capacity. In summary, the degree of hydrolysis (d.h.) attained in PBR-1 and PBR-2 using non-permeabilized bone charcoal biocatalyst was below 7% throughout the process.

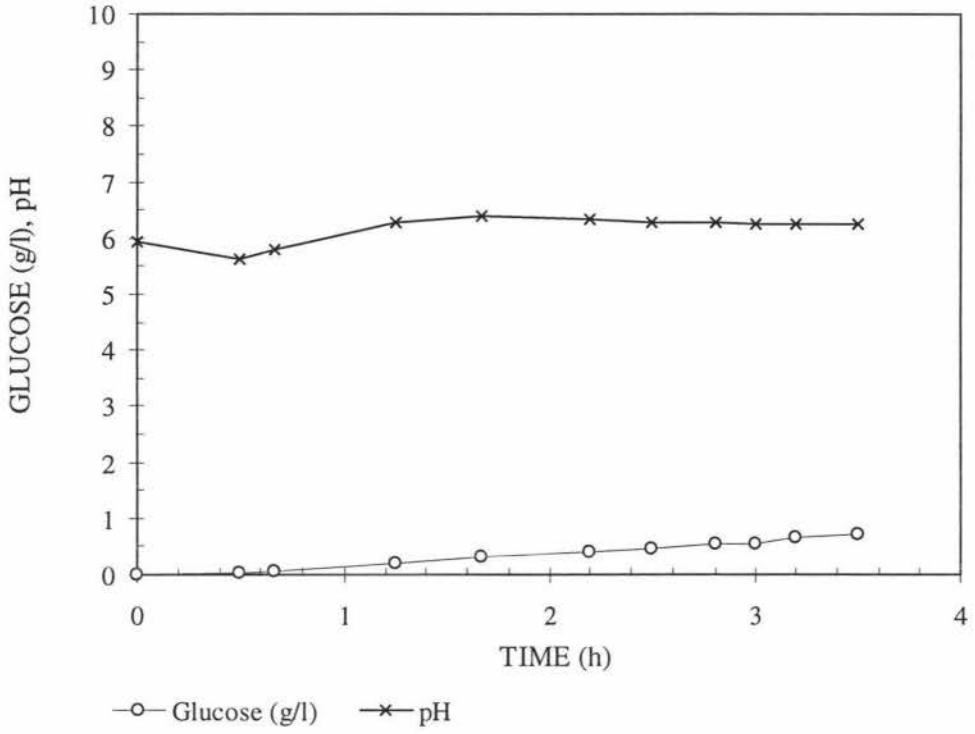


Figure 4.3 Product concentration profile for PBR-1 using bone charcoal without being permeabilized. Inlet glucose concentration = 50 g/l.

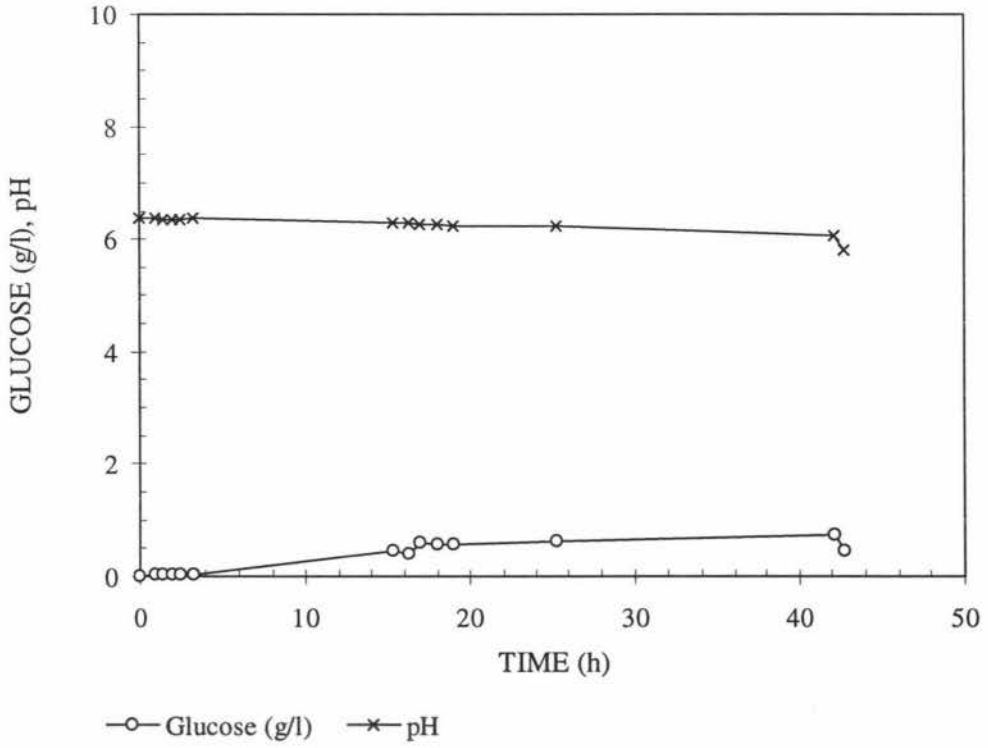


Figure 4.4 Product concentration profile for PBR-2 using bone charcoal biocatalyst without being permeabilized. Inlet glucose concentration = 20 g/l.

4.3.2 Packed-bed Process Using Corn Biocatalyst

It has been reported that d.h. greater than 80% is required to be industrially acceptable (Tomaska, et al., 1995b). In an attempt to enhance the d.h., an alternative system was investigated. The system consisted of *K. lactis* adsorbed onto corn grits. β-Galactosidase *K. lactis* cells have been immobilized on corn grits through covalent bonding followed by permeabilization with ethanol (Siso & Doval, 1994). According to Siso & Doval (1994), for direct adsorption to occur, the pore size dictates the ability of cell to penetrate into the support. Since the pore size of commercial corn grits used was unknown, a successive cultivation stage comprising solid state and liquid growth was performed to get high cell density inside corn particles.

A preliminary test of lactose hydrolysis in batch mode showed that corn biocatalyst prepared through a "single liquid cultivation stage" without being firstly permeabilized exhibited lower activity (0.19 μmol lactose hydrolyzed/min/g biocatalyst) than the permeabilized biocatalyst. However, this activity value is about 2.6 times greater than that of bone charcoal biocatalyst. According to Siso & Doval (1994) there were two advantages of using permeabilized cells of *K. lactis* over untreated cells for lactose hydrolysis. Firstly, the reaction rate proceeds more rapidly. Secondly, further metabolism of the products of hydrolysis (glucose and galactose) into ethanol has been terminated.

The linear plots of activity test for permeabilized corn biocatalysts prepared with a "single liquid cultivation stage" are shown in Figure 4.5 and 4.6, respectively. This corn biocatalyst was not stabilized with GA. The activity obtained was 0.44 and 0.51 μmol lactose hydrolyzed /min/g biocatalyst of which were used for PBR-3 and PBR-4, respectively. From the slope data, it is shown that permeabilization was able to promote biocatalyst activity between 2.3 to 2.8 times higher than the case without permeabilization. Siso & Doval (1994) reported that permeabilization of *K. lactis* cells covalently immobilized in corn grits promoted biocatalyst activity 240-fold higher than that shown by control cells (non-permeabilized free cells). However, instead of using lactose, these authors used ONPG to measure the activity. Later, Kim et al. (1997) reported that the extent of hydrolysis activity of β-galactosidase toward ONPG is not

always consistent with that toward lactose under a given condition, which may cause inaccurate estimation of the enzyme activity.

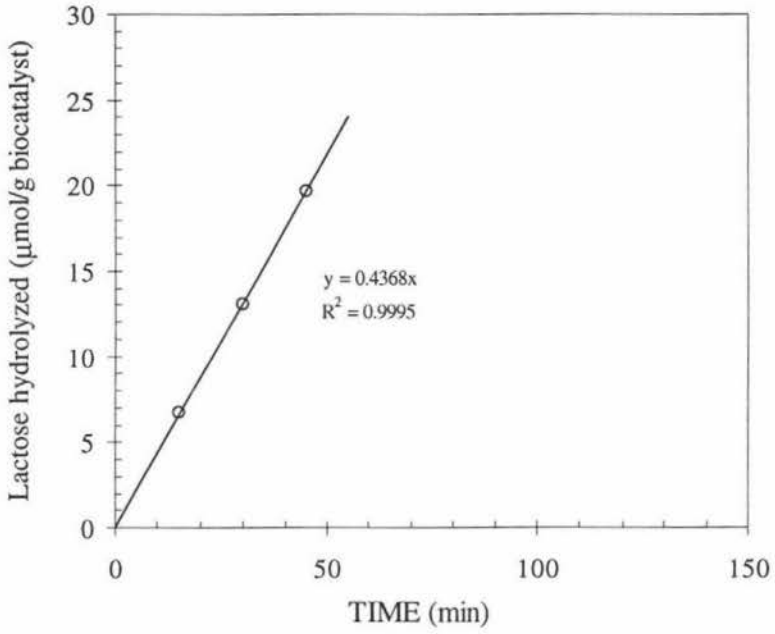


Figure 4.5 Activity test of corn biocatalyst used for PBR-3. The activity (slope) = 0.44 µmol/min/g biocatalyst.

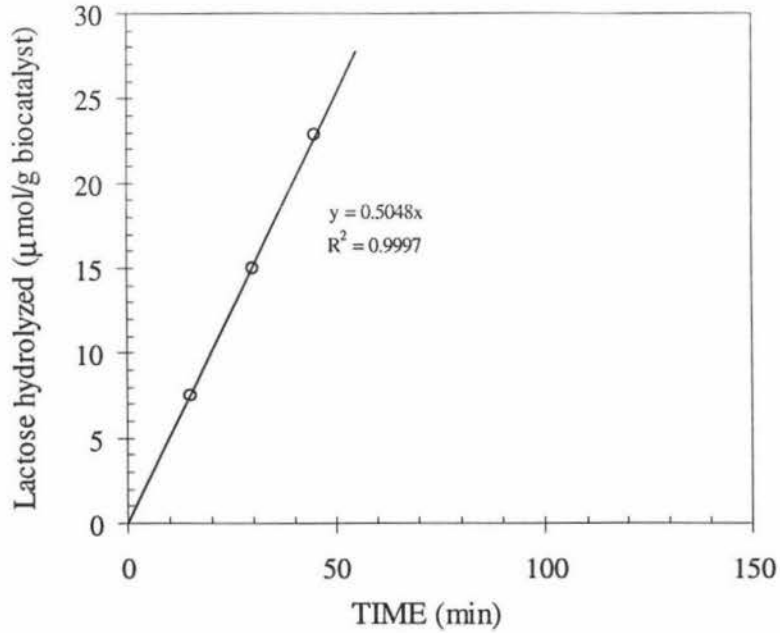


Figure 4.6 Activity test of corn biocatalyst used for PBR-4. The activity (slope) = 0.51 µmol/min/g biocatalyst.

Figures 4.7 and 4.8 display the trend of substrate conversion, product formed and pH profile for PBR-3 and PBR-4, respectively. Their operating conditions are shown in Table 4.2. As can be seen from Figure 4.7 and 4.8, there were marked increases in the outlet glucose concentration during the first hour. This period was a pre-equilibrium phase. In this period, lactose concentration in the reactor was increasing because of the supply from the feed substrate. As the lactose concentration rose, the rate of lactose hydrolysis also increased and therefore more glucose was liberated. Figures 4.7 and 4.8 show that the outlet glucose concentration peaked at 2.2 g/l (d.h. = 22 %) for both PBR-3 or PBR-4 after 1.5-2 hours of operation (See Appendix 5 Tables A5.6 and A5.7). Productivity calculated based on peak glucose concentration for PBR-3 and PBR-4 were 1.99 g/l/h and 2.10 g/l/h, respectively.

Since the outlet glucose concentration for most of the packed-bed processes conducted markedly dropped after the peak point was reached, free enzymes might be present in the reactor, especially during the first 3 hours. The availability of free enzyme was confirmed through prolonged batch incubation of samples in test tube for 1 hour at 37 ^{o}C. After incubation, the total glucose concentration was measured. If the total glucose concentration after incubation was higher than the outlet glucose concentration, free enzyme might have been released from the biocatalyst. This measurement was conducted for PBR-4 and PBR-9 as shown in Figure 4.8 and 4.17, respectively (refer the trends of glucose concentration after incubation in both Figures). According to Figure 4.8 (PBR-4), the total glucose after incubation increased dramatically during the first hour until it peaked at 2.85 g/l. This peak value was 1.2 times higher than that of the outlet glucose concentration (See Appendix 5, Table A5.7). From those peak points onwards, the glucose concentrations depleted. They were then coincided at about 5 hours. These trends implied that a certain amount of β-galactosidase was present in the bioreactor during the first 5 hours. The free enzyme available in the liquid phase might be due to the solubilization of enzyme from the biocatalyst (Breddam and Beenfeldt, 1991; Tomaska et al., 1995b; Flores, et al., 1995). Microscopic examination did not reveal yeast cells in the liquid phase. In addition, no yeast growth was observed in the viability tests. Siso (1994) demonstrated that cells were bound to corn grits by an adsorption mechanism. These authors covalently immobilized the cells to corn grits because they considered the adsorption bond not strong enough to retain the cells inside the support for a long time. However, the absence of free cells in the samples and the

disappearance of the free enzyme activity in the effluent after 5h of operation as shown in PBR-4 suggests that direct adsorption might have rendered a good cell confinement inside the corn particles.

Table 4.2 Operating conditions for PBR-3 and PBR-4

Operating conditions	PBR-3	PBR-4
Feed Lactose	20 g/l (pH 6.5)	20 g/l (pH 7.0)
Temp	37 °C	37 °C
Biocatalyst	corn	corn
Permeabilization	yes	yes
GA stabilization	no	no
Biocatalyst diameter	0.60 – 0.85 mm	0.60 – 0.85 mm
Liquid height from the bottom	26 cm	25 cm
Column diameter	1.5 cm	1.5 cm
Bed height	20.5 cm	17 cm
Feed rate	0.98 ml/min	0.90 ml/min
Porosity	0.45	0.47
Biocatalyst activity (μmol/ min/g biocatalyst)	0.44	0.50

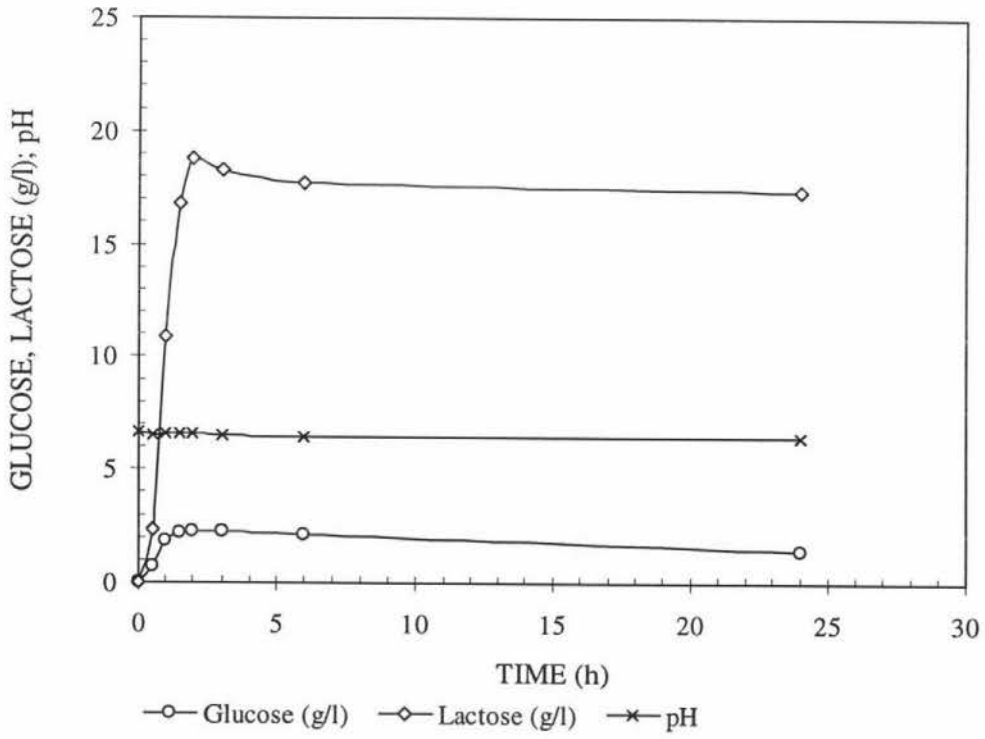


Figure 4.7 Substrate and product concentrations profile for PBR-3 using permeabilized corn biocatalyst. Inlet glucose concentration = 20 g/l

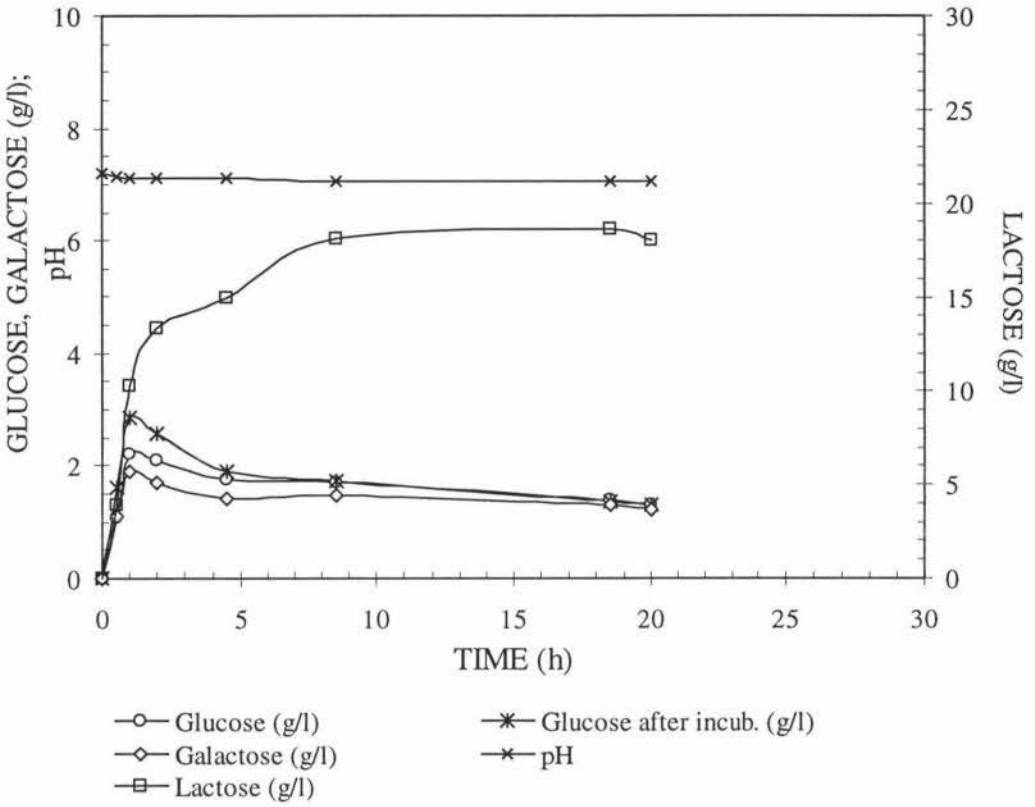


Figure 4.8 Substrate and product concentrations profile for PBR-4 using permeabilized corn biocatalyst. Inlet glucose concentration = 20 g/l

Since the hydrolysis of lactose by biocatalyst and free enzymes took place concurrently during the first 5 h, the actual value of outlet glucose concentration as the action of solid phase biocatalyst alone is superimposed with that resulting from free enzyme action. As a consequence, quantification of the outlet glucose concentration at the peak point initially achieved was developed through data extrapolation. The analysis of substrate and products concentration trends of all PBR processes revealed that the enzyme was deactivated during the process. Tomaska, et al. (1995b) pointed out that yeast β-galactosidases are labile to the temperature close to their optima. Hence, we need to know the glucose concentration at the time the peak point was achieved. Based on the overall packed-bed data, the peak point of the outlet glucose concentration was already achieved at 3 hours, the time at which the gradient concentration and substrate conversion suggested to be in the equilibrium. For data extrapolation purposes, firstly,

it is assumed that the enzyme deactivation follows first order decay, leading to the simplest model as proposed by Peterson (1991) for β-galactosidase:

$$E \xrightarrow{k_d} E_d$$

where E is the active enzyme; E_d is the deactivated enzyme; k_d is the deactivation rate constant. Deactivation of enzyme activity follows exponential decay (Peterson, 1991):

$$A / A_o = e^{-k_d t}$$

where A is the total activity at time t; A_o is the total activity at time t = 0. Secondly, based upon the above deactivation model a plot was re-built exponentially from the data point taken beyond 3 h of PBR data. The least squares fit through the points according to the following equation:

$$y = ce^{bx}$$

where c and b are constants, y is outlet glucose concentration (g/l) and x is time (h). The constant c in this case is the initial outlet concentration of glucose at the time zero.

Figure 4.9 compares the trend of exponential plot lines of outlet glucose concentration for PBR-3 and PBR-4. As can be seen from Figure 4.9, the value of R^2 shows the first order deactivation model describes the experimental data very well. The biomass loaded into the reactor for PBR-3 was higher than PBR-4, as shown in Table 4.2. Accordingly, the outlet glucose concentration for PBR-3 was higher than that of PBR-4 during the initial stage of operation; but then it decreased more rapidly afterwards. The difference in the deactivation rate might be due to the difference of the pH values operated for both processes as shown in Table 4.2. Mahoney and Whitaker (1978) reported that partially purified β-galactosidase from *Kluyveromyces fragilis* was stable for 30 min at 25 °C in the pH range of 6-8.5. When studying the effect of pH on the activity of β-galactosidase from *K. fragilis*, they found that the pH exhibits an important effect on the ionization of

the groups on the active site of the enzyme, which in turn affected the conversion of substrate to product.

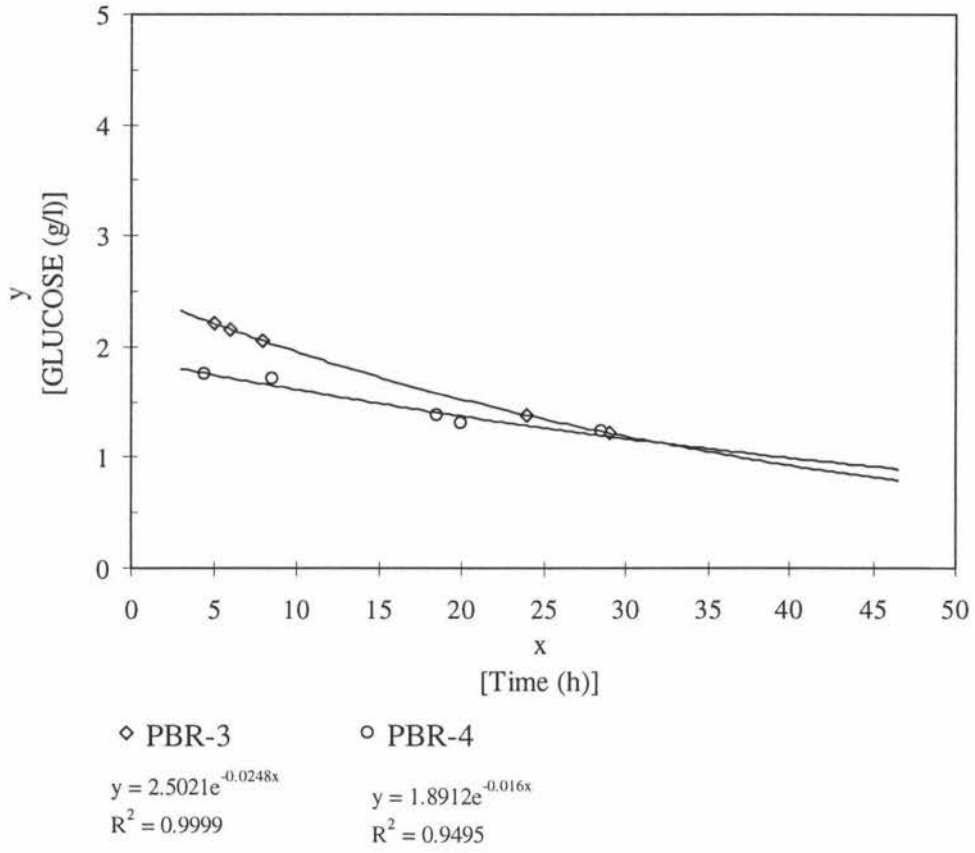


Figure 4.9 The exponential plot of outlet glucose concentration for PBR-3 and PBR-4. R^2 values indicate a good agreement of data fit.

The linear plot of the activity test for permeabilized corn biocatalyst with the higher particle size (0.85-1.00 mm) prepared with a "double liquid cultivation stage" is shown in Figure 4.10. The activity obtained was 0.84 µmol lactose hydrolyzed /min/g biocatalyst. This value was double that obtained through single liquid cultivation stage with permeabilization, and was about 5 times higher than the non-permeabilized corn biocatalyst.

To test the stability over a long period, corn biocatalyst was stabilized with GA. For stabilization, 80 g of wet corn biocatalyst was mixed with 150 ml of Mn-buffer pH 7.0.

Then 60 µl 25% GA solution was added. In order to prevent cell leakage from the support, the mixture was shaken gently up and down in a reciprocal motion for 60 min. The biocatalyst was then washed repeatedly with sterile Mn-Buffer times before use.

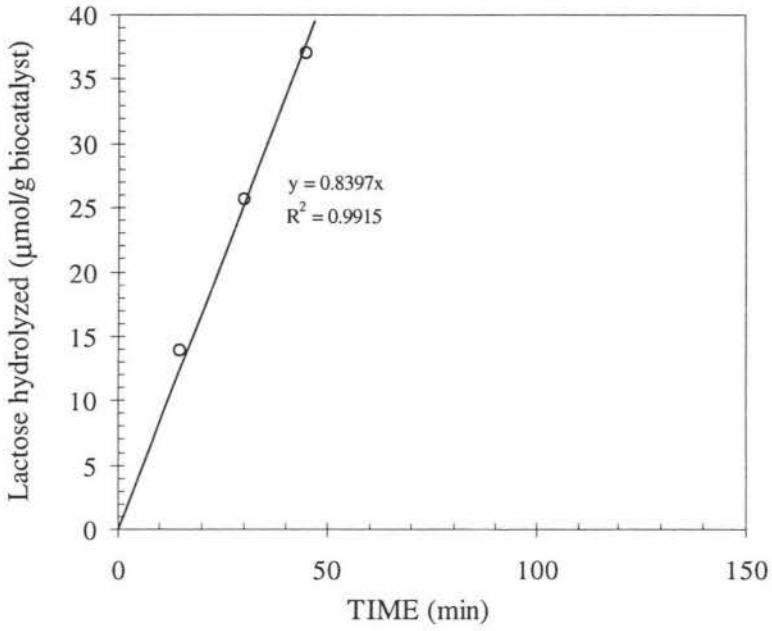


Figure 4.10 Activity test of corn biocatalyst used for PBR-5, PBR-6, PBR-7 and PBR-8. The activity (slope) = 0.84 µmol/min/g biocatalyst.

Packed-bed lactose conversions using GA-stabilized corn biocatalyst are shown in Figure 4.11 and 4.12 for PBR-5 and PBR-6, respectively. PBR-5 and PBR-6 were run in a consecutive process in a packed-bed reactor column. The higher average liquid flow rate of 2.07 ml/min was applied for the first 31 h (PBR-1), followed by a lower flow rate of 0.38 ml/min up to 144 h (PBR-2). The details of the operating conditions are shown in Table 4.3.

Unlike what has been observed in the process involving corn biocatalyst without GA stabilization, Figure 4.11 (PBR-5) shows that there was no sharp peak formed in the plots of glucose, galactose and lactose concentrations during the first two hours of operation. The glucose concentration in the reactor outlet showed a sharp increase within the first 2 hours, peaking at about 2 g/l (d.h. = 20%) and then declined slightly afterwards (see Table A5.9 in the Appendix 5). As shown in Figure 4.12, changing the liquid flow rate after 31 h to about one-fourth the original flow rate (from 2.07 ml/min

down to 0.38 ml/min) could boost the outlet glucose concentration up to 3.5 g/l (d.h.= 35%) at 49 h and then steadily decreased to 23% after 6 days (144 hours). In accordance to PBR-5, PBR-6 that utilized GA-stabilized corn biocatalyst also showed a relatively more stable enzyme system. Trying to improve the stability of whole cell β-galactosidase, Tomaska, et al. (1995a) stabilized the calcium alginate and calcium pectate biocatalyst beads with polyethyleneimine and glutaraldehyde. In a semi-continuous hydrolysis of 5% lactose in a batch reactor, these authors found the immobilized whole cell β-galactosidase system was relatively stable to perform 25 hydrolysis cycles with the d.h. varied between 80-88%. A continuous hydrolysis of 5% lactose in a packed-bed reactor (1.8 cm internal diameter x 10 cm length) at 35 °C with a flowrate 0.37 ml/min revealed that the hydrolysis using alginate biocatalyst decreased by 10% and 20% after 5 and 6 days, respectively. When using calcium pectate biocatalyst, the stability was improved significantly. The hydrolysis with pectate biocatalyst reduced by 10% and 20% after 9 and 11 days.

Table 4.3 Operating conditions for PBR-5 and PBR-6

Operating conditions	PBR-5	PBR-6
Feed Lactose	20 g/l (pH 7.0)	20 g/l (pH 7.0)
Temp	37 °C	37 °C
Biocatalyst	corn	corn
Permeabilization	yes	yes
GA stabilization	yes	yes
Biocatalyst diameter	0.85 – 1.00 mm	0.85 – 1.00 mm
Liquid height from the bottom	25 cm	25 cm
Column diameter	1.5 cm	1.5 cm
Bed height	14 cm	14 cm
Feed rate	2.07 ml/min	0.38 ml/min
Porosity	0.50	0.50
Biocatalyst activity (μmol/min/g biocatalyst)	0.84	0.84

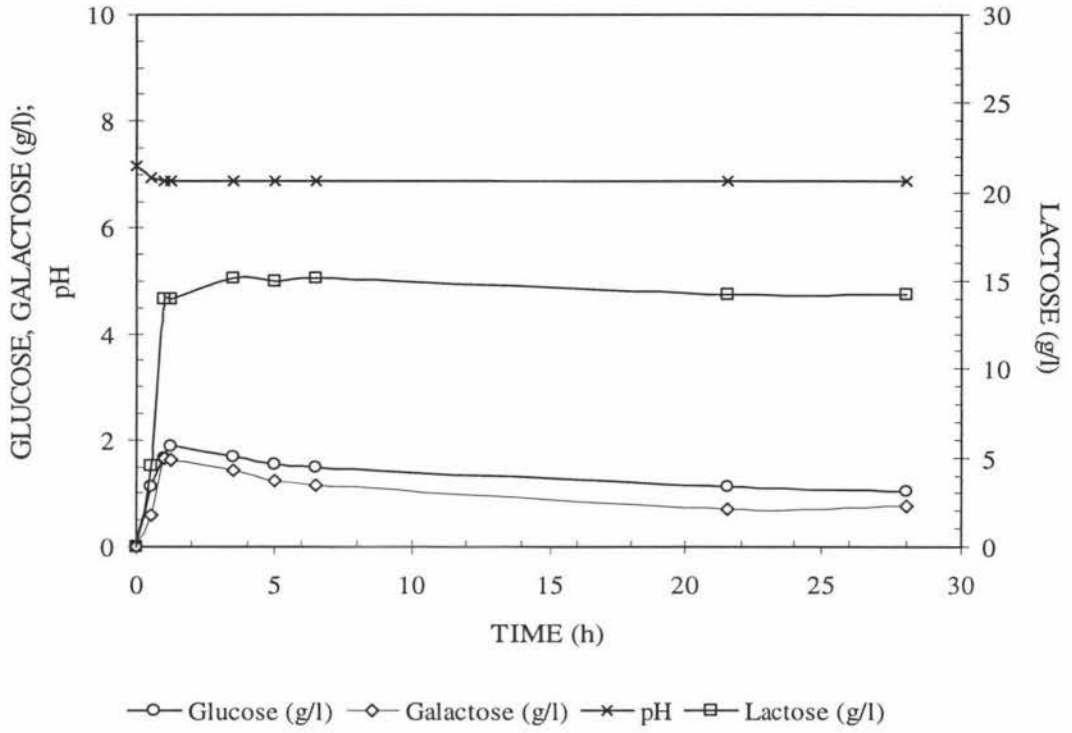


Figure 4.11 Substrate and product concentrations profile for PBR-5 using permeabilized corn biocatalyst with GA stabilization. Inlet glucose concentration = 20 g/l

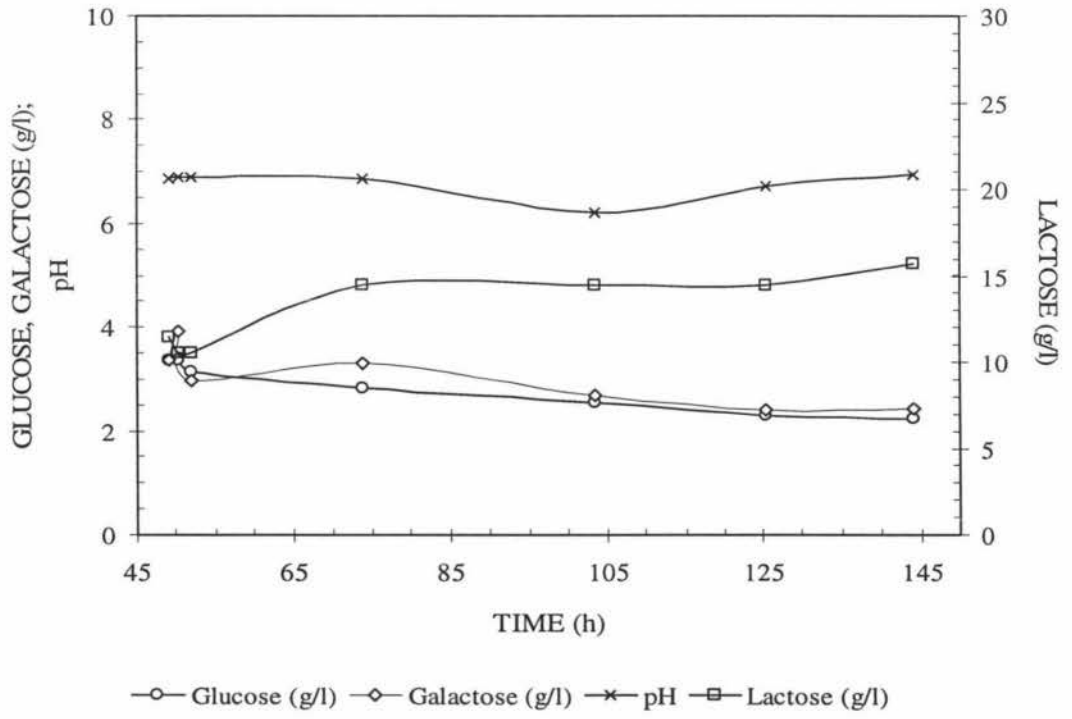


Figure 4.12 Substrate and product concentrations profile for PBR-6 using permeabilized corn biocatalyst with GA stabilization. Inlet glucose concentration = 20 g/l

Figure 4.13 shows a packed-bed process (PBR-7) using corn biocatalyst without GA stabilization. Again, as previously shown for PBR-3 and PBR-4, the concentration profile for PBR-7 showed a similar trend of having a sharp drop just after reaching a peak. In this process even though the conversion reached a maximum outlet glucose concentration of 4.78 g/l at 3.5 h (d.h.=47.8%), which was the highest value obtained, it remained at only half of that after 20 hours (see Table A5.10 in Appendix 5). In other words, apart from enzyme solubilization, an unstable enzyme system was also observed for corn biocatalyst without GA stabilization. Flores at al., (1994 & 1995) reported that a proper permeabilization applied to the yeast could retain the enzyme within cell boundaries during the treatment, but as the cell is incubated the enzyme is slowly released. Flores et al. (1995) suggested that solubilization was the major factor for the disappearance of β-galactosidase activity from a permeabilized cell of *K. lactis*

Siso and Doval (1994) has conducted similar work. Even without GA stabilization, these authors reported that they converted 5% lactose with d.h. 90% using corn biocatalyst, which was prepared through covalent attachment, in a packed-bed reactor. However they did not mention for how long that d.h. value was maintained. Besides, they did not report the flowrate they applied. Furthermore, the d.h. value was achieved after 6 h when the substrate was re-circulated. Extrapolation data presented in Table 4.5 shows that the d.h. of 45.5% could be achieved after 3 h operation for PBR-7 with a flowrate 0.96 ml/min. A higher d.h. value could be achieved by applying lower substrate flowrate.

Figure 4.14 shows the results for PBR-8 using permeabilized corn biocatalyst stabilized with GA with a slightly higher liquid flowrate (1.27 ml/min). As can be seen from Figure 4.14, lactose conversion was relatively stable with the d.h. ranging from around 30% at 5 h down to about 25% at 30 h (see Table A5.11 in Appendix 5).

Table 4.4 Operating conditions for PBR-7 and PBR-8

Operating conditions	PBR-7	PBR-8
Feed Lactose	20 g/l (pH 6.5)	20 g/l (pH 7.0)
Temp	37 °C	37 °C
Biocatalyst	corn	corn
Permeabilization	yes	yes
GA stabilization	no	yes
Biocatalyst diameter	0.85 – 1.00 mm	0.85 – 1.00 mm
Liquid height from the bottom	21 cm	23 cm
Column diameter	1.5 cm	1.5 cm
Bed height	18 cm	17.5 cm
Feed rate	0.96 ml/min	1.27 ml/min
Porosity	0.5	0.5
Biocatalyst activity (μmol/min/g biocatalyst)	0.84	0.84

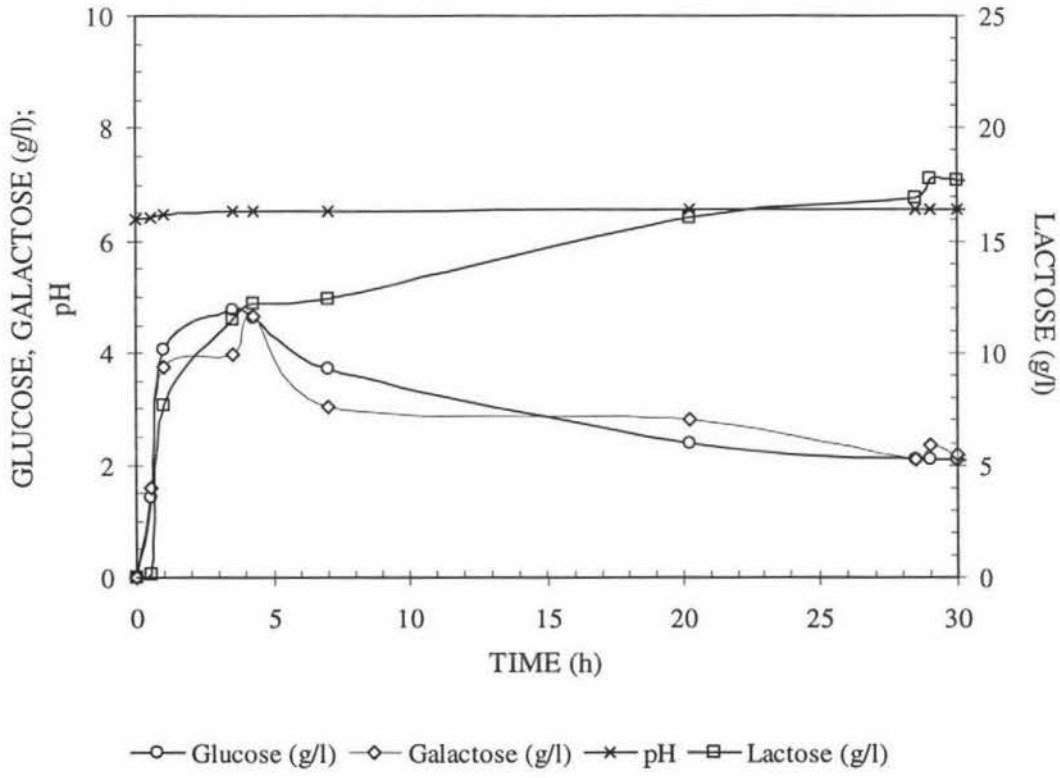


Figure 4.13 Substrate and product concentrations profile for PBR-7 using permeabilized corn biocatalyst without stabilization. Inlet glucose concentration = 20 g/l

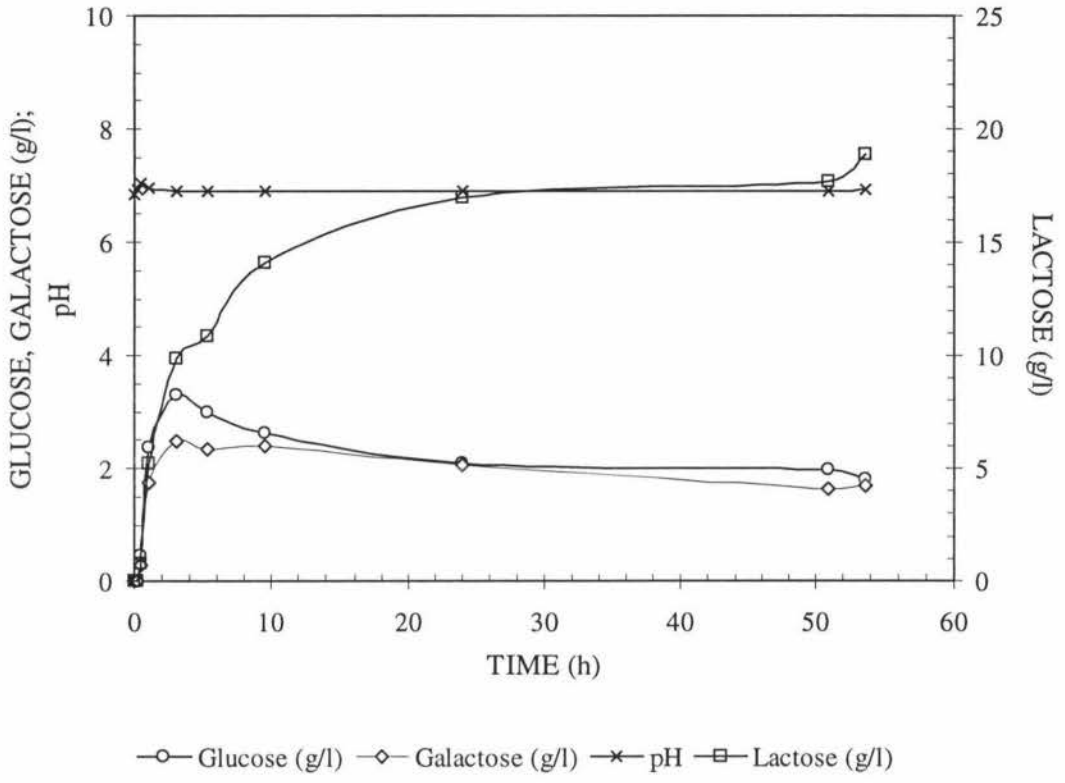


Figure 4.14 Substrate and product concentrations profile for PBR-8 using permeabilized corn biocatalyst with GA stabilization. Inlet glucose concentration = 20 g/l

Figure 4.15 shows the exponential plots of PBR-5, PBR-6, PBR-7 and PBR-8. According to Figure 4.15, it is clearly that corn biocatalyst without GA stabilization produces a dramatic exponential fall in outlet glucose concentration throughout the process (PBR-7). On the other hand, those treated with GA (PBR-5, PBR-6 and PBR-8) exhibited a slow deactivation of the enzyme activity. This implies that GA treatment has improved the biocatalyst stability; however, further optimization of stabilization is still required.

In order to calculate the outlet glucose concentration at the peak point initially achieved, the concentration at 3 hours was considered the best point. The packed-bed reactor productivity is calculated using the relationship:

$$Productivity = [Glu] \times F/\varepsilon V_b$$

where F is flow rate (l/h), ε is bed porosity and V_b is bed volume (l). Table 4.5 summarized the process data of PBR-5, PBR-6, PBR-7 and PBR-8.

Table 4.5 Summary of extrapolated data at t = 3 h for PBR-5, PBR-6, PBR-7 and PBR-8. Lactose in feed substrate = 20 g/l; particle size 0.85-1.00 mm.

No	Process	GA Stabilization (yes/no)	pH of the feed substrate	Flow rate (ml/min)	Bed height (cm)	Outlet Glucose (g/l)	d.h. (%)	Productivity (g/l/h)
1	PBR-6	Yes	7.0	0.38	14.0	3.99	39.89	5.11
2	PBR-8	Yes	7.0	1.27	17.5	2.54	25.36	8.68
3	PBR-5	Yes	7.0	2.07	14.0	1.62	16.22	11.31
4	PBR-7	No	6.5	0.96	18.0	4.55	45.51	11.45

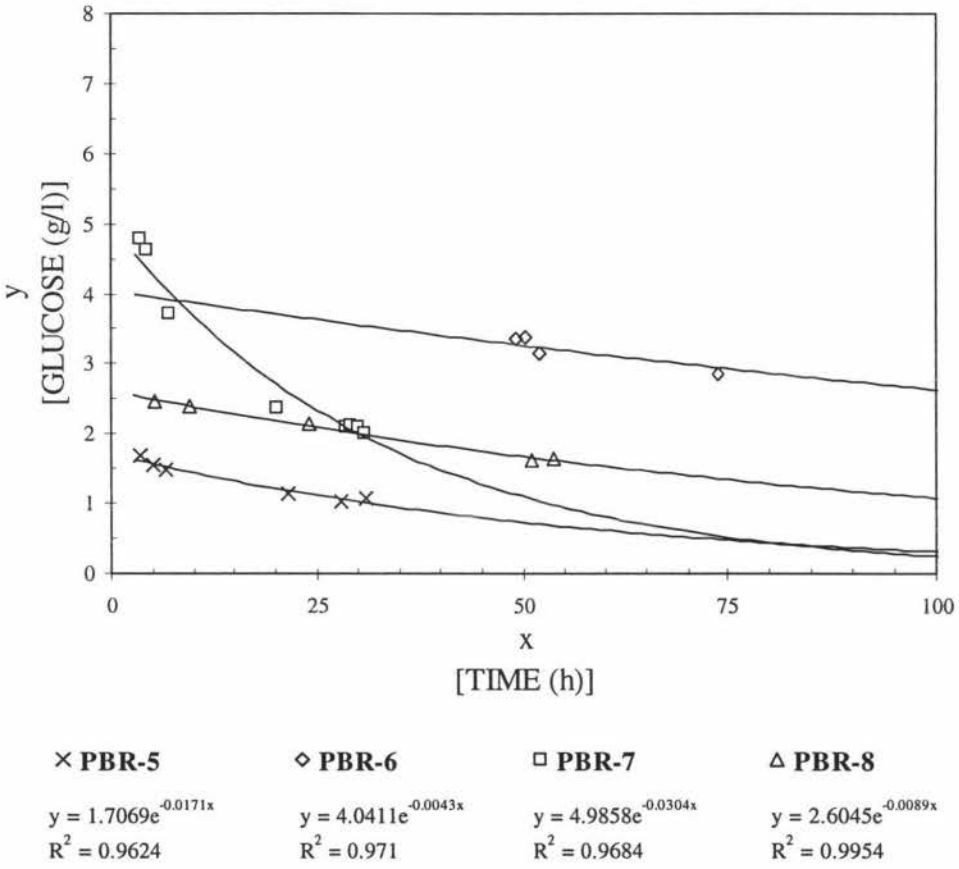


Figure 4.15 The exponential plot of outlet glucose concentration for PBR-5, PBR-6, PBR-7 and PBR-8. R^2 values gave a good agreement of data fit.

Table 4.5 shows that for packed-bed processes utilizing GA-stabilized corn biocatalyst (PBR-5, PBR-6 and PBR-8) operated at pH 7, the reactor productivity is inversely proportional to the flowrate. Generally, low flowrate causes low productivity, but a higher d.h. value.

Another packed-bed process (PBR-9) was also run in order to evaluate corn biocatalyst performance at higher inlet substrate concentrations (5% lactose). On the activity test of newly-prepared corn biocatalyst, the plot of lactose hydrolyzed against time gave a straight-line as shown in Figure 4.16, with an activity of 0.82 μmol lactose hydrolyzed/min/g biocatalyst. This value is comparable to that of corn biocatalyst used for PBR-5, PBR-6, PBR-7 and PBR-8 whose activity value is 0.84 μmol/min/g biocatalyst.

Figure 4.17 shows substrate and product concentration profiles for PBR-9. The total glucose determination in the samples after incubation for 1 h was also conducted for PBR-9. Figure 4.17 demonstrates that the samples taken from 0 h up to 4 h showed the presence of free enzyme measured as glucose liberated upon incubation (see Table A5.13 in Appendix 5). Beyond 4 h, free enzyme was no longer observed; but the enzyme was markedly deactivated during the process. The extrapolation of outlet glucose concentration at 3 hours, as shown in Figure 4.18, gave the peak value of 6.77 g/l (d.h. = 27%). The reactor productivity was 17.04 g of glucose/l/h.

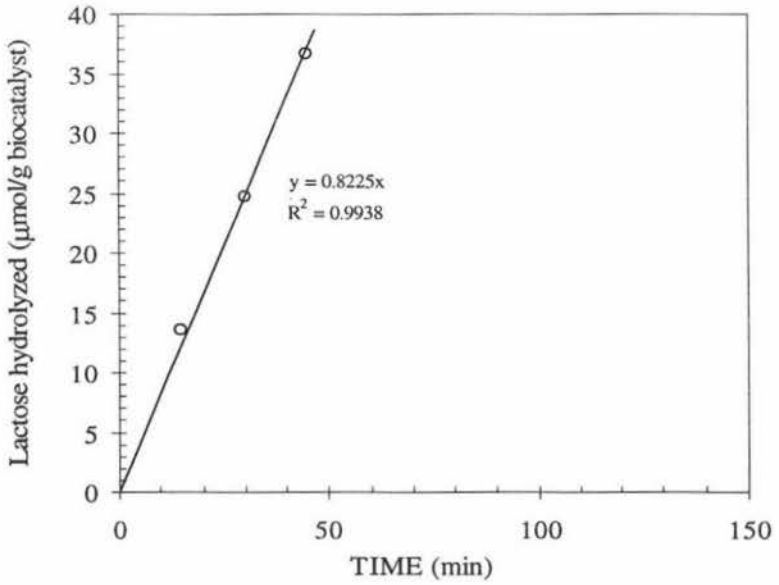


Figure 4.16 Activity test of corn biocatalyst used for PBR-9. The activity (slope) = 0.82 μmol/min/g biocatalyst.

Table 4.6 Operating conditions for PBR-9

Operating conditions	PBR-9
Feed Lactose	50 g/l (pH 6.5)
Temp	37 °C
Biocatalyst	corn
Permeabilization	yes
GA stabilization	no
Biocatalyst diameter	0.85 – 1.00 mm
Liquid height from the bottom	25 cm
Column diameter	1.5 cm
Bed height	16.5 cm
Feed rate	0.88 ml/min
Porosity	0.5
Biocatalyst activity (µmol/min/g biocatalyst)	0.84

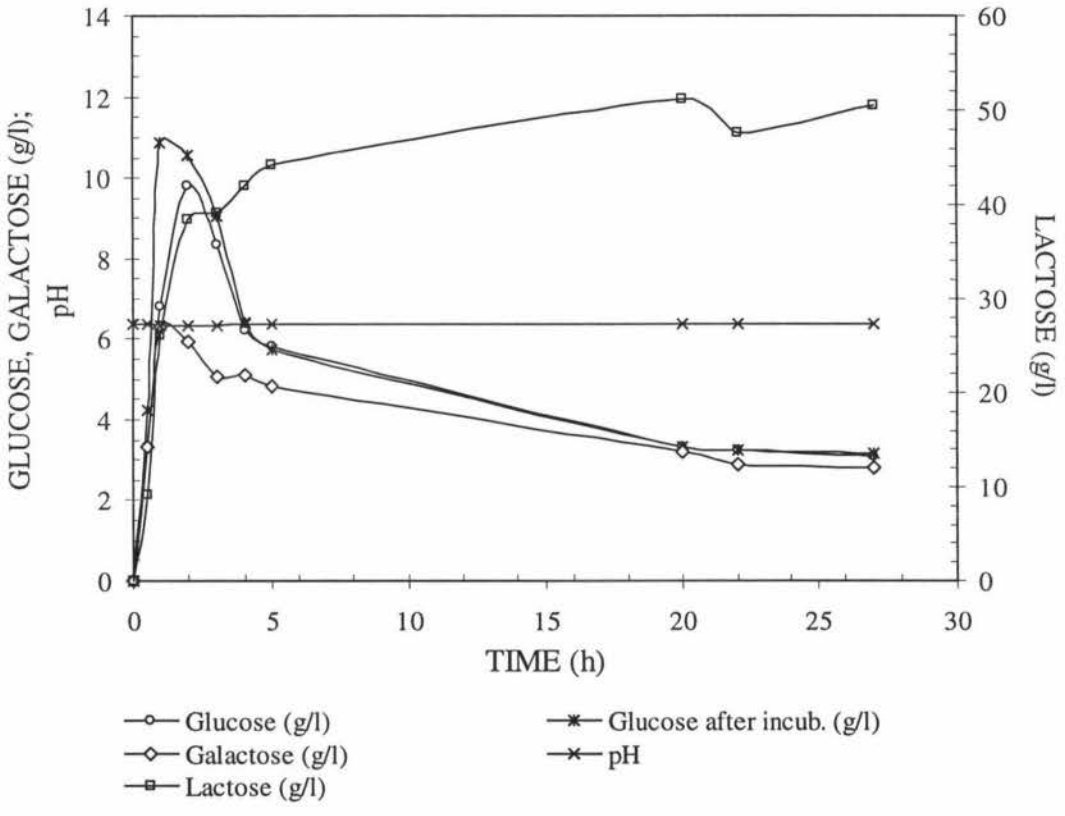


Figure 4.17 Substrate and product concentrations profile for PBR-9 using corn biocatalyst without GA stabilization. Inlet glucose concentration = 50 g/l

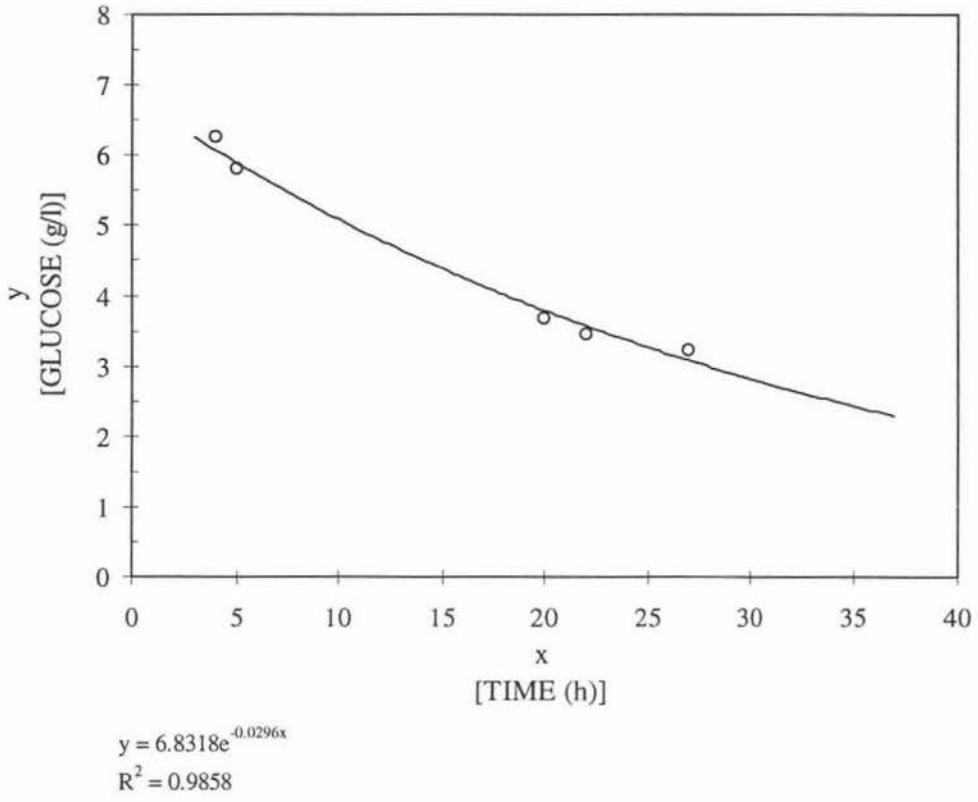


Figure 4.18 The exponential plot of outlet glucose concentration for PBR-9. R^2 values indicate a good agreement of data fit.

4.4 CONCLUSIONS

In the first part of this study, the use of bone charcoal as the support for immobilization of *K. lactis* was investigated. It was demonstrated that bone charcoal biocatalyst prepared through "one stage liquid cultivation stage" without immobilization rendered low activity for lactose hydrolysis. The activity of the biocatalyst produced was only 0.07 μmol lactose hydrolyzed/min/g biocatalyst. When tested in the packed-bed reactor, the maximum degree of hydrolysis (d.h.) attained was below 7% throughout the process under the operating condition tested.

In the second part, a preliminary test showed that corn biocatalyst prepared through single liquid cultivation stage without permeabilization exhibited the activity of 0.19

µmol lactose hydrolyzed/min/g biocatalyst. This activity value is about 2.7 times higher than that of bone charcoal biocatalyst.

In the third part, it was shown that the adsorption of *K. lactis* on corn particles through "double liquid cultivation stage" followed by permeabilization promoted higher biocatalyst activity. The activity obtained was 0.84 µmol lactose hydrolyzed /min/g biocatalyst. This activity was about 5 times higher than the case without permeabilization; about 2 times higher permeabilized biocatalyst prepared with one liquid cultivation stage; and about 12 times greater than that of bone charcoal biocatalyst. When tested in the packed-bed reactor, during the initial stage d.h. could reach as high as 45% within the operational conditions tested. Free enzyme was detected during the first 5 h of operation mainly in the process utilizing non-stabilized corn biocatalyst. After 5 h of operation free enzyme was no longer detected in the samples suggesting that direct adsorption might have rendered a good cell confinement inside corn particles. Furthermore, it is possible to obtain higher conversion when applying a lower substrate flow rate.

The analysis of substrate and product concentration trends of all PBR processes revealed that the enzyme was exponentially deactivated during the process. The stabilization of corn biocatalyst with GA significantly stabilized the enzyme from deactivation. However, further optimization of the process parameters, immobilization and stabilization procedures are still required to improve the biocatalyst performance to use for extended period of time.

Chapter 5
GENERAL DISCUSSION AND CONCLUSIONS

In yeasts, the enzyme β-galactosidase resides within the cellular compartment (Dickson et al., 1979). Certain yeast strains are safe for humans (Gekas & Lopez-Leiva, 1985), and therefore the use of yeast cells for lactose hydrolysis in food processing deserves evaluation. The use of whole cell generally aims at optimizing process technology to be more economically feasible. But many reports show that while the cells can be produced abundantly and cheaply, methods of employing the cells to function properly as an efficient "mini bioreactor" system which can retain their activity for long periods of process operation have been proven to be somewhat difficult to attain. There are, however, recent reports which describe stable preparations of immobilized whole cells for β-galactosidase activity (Tomaska, 1995a).

In this study, several yeast strains were considered for immobilization for lactose hydrolysis. The selection procedure showed the β-galactosidase activity of *K. lactis* CBS 2357 was about 12 times greater than that of *K. marxianus* CBS 712. Therefore *K. lactis* CBS 2357 was investigated for stability and performance in a packed-bed reactor.

In the second part of the work, the cells of *K. lactis* were permeabilized and used in kinetic parameter determination. It has been proven that permeabilization can break the barrier in the yeast cell wall to allow substrate to penetrate freely into the cells. In packed-bed reactor systems, external mass transfer resistance can play a significant role in determining the net reaction rate. But the absence of external diffusion limitation for lactose to reach the active site within the cell boundaries can be observed from plot methods (Horvath & Engasser, 1974). As shown in Table 3.3 (Chapter 3), all methods tested (Langmuir, Lineweaver-Burk and Eadie-Hofstee) based on glucose analysis gave high confidence (more than 95%). According to Horvath & Engasser (1974), the presence of diffusion limitation causes a curvature in the plot for the lowest substrate concentration. Therefore, this finding suggests that the reaction rate of lactose hydrolysis by permeabilized free cells of *K. lactis* is solely governed by the enzymatic

reaction. Compared to the value obtained by Flores, et al. (1995) as shown in Table 1.4 (Chapter 1), the value of K_M obtained in this study (59.34 μmol/ml) was approximately two times higher at 37 °C. Also, V_{max} of this study of 54.64 μmol/min/10^9 cells was about 1.7 times higher that obtained by Flores, et al. (1995) of 33.1 μmol/ml. From the Michaelis-Menten kinetic equation, it can be seen that the higher the V_{max} value, the higher the reaction rate. In contrast, the effect of K_M value to the reaction rate is inversely proportional. These imply that the biocatalyst prepared by Flores et al. (1995) was more active than the permeabilized cells prepared in this experiment. The most acceptable reasons to explain those differences are:

- The yeasts used were of different strains. Flores et al. (1995) employed *K. lactis* NRRL 1118, but this project utilized *K. lactis* CBS 2357.
- In preparing the cell culture, Flores et al. (1995) used a synthetic medium containing C-source, N-source and complete mineral elements supplemented with 8 different growth factors (vitamin and essential compounds such as inositol) that altogether can stimulate higher enzyme production. This might have led to more β-galactosidase synthesis in the cell.

In the third part, the study was focused on testing the stability of permeabilized cells *K. lactis* after GA treatment. The results showed that the stability of biocatalyst was significantly improved. However, the stability of GA-treated cells, in fact, was strongly influenced also by the presence of Mn-Buffer. In the absence of the buffer solution, the activity was markedly lower, but the external enzyme activity was also low, an indication of the absence of enzyme leakage. These facts suggested the buffer used played a significant role in stabilizing the whole cell biocatalyst. In the absence of Mn-Buffer, the enzyme of GA-treated cells was deactivated internally. When the whole cells were incubated in the 0.89% saline containing manganese ion, it still showed dramatic fall in activity. Mahoney and Whitaker (1978) reported that manganese alone is not effective in stabilizing the enzyme. They also showed that the presence of potassium ion is essential for the enzyme stability. Therefore, if the whole cells are to be used for longer process times, further improvement of the stability of the enzyme is still required.

In the fourth part, immobilization in alginate beads of permeabilized *K. lactis* cells was studied. The direct treatment of the alginate biocatalyst with GA did not give results as

good as GA-treated free cells. Free cell treatment with GA rendered good cell confinement of the enzyme. Since alginate molecule can react with GA (Birnbaum, et al., 1981), it is suggested to firstly stabilize permeabilized free cells with GA followed by gel entrapment to attain high biocatalyst activity and stability. However, the alginate bead might be dissolved by the action of phosphate available in the buffer solution used for lactose hydrolysis. Alternatively, the alginate beads can be hardened with polyethyleneimine (PEI) and GA (Tomaska, et al. 1995a). Tomaska, et al. (1995a) found that the biocatalyst retained the activity of lactose hydrolysis in the Mn-buffer media at pH 6.5 for 5 days. In addition, leakage of enzyme was not detected.

Finally, the immobilization through direct cultivation of *K. lactis* in corn grits was conducted in preparing immobilized whole cell biocatalyst. It was shown that the corn biocatalyst exhibited a moderate activity of lactose hydrolysis. Under the conditions tested, the packed-bed process yielded the d.h. as high as 45%. Higher d.h. values are possible by applying a lower substrate flowrate. Further investigation of the nature of the cells immobilized on the corn particles is required in order to improve the biocatalyst performance. Direct immobilization through cultivation offers the following advantages:

- The cells are grown concurrently with immobilization thereby reducing time and cost;
- The support is strong enough that enables its use in the bioreactor for long time process;
- The support material is easily obtained and cheap;

The whole cells of *K. lactis* offer an alternative way for preparing immobilized biocatalyst system for lactose hydrolysis. Cells permeabilization is an important step to increase the substrate accessibility into the cells, which in turn, increases the overall activity. Immobilization of whole cells on corn particles provides an alternative way of producing whole cell biocatalyst; but further work on optimization of the reactor system is required to achieve a commercially viable operation.

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APPENDIX 1

The Measurement of Cell Dry Weight (DW)

Procedure:

- After permeabilization, the cells were suspended in Mn-Buffer pH 6.5 to get the cell number between $2x10^9$ to $8x10^9$ cells/ml
- Count the cell number
- 0.5 ml permeabilized cell suspension was dried overnight at 105 °C in a pre-weighed aluminum foil dish
- Weigh up the dish with dried cells.

DW Measurement:

I. From stock suspension 1 : cell number = 7.0×10^9 cells/ml

# Dish	Empty weight (g)	Weight after drying (g)	DW (g)
1	0.2604	0.3088	0.0484
2	0.4587	0.5059	0.0472
3	0.2221	0.2708	0.0481
Average			0.0481

Hence, DW = 48.1 mg/0.5 ml = 96.2 mg/ml
= 96.2 mg/$7x10^9$ cells
= 13.74 mg/10^9 cells

II. From stock suspension 2 : cell number = 5.5×10^9 cells/ml

# Dish	Empty weight (g)	Weight after drying (g)	DW (g)
1	0.1499	0.1893	0.0394
2	0.1466	0.1864	0.0390
3	0.2207	0.2609	0.0402
Average			0.0395

Hence, DW = 39.5 mg/0.5 ml = 79 mg/ml
= 79 mg/$5.5x10^9$ cells
= 14.36 mg/10^9 cells

Take average from Measurement I and II:

DW = 14 mg/10^9 cells

APPENDIX 2

The Data for Kinetic Parameters Measurement

Example of calculation with the reaction conditions:

Initial lactose.H_2O concentration (M.wt 360) = 5 g/l = 13.89 µmol/ml
Cell number in the reaction mixture = $6x10^7$ cells/ml
Time of hydrolysis = 15 min
The amount of lactose hydrolyzed after 15 min = 9.17 µmol/ml

The rate of hydrolysis = 9.17 (µmol/ml) / 15 min / $6x10^7$ (cells/ml)
= 10.19 µmol/min/10^9 cells

Table A2.1 The hydrolysis of lactose in the reaction mixtures contains $6x10^7$ cells/ml for 15 min (The data is based on the enzymatic assay of glucose liberated).

Initial Lactose.H_2O		Glucose Produced				Lactose hydrolyzed (µmol/ml)	Rate of lactose hydrolysis (µmol/min/ 10^9 cells)
		Assay-1 (g/L)	Assay-1 (g/L)	Mean value			
g/L	µmol/ml			(g/L)	(µmol/ml)		
5	13.89	1.62	1.68	1.65	9.17	9.17	10.19
10	27.78	2.77	2.85	2.81	15.61	15.61	17.35
15	41.67	3.62	3.72	3.67	20.39	20.39	22.65
20	55.56	4.25	4.37	4.31	23.94	23.94	26.60
25	69.44	4.68	4.82	4.75	26.39	26.39	29.32
30	83.33	5.12	5.29	5.21	28.92	28.92	32.13
40	111.11	5.67	5.94	5.81	32.25	32.25	35.83
50	138.89	6.02	6.26	6.14	34.11	34.11	37.90

Table A2.2 Re-arrangement of Michaelis-Menten variables from Table A2.1 as data plot for kinetic parameter determinations

S		*v*	*S/v*	*1/S*	*1/v*	*v/S*
g/L	µmol/ml					
5	13.89	10.19	1.364	0.072	0.098	0.733
10	27.78	17.35	1.601	0.036	0.058	0.624
15	41.67	22.65	1.839	0.024	0.044	0.544
20	55.56	26.60	2.088	0.018	0.038	0.479
25	69.44	29.32	2.368	0.014	0.034	0.422
30	83.33	32.13	2.594	0.012	0.031	0.386
40	111.11	35.83	3.101	0.009	0.028	0.323
50	138.89	37.90	3.664	0.007	0.026	0.273

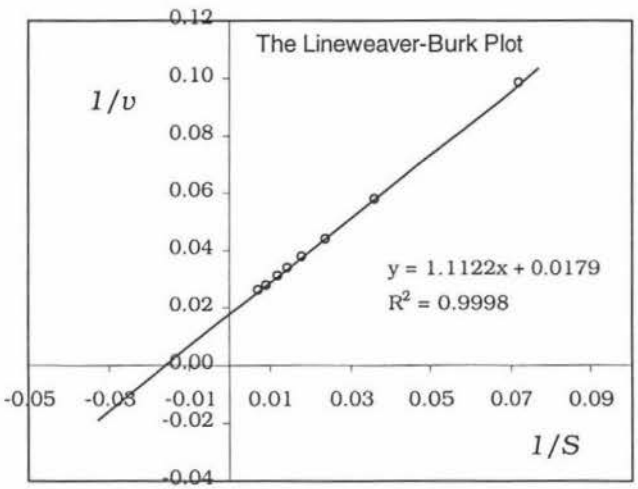

Figure A2.1 The Lineweaver-Burk plot of lactose hydrolysis based on enzymatic assay of glucose.

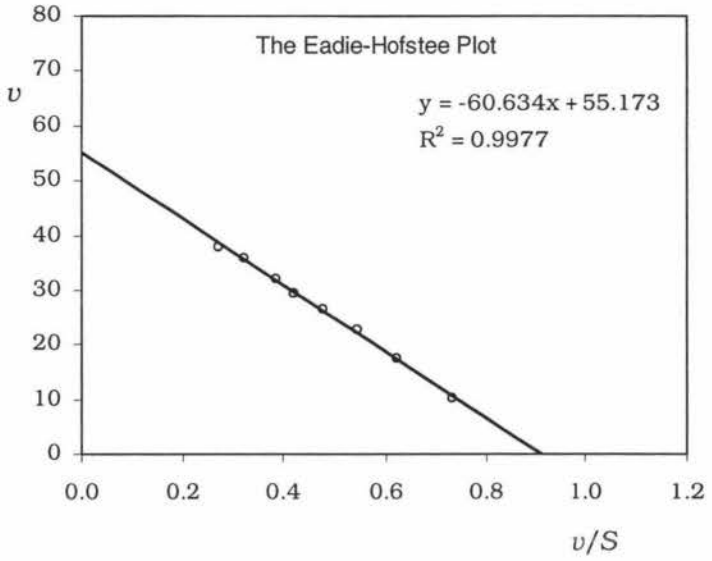

Figure A2.2 The Eadie-Hofstee plot of lactose hydrolysis based on enzymatic assay of glucose.

Table A2.3 The hydrolysis of lactose in the reaction mixtures contains $6x10^7$ cells/ml for 15 min (The data is based on HPLC assay of residual lactose).

Initial Lactose.H_2O		Residual Lactose		Lactose Hydrolyzed		Mean of Conversion		Rate of lactose hydrolysis (μmol/min/ 10^9 cells)
g/L	μmol/ml	Assay-1 (g/l)	Assay-2 (g/l)	Assay-1 (g/l)	Assay-2 (g/l)	g/l	μmol/ml	
5	13.89	1.61	1.88	3.39	3.12	3.25	9.03	10.03
10	27.78	5.90	5.76	4.11	4.24	4.17	11.59	12.88
15	41.67	13.19 *	8.43	--	6.57	6.57	18.25	20.28
20	55.56	14.07	11.58	5.93	8.42	7.18	19.94	22.15
25	69.44	17.00	18.18	8.00	6.82	7.41	20.58	22.86
30	83.33	20.64	26.41	9.36	3.59	9.36	26.00	28.89
40	111.11	27.89	32.70	12.11	7.30	9.71	26.97	29.97
50	138.89	39.78	53.48 *	10.22	--	10.22	28.39	31.54

* These data are not included in the calculation because they cause big deviation.

Table A2.4 Re-arrangement of Michaelis-Menten variables from Table A2.3 as data plot for kinetic parameter determinations

S		*v*	*S/v*	*1/S*	*1/v*	*v/S*
g/L	µmol/ml					
5	13.89	10.03	1.385	0.072	0.100	0.722
10	27.78	12.88	2.157	0.036	0.078	0.464
15	41.67	20.28	2.055	0.024	0.049	0.487
20	55.56	22.15	2.508	0.018	0.045	0.399
25	69.44	22.86	3.038	0.014	0.044	0.329
30	83.33	28.89	2.885	0.012	0.035	0.347
40	111.11	29.97	3.708	0.009	0.033	0.270
50	138.89	31.54	4.403	0.007	0.032	0.227

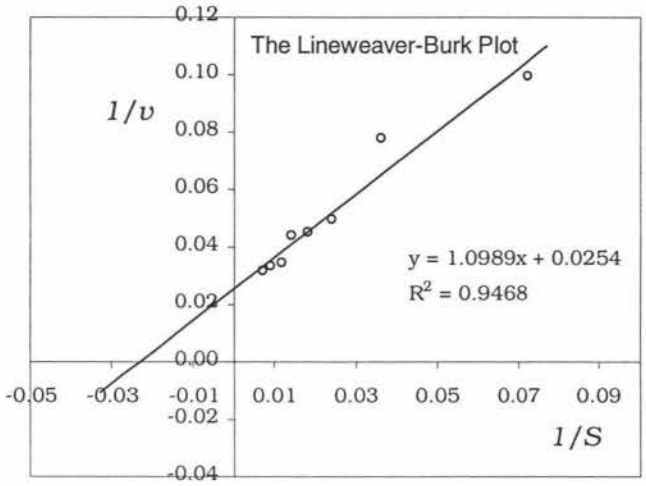

Figure A2.3 The Lineweaver-Burk plot of lactose hydrolysis based on HPLC assay of lactose.

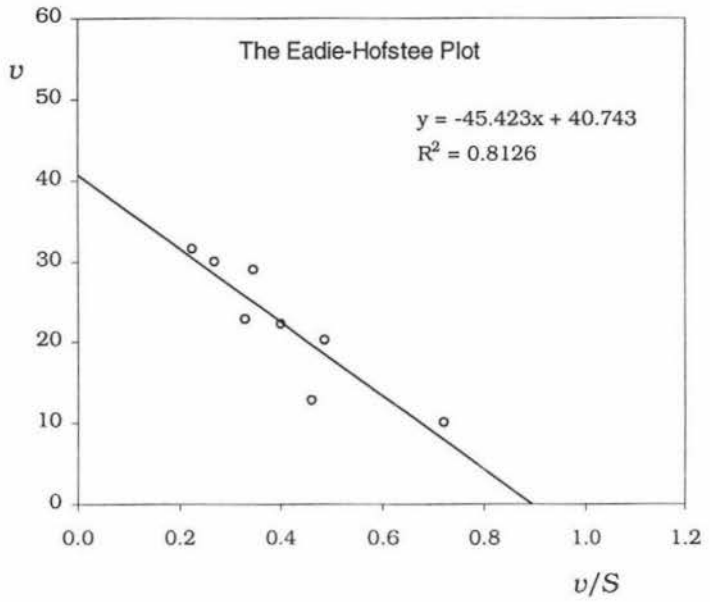

Figure A2.4 The Eadie-Hofstee plot of lactose hydrolysis based on HPLC assay of lactose.

APPENDIX 3

The Data for Stability Test of Free Cells β-Galactoseidase

Table A3.1 The absorbance data of *o*-nitrophenol for the assays of total and biomass activity cell suspension in different media stored at 30 °C for 24, 48 and 72 h

Treatment	Cell number in The Stored Suspension (cells/ml)	Total Cell number in The reaction mixture (cells)	Absorbance after 5 min hydrolysis (λ = 416 nm)					
			After 24 h		After 48 h		After 72 h	
			Assay-1	Assay-2	Assay-1	Assay-2	Assay-1	Assay-2
A. Total Activity								
CB-30	5.5×10^8	2.2×10^6	0.461	0.493	0.315	0.315	0.158*	0.151*
CSM-30	5.5×10^8	2.2×10^6	0.034	0.032	0.009*	0.005*	0.010*	0.019*
GB-30	5.0×10^8	2.0×10^6	0.572	0.578	0.585	0.595	0.545	0.460
GS-30	5.0×10^8	2.0×10^6	0.229	0.271	0.039	0.010	0.032*	0.026*
GSM-30	5.0×10^8	2.0×10^6	0.335	0.327	0.028	0.029	0.029*	0.035*
B. Biomass Activity								
CB-30	5.5×10^8	2.2×10^6	0.141	0.144	0.216	0.210	0.114*	0.114*
CSM-30	5.5×10^8	2.2×10^6	0.041	0.042	0.000	0.000	0.000	0.000
GB-30	5.0×10^8	2.0×10^6	0.527	0.491	0.550	0.585	0.428	0.416
GS-30	5.0×10^8	2.0×10^6	0.215	0.214	0.065*	0.118*	0.019*	0.023
GSM-30	5.0×10^8	2.0×10^6	0.273	0.247	0.092*	0.081*	0.049*	0.027*

* Activity test was carried out fot 15 min

CB-30 : Untreated Cells in Mn-buffer, stored at 30 °C

CSM-30 : Untreated Cells in 0.89% saline containing $MnCl_2$, stored at 30 °C

GB-30 : GA-treated Cells in Mn-Buffer, stored at 30 °C

GS-30 : GA-treated Cells in 0.89% saline, stored at 30 °C

GSM-30 : GA-treated Cells in 0.89% saline containing $MnCl_2$, stored at 30 °C

Table A3.2 The total activity of cell suspensions calculated based on the data from Table A3.1

Treatment	Total Activity (μmol ONPG hydrolyzed/min/10^9 cells) at								
	24 h			48 h			72 h		
	Assay-1	Assay-2	Mean	Assay-1	Assay-2	Mean	Assay-1	Assay-2	Mean
CB-30	27.932	29.871	28.902	19.086	19.086	19.086	13.088	12.724	12.906
CSM-30	2.060	1.939	2.000	0.182	0.101	0.141	0.202	0.384	0.293
GS-30	15.263	18.062	16.663	2.599	0.667	1.633	0.711	0.578	0.644
GB-30	38.124	38.524	38.324	38.990	39.723	39.357	36.658	38.990	37.824
GSM-30	22.328	21.795	22.061	1.866	1.933	1.900	0.644	0.778	0.711

Table A3.3 The total activity summarized from Table A3.2 as the data for Figure 3.3

Treatment	0 h	24 h	48 h	72 h
CB-30	40.370	28.902	19.086	12.906
CSM-30	40.370	2.000	0.141	0.293
GS-30	39.338	16.663	1.633	0.644
GB-30	39.338	38.324	39.357	37.824
GSM-30	39.338	22.061	1.900	0.711

Table A3.4 The percentage of total activity remained as the data for Figure 3.4

Treatment	0 h	24 h	48 h	72 h
CB-30	100.00	71.59	47.28	31.97
CSM-30	100.00	4.95	0.35	0.73
GS-30	100.00	42.36	4.15	1.64
GB-30	100.00	97.42	100.05	96.15
GSM-30	100.00	56.08	4.83	1.81

Table A3.5 The biomass activity of cell suspensions calculated based on the data from Table A3.1

Treatment	Biomass Activity (μmol ONPG hydrolyzed/min/10^9 cells)								
	24 h			48 h			72 h		
	Assay-1	Assay-1	Mean	Assay-1	Assay-2	Mean	Assay-1	Assay-2	Mean
CB-30	8.543	8.725	8.634	9.573	9.149	9.361	2.302	2.302	2.302
CSM-30	2.484	2.545	2.515	0.000	0.000	0.000	0.000	0.000	0.000
GS-30	14.330	14.263	14.296	1.444	2.622	2.033	0.422	0.511	0.467
GB-30	35.125	32.725	33.925	36.324	30.659	33.492	28.526	27.726	28.126
GSM-30	18.195	16.463	17.329	2.044	1.800	1.922	1.089	0.600	0.844

Table A3.6 The biomass activity summarized from Table A3.2 as the data for Figure 3.5

Treatment	0 h	24 h	48 h	72 h
CB-30	40.370	8.634	9.361	2.302
CSM-30	40.370	2.515	0.000	0.000
GS-30	39.338	14.296	2.033	0.467
GB-30	39.338	33.925	33.492	28.126
GSM-30	39.338	17.329	1.922	0.844

Table A3.7 The percentage of biomass activity remained as the data for Figure 3.6

Treatment	0 h	24 h	48 h	72 h
CB-30	100.00	21.39	23.19	5.70
CSM-30	100.00	6.23	0.00	0.00
GS-30	100.00	36.34	5.17	1.19
GB-30	100.00	86.24	85.14	71.50
GSM-30	100.00	44.05	4.89	2.15

Table A3.8 The absorbance data of *o*-nitrophenol for the assays of total and biomass activity cell suspension in different media stored at 37 °C for 24, 48 and 72 h

Treatment	Cell number in The Stored Suspension (cells/ml)	Total cell number in The reaction mixture (cells)	Absorbance after 5 min hydrolysis (λ = 416 nm)					
			After 24 h		After 48 h		After 72 h	
			Assay-1	Assay-2	Assay-1	Assay-2	Assay-1	Assay-2
A. Total Activity								
CS-37	5.5×10^8	2.2×10^6	0.010*	0.014*	0.034	0.024	0.024*	0.026*
CB-37	5.5×10^8	2.2×10^6	0.273	0.281	0.100	0.094	0.045*	0.050*
CSM-37	5.5×10^8	2.2×10^6	0.020	0.020	0.025*	0.012*	0.008*	0.009*
GB-37	5.0×10^8	2.0×10^6	0.469	0.566	0.613**	0.543**	0.448**	0.425**
GS-37	5.0×10^8	2.0×10^6	0.168	0.160	0.041	0.045	0.044*	0.054
GSM-37	5.0×10^8	2.0×10^6	0.185	0.258	0.048	0.045	0.013*	0.028*
B. Biomass Activity								
CS-37	5.5×10^8	2.2×10^6	0.000	0.001*	0.026	0.081	0.026*	0.024*
CB-37	5.5×10^8	2.2×10^6	0.112*	0.136*	0.031	0.039	0.029*	0.029
CSM-37	5.5×10^8	2.2×10^6	0.019	0.051	0.000	0.000	0.000	0.000
GB-37	5.0×10^8	2.0×10^6	0.511	0.449	0.398	0.397	0.364	0.365
GS-37	5.0×10^8	2.0×10^6	0.116	0.152	0.128*	0.151*	0.029*	0.026*
GSM-37	5.0×10^8	2.0×10^6	0.121	0.118	0.136*	0.126*	0.074*	0.076*

* Activity test was carried out for 15 min

* * Total cell number in the reaction mixture = 2.5×10^6 cells

CB-30 : Untreated Cells in Mn-buffer, stored at 30 °C

CSM-30 : Untreated Cells in 0.89% saline containing $MnCl_2$, stored at 30 °C

GB-30 : GA-treated Cells in Mn-Buffer, stored at 30 °C

GS-30 : GA-treated Cells in 0.89% saline, stored at 30 °C

GSM-30 : GA-treated Cells in 0.89% saline containing $MnCl_2$, stored at 30 °C

Table A3.9 The total activity of cell suspensions calculated based on the data from Table A3.8

Treatment	Total Activity (μmol ONPG hydrolyzed/min/10^9 cells) at								
	24 h			48 h			72 h		
	Assay-1	Assay-2	Mean	Assay-1	Assay-2	Mean	Assay-1	Assay-2	Mean
CS-37	0.202	0.283	0.242	0.687	0.525	0.606	0.525	1.636	1.081
CB-37	16.541	17.026	16.784	6.059	5.696	5.877	1.878	2.363	2.121
CSM-37	1.212	1.212	1.212	0.505	0.242	0.374	0.162	0.182	0.172
GS-37	11.197	10.664	10.931	2.733	2.999	2.866	0.978	1.200	1.089
GB-37	31.259	32.858	32.059	32.685	28.953	30.819	26.527	26.460	26.493
GSM-37	12.330	17.196	14.763	3.199	2.999	3.099	0.289	0.622	0.455

Table A3.10 The total activity summarized from Table A3.9 as the data for Figure 3.7

Treatment	0 h	24 h	48 h	72 h
CS-37	40.370	0.242	0.606	1.081
CB-37	40.370	16.784	5.877	2.121
CSM-37	40.370	1.212	0.374	0.172
GS-37	39.338	10.931	2.866	1.089
GB-37	39.338	32.059	30.819	26.493
GSM-37	39.338	14.763	3.099	0.455

Table A3.11 The percentage of total activity remained as the data for Figure 3.8

Treatment	0 h	24 h	48 h	72 h
CS-37	100.00	9.91	1.50	2.68
CB-37	100.00	41.57	14.56	5.25
CSM-37	100.00	8.67	0.93	0.43
GS-37	100.00	27.79	7.29	2.77
GB-37	100.00	81.50	78.34	67.35
GSM-37	100.00	37.53	7.88	1.16

Table A3.12 The biomass activity of cell suspensions calculated based on the data from Table A3.8

Treatment	Total Activity (μmol ONPG hydrolyzed/min/10^9 cells) at								
	24 h			48 h			72 h		
	Assay-1	Assay-2	Mean	Assay-1	Assay-2	Mean	Assay-1	Assay-2	Mean
CS-37	0.000	0.222	0.111	0.485	0.525	0.505	0.525	0.485	0.505
CB-37	2.262	2.747	2.504	0.909	1.010	0.959	0.586	0.586	0.586
CSM-37	1.151	3.090	2.121	0.000	0.000	0.000	0.000	0.000	0.000
GS-37	7.731	10.131	8.931	2.844	3.355	3.099	0.644	0.578	0.611
GB-37	34.058	29.926	31.992	29.859	28.326	29.093	24.261	24.327	24.294
GSM-37	8.065	7.865	7.965	3.021	2.799	2.910	1.644	1.688	1.666

Table A3.13 The biomass activity summarized from Table A3.12 as the data for Figure 3.9

Treatment	0	24	48	72
CS-37	40.370	0.111	0.505	0.505
CB-37	40.370	2.504	0.959	0.586
CSM-37	40.370	2.121	0.000	0.000
GS-37	39.338	8.931	3.099	0.611
GB-37	39.338	31.992	29.093	24.294
GSM-37	39.338	7.965	2.910	1.666

Table A3.14 The percentage of biomass activity remained as the data for Figure 3.10

Treatment	0 h	24 h	48 h	72 h
CS-37	100.00	0.28	1.25	1.25
CB-37	100.00	6.20	2.38	1.45
CSM-37	100.00	5.25	0.00	0.00
GS-37	100.00	22.70	7.88	1.55
GB-37	100.00	81.33	73.96	61.76
GSM-37	100.00	20.25	7.40	4.24

APPENDIX 4

The Data for Immobilized Whole Cell in Alginate Bead

Table A4.1 Absorbance data of *o*-nitrophenol for activity tests of bead biocatalyst with 2% sodium alginate treated with different GA concentration

GA (mM)	Abs. (416 nm)				Abs. (416 nm)			
	Assay-1				Assay-2			
	0 min	2 min	4 min	6 min	0 min	2 min	4 min	6 min
0 (control)	0.0	0.290	0.651	0.863	0.0	0.289	0.511	0.705
0.5	0.0	0.136	0.317	0.599	0.0	0.096	0.263	0.410
1	0.0	0.122	0.332	0.506	0.0	0.108	0.279	0.440
2	0.0	0.078	0.225	0.359	0.0	0.074	0.207	0.406
4	0.0	0.072	0.040	0.079	0.0	0.026	0.091	0.092

Table A4.2 The bead activity calculated based on data from Table A4.1 (the activity is obtained from the slope of the graph)

GA (mM)	ONPG Hydrolyzed (µmol)				Activity of Assay-1 (µmol ONPG/ min/g bead)	ONPG Hydrolyzed (µmol)				Activity of Assay-2 (µmol ONPG/ min/g bead)	Mean of Activity (µmol ONPG/ min/g bead)
	Assay-1					Assay-2					
	0 min	2 min	4 min	6 min		0 min	2 min	4 min	6 min		
0 (control)	0.0	2.321	5.211	6.907	1.1952	0.0	2.312	4.088	5.64	0.9789	1.0871
0.5	0.0	1.089	2.537	4.794	0.7338	0.0	0.768	2.104	3.28	0.5292	0.6315
1	0.0	0.976	2.657	4.05	0.6586	0.0	0.864	2.232	3.52	0.5675	0.6131
2	0.0	0.624	1.801	2.873	0.4588	0.0	0.592	1.656	3.248	0.4875	0.4732
4	0.0	0.576	0.320	0.632	0.1112	0.0	0.208	0.728	0.736	0.1383	0.1248

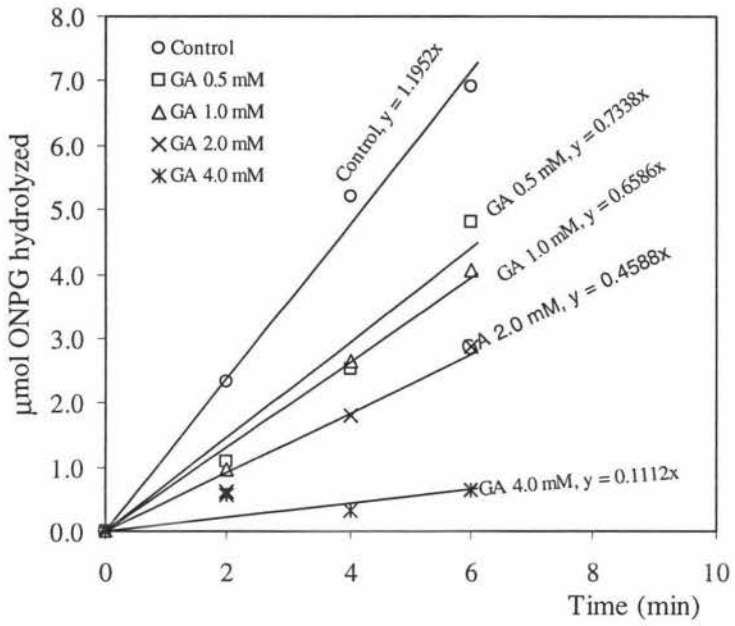

Figure A4.1 The graph of ONPG hydrolyzed vs time of bead with 2% alginate for assay-1 (the slope is the activity).

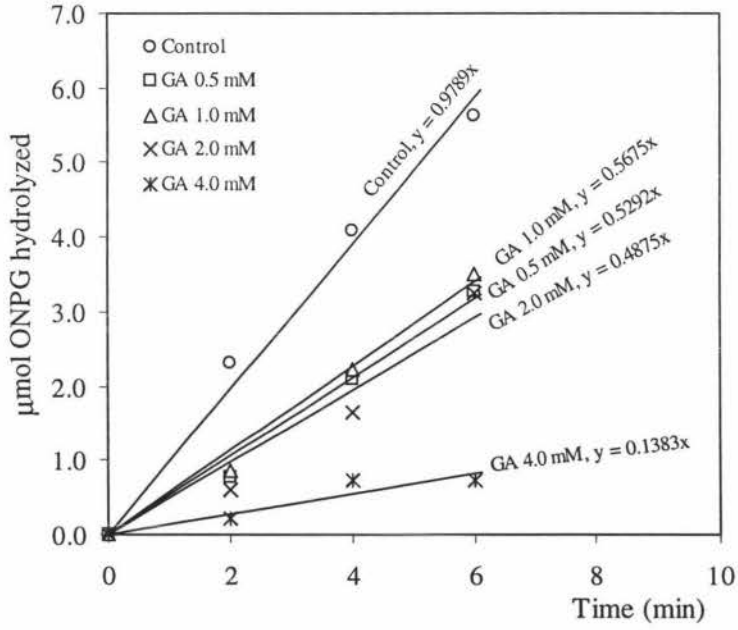

Figure A4.2 The graph of ONPG hydrolyzed vs time of bead with 2% alginate for assay-2 (the slope is the activity).

Table A4.3 Absorbance data of *o*-nitrophenol for activity tests of bead biocatalyst with 2.35% sodium alginate treated with different GA concentration

GA (mM)	Abs. (416 nm)				Abs. (416 nm)			
	Assay-1				Assay-2			
	0 min	2 min	4 min	6 min	0 min	2 min	4 min	6 min
0 (control)	0.0	0.205	0.434	0.602	0.0	0.211	0.494	0.637
0.5	0.0	0.109	0.280	0.426	0.0	0.125	0.316	0.435
1	0.0	0.071	0.251	0.326	0.0	0.094	0.249	0.403
2	0.0	0.092	0.270	0.317	0.0	0.053	0.174	0.282
4	0.0	0.234	0.318	0.311	0.0	0.088	0.241	0.428

Table A4.4 The bead activity calculated based on data from Table A4.3 (the activity is obtained from the slope of the graph)

GA (mM)	ONPG Hydrolyzed (µmol) Assay-1				Activity of Assay-1 (µmol ONPG/ min/g bead)	ONPG Hydrolyzed (µmol) Assay-2				Activity (µmol ONPG/ min/g bead)	Mean of Activity (µmol ONPG/ min/g bead)
	0 min	2 min	4 min	6 min		0 min	2 min	4 min	6 min		
0 (control)	0.0	1.641	3.474	4.818	0.8230	0.0	1.688	3.952	5.096	0.8886	0.8558
0.5	0.0	0.872	2.241	3.41	0.5566	0.0	1	2.528	3.48	0.5892	0.5729
1	0.0	0.568	2.009	2.609	0.4434	0.0	0.752	1.992	3.224	0.5146	0.4790
2	0.0	0.736	2.161	2.537	0.4525	0.0	0.424	1.392	2.256	0.3563	0.4044
4	0.0	1.873	2.545	2.489	0.5154	0.0	0.704	1.928	3.424	0.5297	0.5226

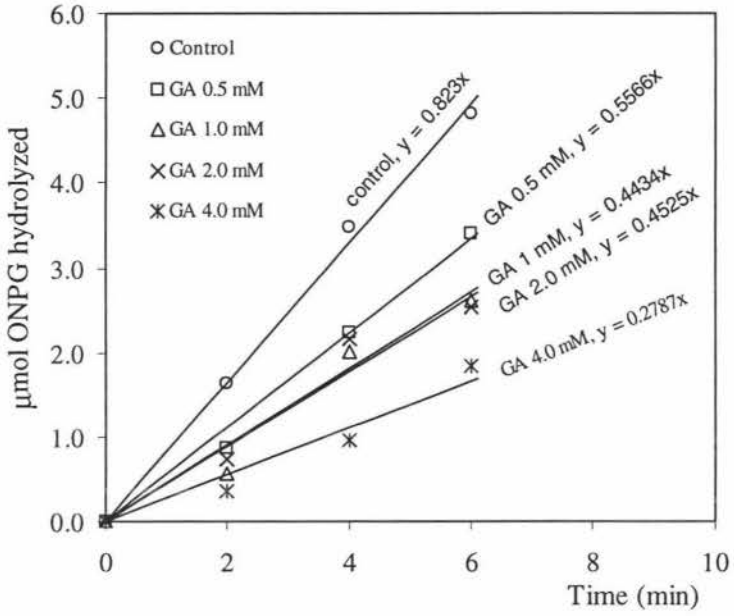

Figure A4.3 The graph of ONPG hydrolyzed vs time of bead with 2.35% alginate for assay-1 (the slope is the activity).

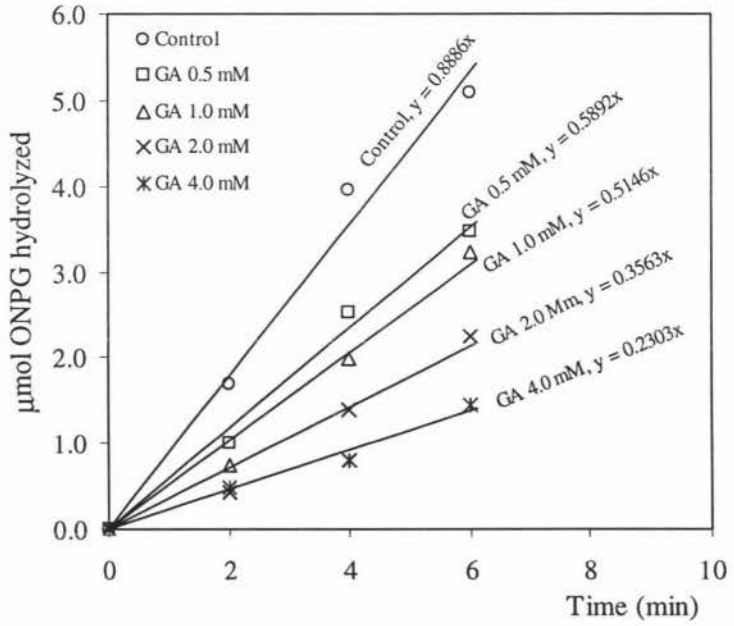

Figure A4.4 The graph of ONPG hydrolyzed vs time of bead with 2.35% alginate for assay-2 (the slope is the activity).

Table A4.5 Absorbance data of *o*-nitrophenol for activity tests of bead biocatalyst with 3% sodium alginate treated with different GA concentration

GA (mM)	Abs. (416 nm)				Abs. (416 nm)			
	Assay-1				Assay-2			
	0 min	2 min	4 min	6 min	0 min	2 min	4 min	6 min
0 (control)	0.0	0.116	0.297	0.478	0.0	0.082	0.243	0.388
0.5	0.0	0.064	0.213	0.347	0.0	0.051	0.183	0.307
1	0.0	0.062	0.153	0.266	0.0	0.035	0.133	0.244
2	0.0	0.050	0.171	0.232	0.0	0.053	0.158	0.312
4	0.0	0.005	0.040	0.090	0.0	0.003	0.057	0.081

Table A4.6 The bead activity calculated based on data from Table A4.5 (the activity is obtained from the slope of the graph)

GA (mM)	ONPG Hydrolyzed (µmol) Assay-1				Activity of Assay-1 (µmol ONPG/ min/g bead)	ONPG Hydrolyzed (µmol) Assay-2				Activity of Assay-2 (µmol ONPG/ min/g bead)	Mean of Activity (µmol ONPG/ min/ g bead)
	0 min	2 min	4 min	6 min		0 min	2 min	4 min	6 min		
0 (control)	0.0	0.928	2.377	3.826	0.6129	0.0	0.656	1.944	3.104	0.4949	0.5539
0.5	0.0	0.512	1.705	2.777	0.4376	0.0	0.408	1.464	2.456	0.3823	0.4100
1	0.0	0.496	1.225	2.129	0.3333	0.0	0.28	1.064	1.952	0.2952	0.3143
2	0.0	0.4	1.369	1.857	0.3110	0.0	0.424	1.264	2.496	0.3729	0.3420
4	0.0	0.040	0.320	0.720	0.1015	0.0	0.024	0.456	0.648	0.1029	0.1022

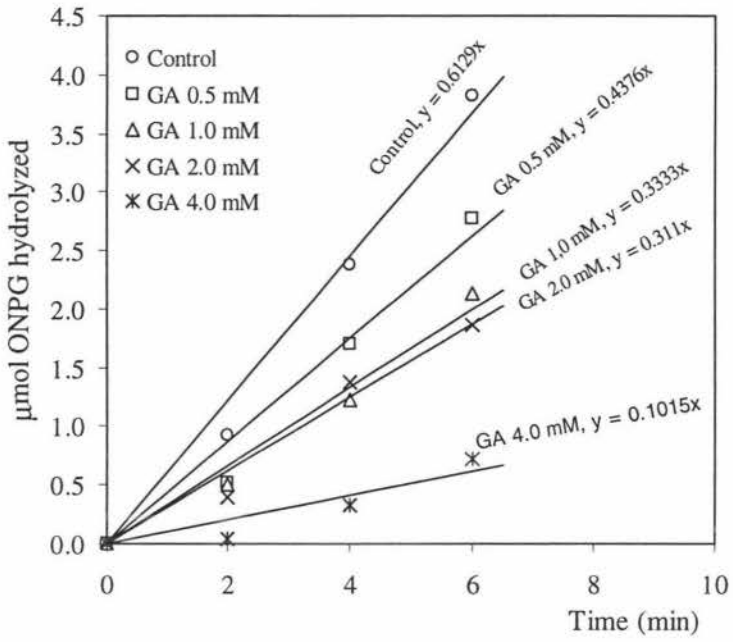

Figure A4.5 The graph of ONPG hydrolyzed vs time of bead with 3% alginate for assay-1 (the slope is the activity).

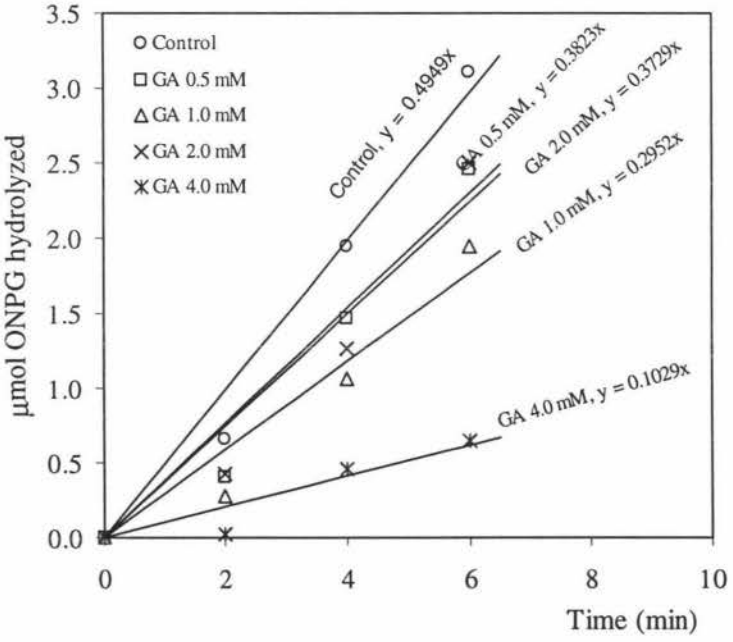

Figure A4.6 The graph of ONPG hydrolyzed vs time of bead with 3% alginate for assay-2 (the slope is the activity).

APPENDIX 5

Packed-Bed Process Data

Note: Unless otherwise stated, the glucose concentrations were measured enzymatically using a YSI-fixed glucose analyzer (Model 27, Yellow Springs Instrument Co., Inc., Yellow Springs, Ohio USA).

Table A5.1 Data for Figure 4.2

Time (min)	Glucose (g/l)			Lactose hydrolyzed (µmol/g bead)
	Assay-1	Assay-1	Mean	
20	0.09	0.11	0.10	1.11
40	0.22	0.24	0.23	2.56
125	0.88	0.72	0.80	8.89

Table A5.2 Data for Figure 4.3

Time (h)	pH	Glucose (g/l)
0.00	5.95	0.000
0.50	5.62	0.040
0.67	5.80	0.070
1.25	6.27	0.190
1.67	6.38	0.300
2.20	6.34	0.400
2.50	6.29	0.460
2.80	6.27	0.540
3.00	6.26	0.550
3.20	6.24	0.640
3.50	6.25	0.720

Table A5.3 Data for Figure 4.4

Time (h)	pH	Glucose (g/l)
0.00	6.38	0.00
1.00	6.37	0.02
1.50	6.34	0.02
2.00	6.34	0.02
2.50	6.34	0.02
3.25	6.38	0.02
15.33	6.29	0.46
16.25	6.29	0.39
17.00	6.26	0.60
18.00	6.25	0.57
19.00	6.24	0.56
25.33	6.24	0.62
42.18	6.05	0.73
42.75	5.81	0.45

Table A5.4 Data for Figure 4.5

Time (min)	Glucose (g/l)			Lactose hydrolyzed (µmol/g bead)
	Assay-1	Assay-2	Mean	
15	0.60	0.61	0.61	6.72
30	1.16	1.18	1.17	13.00
45	1.74	1.80	1.77	19.67

Table A5.5 Data for Figure 4.6

Time (min)	Glucose (g/l)			Lactose hydrolyzed (µmol/g bead)
	flask-1	flask-2	Mean	
15	0.69	0.66	0.68	7.50
30	1.38	1.32	1.35	15.00
45	2.10	2.01	2.06	22.83

Table A5.6 Data for Figure 4.7

Time (h)	pH	Glucose (g/l)	Lactose (g/l)
0.00	6.61	0.00	0.000
0.50	6.53	0.71	2.310
1.00	6.55	1.84	10.907
1.50	6.56	2.20	16.810
2.00	6.54	2.25	18.799
3.00	6.51	2.25	18.281
4.00	6.46	2.19	21.333
5.00	6.46	2.21	16.277
6.00	6.46	2.15	17.746
8.00	6.46	2.06	14.845
24.00	6.45	1.38	17.345
29.00	6.45	1.22	24.092

Table A5.7 Data for Figure 4.8

Time (h)	pH	Glucose (g/l)	Glucose after 1 h sample incubation (g/l)	Glucose (g/l)	Galactose (g/l)	Lactose (g/l)
		Enzymatic Method		HPLC Method		
0.00	7.20	0.00	0.00	0.000	0.000	0.000
0.50	7.14	1.08	1.62	1.106	1.109	3.925
1.00	7.11	2.20	2.85	1.890	1.909	10.324
1.50	7.10	2.21	2.82	2.183	2.080	14.617
2.00	7.10	2.10	2.57	1.805	1.697	13.326
4.50	7.10	1.75	1.90	1.462	1.419	14.941
8.50	7.06	1.71	1.74	1.513	1.487	18.082
18.50	7.04	1.38	1.35	1.396	1.300	18.589
20.00	7.05	1.31	1.30	1.238	1.213	18.054
28.50	7.11	1.24	1.19		1.878	22.411

Table A5.8 Data for Figure 4.10

Time (min)	Glucose (g/l)			Lactose hydrolyzed (μmol/g bead)
	Assay-1	Assay-2	Mean	
15	1.26	1.23	1.25	13.83
30	2.40	2.22	2.31	25.67
45	3.42	3.25	3.34	37.06

Table A5.9 Data for Figure 4.11 (t = 0 to 28 h) and Figure 4.12 (t = 31 to 144 h)

Time (h)	Flow rate* (ml/min)	pH	Glucose (g/l)	Glucose (g/l)	Galactose (g/l)	Lactose (g/l)
			Enzymatic Method	HPLC Method		
0.00	2.128	7.15	0.00	0.000	0.000	0.000
0.50		6.94	1.12	0.515	0.601	4.529
1.00		6.88	1.67	1.455	1.665	13.960
1.25		6.88	1.87	1.380	1.633	14.016
3.50		6.88	1.69	1.174	1.429	15.161
5.00		6.87	1.55	1.105	1.228	15.031
6.50	2.027	6.88	1.49	0.902	1.145	15.175
21.50		6.88	1.13	0.607	0.713	14.240
28.00	2.054	6.88	1.03	0.602	0.769	14.262
31.00	0.377	6.89	1.06	0.632	0.798	14.719
49.00		6.85	3.35	3.536	3.364	11.448
50.25		6.90	3.37	4.076	3.913	10.504
52.00		6.89	3.15	3.116	2.971	10.500
73.75	0.378	6.85	2.84	3.192	3.313	14.486
103.50		6.23	2.55	2.347	2.681	14.491
125.50		6.72	2.31	2.076	2.402	14.460
144.00		6.96	2.24	2.034	2.437	15.755

* The values show the measurement at the time designated. The flow rate was changed to lower value started at 31 h

Table A5.10 Data for Figure 4.13

Time (h)	pH	Glucose (g/l)	Lactose (g/l)	Galactose (g/l)
		Enzymatic	HPLC	
0.00	6.38	0.000	0.000	0.000
0.50	6.43	1.410	0.144	1.586
1.00	6.47	4.070	7.680	3.748
3.50	6.53	4.780	11.532	3.979
4.25	6.52	4.620	12.202	4.666
7.00	6.53	3.720	12.413	3.033
20.25	6.56	2.380	16.042	2.804
28.50	6.56	2.100	16.928	2.096
29.00	6.55	2.110	17.788	2.345
30.00	6.55	2.090	17.685	2.185
30.75	6.55	2.010	17.585	2.166

Table A5.11 Data for Figure 4.14

Time (h)	pH	Glucose (g/l)	Glucose (g/l)	Galactose (g/l)	Lactose (g/l)
		Enzymatic	HPLC		
0.00	6.85	0.00	0.000	0.000	0.000
0.25	6.94	0.01	0.140	0.000	0.000
0.50	7.04	0.46	0.267	0.273	0.743
1.00	6.95	2.37	1.842	1.749	5.230
3.00	6.91	3.29	2.620	2.469	9.854
5.25	6.91	3.00	2.465	2.331	10.855
9.50	6.91	2.61	2.389	2.385	14.056
24.00	6.90	2.09	2.144	2.049	16.943
28.00	6.90	1.97	1.498	1.554	13.091
32.00	6.87	1.92	1.704	1.637	14.328
33.50	6.87	2.07	1.337	1.364	11.984
51.00	6.91	1.97	1.629	1.635	17.705
53.67	6.92	1.79	1.631	1.697	18.872
57.00	6.92	1.61	1.268	1.293	14.039

Table A5.12 Data for Table 4.16

Time (min)	Glucose (g/l)			Lactose hydrolyzed (µmol/g bead)
	Assay-1	Assay-2	Mean	
15	1.19	1.25	1.22	13.56
30	2.25	2.19	2.22	24.67
45	3.31	3.28	3.30	36.61

Table A5.13 Data for Figure 4.17

Time (h)	pH	Glucose (g/l)	Glucose after 1 h sample incubation	Glucose (g/l)	Galactose (g/l)	Lactose (g/l)
		Enzymatic Method		HPLC Method		
0.0	6.38	0.00	0.00	0.000	0.000	0.000
0.5	6.36	3.31	4.24	3.539	3.330	9.229
1.0	6.33	6.82	10.88	7.399	6.282	26.124
2.0	6.33	9.80	10.56	7.248	5.940	38.490
3.0	6.34	8.36	9.04	6.354	5.043	39.124
4.0	6.35	6.20	6.40	6.252	5.103	42.027
5.0	6.35	5.80	5.75	5.813	4.842	44.275
20.0	6.37	3.34	3.34	3.680	3.193	51.267
22.0	6.37	3.25	3.24	3.438	2.894	47.670
27.0	6.35	3.07	3.17	3.229	2.812	50.458