The enzymatic removal of lactose from skimmed milk using a membrane reactor.

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The Enzymatic Removal of Lactose from Skimmed Milk using a Membrane Reactor

by

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DECLARATION

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Summary

Many people are lactose intolerant. The aim of this project was to produce suitable alternative on milk products with low lactose. The hydrolysis of lactose in skimmed milk was investigated using an enzyme reaction employing β-galactosidase in membrane bioreactor (MBR). This process with suitable membrane technology can produce low lactose milks.

Lactobacillus delbrueckii ssp *bulgaricus* NCTC 11778 was selected to be the source of β-galactosidase production since as it already used in fermented foods and produces relatively high levels of intracellular β-galactosidase. Initially, the effects on nutrient composition of growth medium for *L. delbrueckii* were studied in test tube to formulate a medium for high cell productivity. The suitable growth medium consisted of (g/l) yeast extract 10, soy peptone 10, lactose 20, KH_2PO_4 2.5, sodium acetate 5, triammonium citrate 2, $MgSO_4.7H_2O$ 0.2, $MnSO_4.H_2O$ 0.05 and tween80 1 ml/l. The optimum pH for cell growth conducted in 5-l batch fermenter at control temperature 37 °C was range from 5.0 to 5.5. From this study, the pH value at 5.5 was selected for cultivation in 140-l pilot fermenter because growth time to stationary phase was shorter than pH at 5.0.

Harvested the cells from pilot fermenter were washed buffer in microfiltration (MF) membrane system, the cells were disrupted to release the enzyme by using high pressure homogenisation. The pressure at 25 kpsi (172 MPa) was used based on optimal release in high protein and enzyme contents. The enzyme was separated and purified from cell debris by membrane separation apparatus. A 0.2 μm MF membrane was used to remove soluble protein from cell debris and this was dialyzed to remove additional enzyme from debris. The permeate was then concentrated in ultrafiltration (UF) membrane system with MWCO 50 kDa to form the partially purified enzyme used for hydrolysis.

The properties of β-galactosidase from both crude and partially purified enzyme showed good activity on ONPG and lactose at neutral pH range from 6.0 to 7.0. The optimum pH value for both substrates was at 6.5. The activity of β-galactosidase increased with the rising temperature from 20 to 65 °C. However, the stability of the enzyme at temperature above 40 °C decreased rapidly. The calculated V_{max} and K_m values based on the Michaelis-Menten equation and of Lineweaver-Burk transformation plots in two substrates, ONPG and lactose were investigated. The V_{max} values from crude enzyme were 1.25 and 0.91 μmol/min/ml and K_m values were 12.63 mM and 23.23 mM respectively. While the V_{max} values from purified form were 1.04 and 1.34 μmol/min/ml and K_m values were 17.62 mM and 27.58 mM when ONPG or lactose were applied as substrate respectively. The results also indicate that glucose and galactose acted as non-competitive inhibitor for β-galactosidase. Additions of Na^+ in lactose hydrolysis reduced the enzyme activity while Ca^{2+} also strongly inhibited the activity. Only the presence of K^+ seemed to promote β-galactosidase activity.

The enzymatic lactose hydrolysis reaction in synthetic and skimmed milk preparation was carried out in both batch bioreactor and membrane bioreactor (MBR) with 50 kDa MWCO UF membrane. Lactose concentrations and amount of enzyme activity influenced the hydrolysis. The results from batch bioreactor were in agreement with that observed in MBR. High concentration of lactose and enzyme increased the productivity. Low permeate flow rate during operating MBR resulted in greater hydrolysis yield (the obtained product was 67.7 μmol/ml at flow rate 8 ml/min) than when at high permeate flow rate (the obtained product was 44.7 μmol/ml at flow rate 25 ml/min). But in terms of productivity, high flow rates gave greater productivity (1117 μmol/min at flow rate 25 ml/min whereas 541μmol/min at flow rate 8 ml/min). The kinetics of lactose hydrolysis in batch bioreactor could be predicted by mathematical model based on reversible reaction from Haldane equation while in MBR did not correlate well with the model.

Using the results of the work, a basic unoptimised design study showed that 1.5-1.75% lactose milk could be prepared for a cost of 15 pence per litre which suggest that process may be viable.

Nomenclature

Symbol	Description
C_{bottom}	Molecules concentration in the bottom phase
C_{top}	Molecules concentration in the top phase
dA/dt	Rate of change of absorbance with time
-d[Lac]/dt	Lactose utilization rate (mmol $l^{-1}min^{-1}$)
d [Glu]/dt	Glucose production rates (mmol $l^{-1}min^{-1}$)
d[Gal]/dt	Galactose production rates (mmol $l^{-1}min^{-1}$)
D_p	Dilution rate (min^{-1})
Ea	Activation energy (kJ mol^{-1})
E_B	Enzyme activity in the bottom phase
E_T	Enzyme activity in the top phase
F	Flow rate (ml min^{-1})
i	Inflow
J	Permeate flux (l m^{-2} h^{-1})
k_1	Forward reaction rate constant for the formation of ES
k_{-1}	Reverse reaction rate constant for the breakdown of ES to E+S
k_2	Reaction rate constant for the breakdown of ES to E+P.
K	Partition coefficient
K_{eq}	Equilibrium constant
K_{enz}	Partition coefficient of enzyme
K_i	Inhibition constant (mmol l^{-1})
K_m	Michaelis–Menten constant for substrate (mmol l^{-1})
K_p	Michaelis–Menten constant for product (mmol l^{-1})
$K_{protein}$	Partition coefficient of protein

o	Outflow
P	Pressure
P	Product concentration (mmol l^{-1})
P_B	Protein concentration in bottom phase (mg ml^{-1})
P_{inlet}	Inlet pressure
$P_{permeate}$	Permeate pressure.
P_{outlet}	Outlet pressure
P_T	Protein concentration in top phase (mg ml^{-1})
r	Reaction rate (mmol $l^{-1}min^{-1}$)
R	Ideal gas constant, 8.314 (J K^{-1} mol^{-1})
R_B	Recovery from bottom phase
R_m	Membrane resistance (m^{-1})
R_T	Recovery from top phase
R_V	Ratio of volume in top phase to bottom phase
S	Substrate concentration (mmol l^{-1})
$t_{1/2}$	Half-life time of enzyme
T	Absolute temperature (K)
v	Initial rate of reaction (mmol $l^{-1}min^{-1}$)
V	Reactor volume (ml)
V_B	Volume the bottom phase
V_f	Final volume in the cuvette (ml)
V_{max}	Maximum reaction rate (mmol $l^{-1}min^{-1}$)
$V_{max\ r}$	Maximum reaction rate in the reverse direction (mmol $l^{-1}min^{-1}$)
V_s	Amount of enzyme taken from sample (ml)
V_t	Total volume of sample (ml),

V_T	Volume of the top phase

Greek symbols

$\triangle P$	Transmembrane pressure (Pa)
μ_i	Specific growth rates (h^{-1})
μ	Viscosity of liquid (Pa. s)
μ_{max}	Maximum specific growth rate (h^{-1})
ε	Molar extinction coefficient

Abbreviations used

ATPS	Aqueous two phase system
[Gal]	Galactose concentration (mmol l^{-1})
GalOS	Galacto-oligosaccharide
[Glu]	Glucose concentration (mmol l^{-1})
[Lac]	Lactose concentration (mmol l^{-1})
MBR	Membrane bioreactor
MF	Microfiltration
MWCO	Molecular weight cut-off
OD	Optical Density
OD_{max}	Maximum optical density
ONP	Ortho-nitrophenol
ONPG	Ortho-nitrophenol-β-galactosidase
PEG	Polyethylene glycol
PR	Purification ratio
STR	Stirred tank reactor
TMP	Transmembrane pressure
UF	Ultrafiltration

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Chapter 1

β-galactosidase is a widely used enzyme in dairy technologies. It degrades lactose to glucose and galactose. Considerable work has been carried in attempt to reduce lactose in food industry especially lactose intolerance problem from milk. This project investigates the problem and how it could be carried out by using a combination of enzymatic reaction and membrane system for lactose hydrolysis. The β-galactosidase from lactic acid bacteria will be preferred in view of producing the enzyme. The application of membrane on separation process to prepare the partially purified enzyme and filter milk will be studied.

The high molecular weight materials such as protein and fat contents from milk will be filtered by microfiltration and followed by ultrafiltration membrane. Then the permeate, containing low molecular weight materials, pass through a membrane reactor containing β-galactosidase where the lactose will be hydrolysed. Membrane bioreactor is a good model process and could have several advantages over previous work in that enzyme productivity can be significantly improved while not contaminating products with the active enzyme.

1.1 Sources of β-galactosidase

The β-galactosidase derived from various sources, microorganisms, plants and animals, provides different properties in molecular weight, protein chain length and the position of the active site. Apart from physical properties, different sources of the enzyme also show different chemical properties such as activity and stability. In general the enzymes from fungi are active at pH in range 2.5-4.5 which is suitable to use for acid whey hydrolysis. The enzyme from yeast and bacteria show pH optima in the neutral region 6-7 and 6.5-7.5 respectively thus used in milk and sweet whey (Ladero *et al.*, 2002).

Table 1.1 *Sources of β-galactosidase and their properties*

Microorganisms	Molecular weight (Da)	optimum pH activity	optimum temperature (°C)	Reference
Lactobacillus lactis	540,000	6.2	55	Premi *et al.*, 1972
Lactobacillus bulgaricus		5.5	55	Ohmiya *et al.*, 1977
Kluyveromyces lactis	117,618	6.3	37	Ohmiya *et al.*, 1977; Zhou and Chen, 2001
Escherichia coli	116,351	7.5	50	Ohmiya *et al.*, 1977; Zhou and Chen, 2001
Aspergillus oryzae	270,000	4.0	30	Cruz *et al.*, 1999
Aspergillus niger	119,160	4.6		Cruz *et al.*, 1999; Zhou and Chen, 2001
Penicillium chrysogenum	270,000	4.0	30	Nagy *et al.*, 2001
Pseudoalteromonas sp.		9.0	26.0	Fernandes *et al.*, 2001
Bacillus coagulans RCS3		6.0-7.0	63	Batra *et al.*, 2002
Kluyveromyces fragilis	200,000	6.5-6.8	25	Ladero *et al.*, 2002
Thermus sp T2	550,000	5.0	70	Ladero *et al.*, 2002
Gene of *Thermotoga maritima* expressed in *E.coli*	120,000	6.5	80-85	Kim *et al.*, 2004

The β-galactosidase sources and their properties are shown in Table 1.1. The sources of the enzyme are roughly grouped into 3 sources for the ease of consideration in this study. These are from lactic acid bacteria, other microbes and from plants and animals.

1.1.1 β-galactosidase from lactic acid bacteria

Lactic acid bacteria (LAB) refer to a large group of beneficial bacteria that have similar properties and provide lactic acid as the major end product in carbohydrate fermentation process. They can be found in nature and human digestive system. The genera important members of this group are *Lactobacillus, Leuconostoc, Pediococcus and Streptococcus.* These organisms are heterotrophic and generally have complex nutritional requirements

due to lacking of many biosynthetic capabilities. Consequently, most species have multiple requirements for amino acids and vitamins.

Generally, lactic acid bacteria are non motile gram positive, non spore forming, micro-aerophilic bacteria and vary in morphology from long, slender rods to short coccobacilli. These bacteria can be categorized according to principal metabolites into two groups, homo- and hetero-fermentative. The pathways of lactic acid production are difference, the homofermenters produce mainly lactic acid via the glycolytic (Embden-Meyerhof pathway) while the end products of heterofermenters are lactic acid plus amounts of ethanol, acetate and carbon dioxide via the 6-phosphogluconaste/phosphoketolase pathway (Figure 1.1).

A group of these beneficial bacteria are broadly used in fermented foods such as pickled vegetables, sorghum beer, sour dough bread, winemaking, curing fish, meats and sausages. Moreover, they are well known for their role in the preparation of fermented dairy product, including yoghurt, cheese, butter, buttermilk and kefir. They are also the important living bacteria in connection with lactose hydrolyzed in the present of β-galactosidase.

β-galactosidase enzyme from lactic acid bacteria have been attractively attended because this bacteria group is normally considered safe so the enzyme derived from them might be used with no need of extensive purification and there is little or no adverse effects on fermented dairy products.

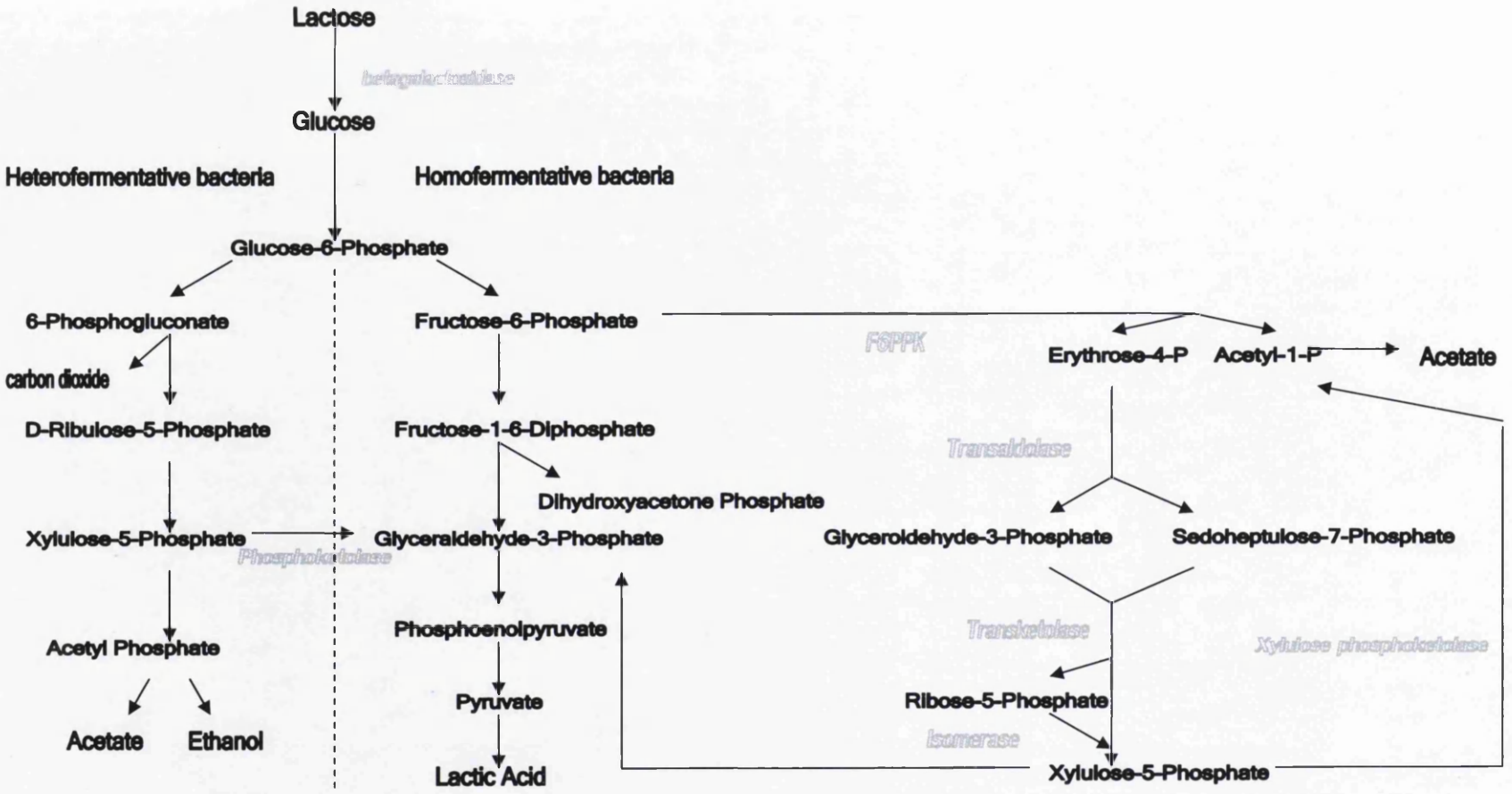


Figure 1.1 *Overview of the carbohydrate metabolism and the end metabolites of hetero- and homofermentative lactic acid bacteria (De Vuyst, 2000).*

Various strains of lactic acid bacteria have been recently researched for the enzyme for example;

- *Lactobacillus acidophilus* (Lin *et al.*, 1991; Noh and Gilliland, 1993; Gupta *et al.*, 1994; Lin, 1995; Montes *et al.*, 1995; Wang and Sakakibara, 1997)

- *Lactobacillus delbrueckii* subsp. *bulgaricus* (Ohmiya *et al.*, 1977; Wang and Sakakibara, 1997)

- *Streptococcus thermophilus* (Greenberg and Mahoney, 1982)

- *Lactococcus lactis* subsp. *cremoris* (Shah and Jelen, 1991)

- *Lactobacillus kefiranofaciens* K-1 (Itoh *et al.*, 1992)

- *Lactobacillus helveticus* (Wang and Sakakibara, 1997)

- *Lactobacillus plantarum* (Fernandez *et al.*, 1999; Montanari *et al.*, 2000)

- *Lactobacillus brevis* release the enzyme immediately after the end of cell multiplication and is connected to cell autolysis and breakage of the cell wall (Montanari *et al.*, 2000)

- *Lactobacillus crispatus* (Kim and Rajagopal, 2000)

In comparison to lactic acid bacteria, the strain of *Lactobacillus delbrueckii* subsp. *bulgaricus* produces relatively high level of intracellular β-galactosidase (Shah and Jelen 1990, 1991). Wang and Sakakibara (1997) studied the production of β-galactosidase from four strains of lactic acid bacteria, *L. delbrueckii* ssp *bulgaricus* B-5b, *L. helveticus* LH-17, *L. delbrueckii* ssp *lactis* SBT-2080 *and L. acidophilus* SBT-2068. The amount of enzyme released after sonication was detected with ONPG. The highest enzyme activity was found about 1.5 U/cm^3 from *L. delbrueckii* ssp *bulgaricus* B-5b while the lowest one was obtained at 0.05 U/cm^3 from *L. acidophilus*. This bacteria type is one component of thermophilic starter cultures widely used in yoghurt.

1.1.2 β-galactosidase from other microbes

In addition to lactic acid bacteria, β-galactosidase has been discovered in other microorganisms. Goodman and Pederson (1976) indicated that several strains of thermophilic aerobic spore forming bacilli produce a β-galactosidase such as *Bacillus circulans* (Boon *et al.*, 1999) and *Bacillus stearothermophilus* (Goodman and Pederson, 1976).

The enzyme also found in *Escherichia coli* (Higgins *et al.*, 1978; Ladero *et al.*, 2001). Ohmiya *et al.* (1977) found the enzyme from *E. coli* performed higher activity and released glucose from lactose hydrolysis better than from lactobacillus group.

Prenosil *et al.* (1987) said that β-galactosidase from fungi for example *Aspergillus niger* and *Aspergillus oryzae* are normally used for the hydrolysis of lactose in whey because their optimum pH are generally at acid range. In industrial penicillin production, growing fungus with glucose or other metabolizable sugar, which used as carbon source, suppressed penicillin biosynthesis. The fungus *Penicillium chrysogenum*, which is able to produce β-galactosidase and use lactose as carbon source during growth, were grown with lactose for biosynthesis production instead of glucose (Nagy *et al.*, 2001). Cruz *et al.* (1999) found that *Penicillium simplicissimum* grown in semi solid medium performed good productivity of β-galactosidase with galactosyltransferase activity. When incubate the enzyme with high concentration of lactose up to 60 % at pH 6.5 and 50 °C, lactose was converted to 30% monosaccharide and 30.5% transgalactosylated oligosaccharides.

However, recent studies have focused on yeast source due to providing high enzyme activity. Szczodrak (2000) studied the hydrolysis of lactose in sweet whey permeate by both free enzyme and enzyme immobilized on porous glass modified by glutaraldehyde from *Kluyveromyces fragilis*. It was found that the highly efficient lactose conversion in whey permeate was up to 86-90% from both in a batch process and a recycling packed bed bioreactor. β-galactosidase has also been found in other yeast strains such as *Candida kefir* (Berruga *et al.*, 1997) and *Candida peudotropicalis* (Pedrique and Castillo, 1982) *Candida pseudotropicalis* var *lactosa* (Ohmiya *et al., 1977).*

1.1.3 β-galactosidase from plants and animals

Apart from β-galactosidase found in microorganisms, it has been detected in various plant tissues. The β-galactosidase from jack beans has been found to be located in cell wall (Boller and Kende, 1979) and membrane fractions (Gaudreault and Beevers, 1984). The enzyme of the cell wall in suspension culture is released into medium after cell expansion (Asamizu *et al.*, 1981; Chaubet *et al.*, 1981). β-galactosidase has also been isolated from dry cotton seeds (Shrioya, 1963) and sugar cane leaves (Etcheberrigaray *et al.*, 1986).

β-galactosidase from animals, normally called as lactase, are expressed exclusively in the small intestine of mammals. Koldovsky (1981) said the enzyme activity is high during the suckling period and decreases to a low level after weaning. The enzyme activity of adult rat is influenced by carbohydrate intake (Goda and Koldovsky, 1988). Generally, animals such as cow, goat, cat and marsupial only create enough enzymes to break down the amount of lactose found in their mother's milk.

1.2 Properties of β-galactosidase

The β-galactosidase or β-D-galactoside-galactohydrolase (EC.3.2.1.23) is an enzyme that usually hydrolyses non reducing terminal galactosyl moiety from oligosaccharide including lactose into monosaccharides subunits, glucose and galactose. It normally disrupts substrates at β-1,4 bond however, some is able to break and form other covalent bonds, as β-1,6 more common and β-1,2 rarely (Ladero *et al.*, 2002).

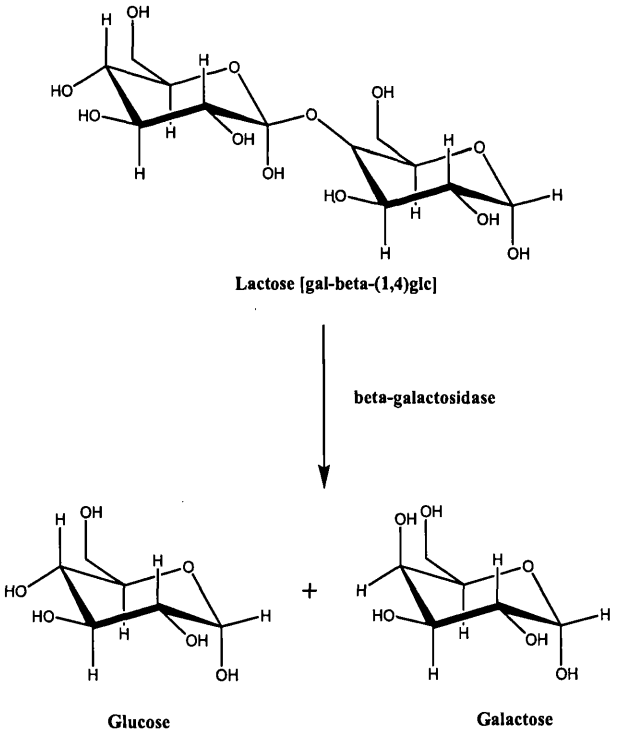


Figure 1.2 *Lactose hydrolysis by β-galactosidase*

The β-galactosidase is also capable of catalysing transferase reactions which can result in both internal rearrangement of the lactose molecule and the formation of different disaccharides, known as transgalactosylation (Vasiljevic and Jelen, 2003). The transferase

reaction can produce a range of di-, tri- and higher oligosaccharides. However, the hydrolysis reaction of oligosaccharides can simultaneously occur during the synthesis.

1.2.1 Biochemical properties

The first purification of β-galactosidase was carried out by Cohn and Monod (Wallenfels and Weil, 1972). The specificity and molecular properties of β-galactosidase for example subunit size, quaternary structure and metal ion requirements are significantly unlike with the source of enzyme.

Appel *et al.* (1965) indicated the functional form of β-galactosidase in *E. coli* is a tetramer of four identical subunits and each subunit consists of 1,023 amino acid residues. Three steps of enzyme activity are taken to completely breakdown the lactose into galactose and glucose. First step, the enzyme cleaves lactose to galactose plus glucose. Second, lactose is converted to allolactose when the enzyme acts as a transglycosylase. Then β-galactosidase finally hydrolyzes allolactose into galactose and glucose (Juers *et al.*, 2000). Karasova *et al.* (2002) said β-galactosidase belongs to the glycosidase because it has two models, hydrolysation and transglycosylation activity. It was found that during hydrolysis of natural substrates they are able to synthesis oligosaccharides with 2 or more galactose units starting from lactose.

1.2.2 β-galactosidase assay

The measuring method for β-galactosidase activity is variable depending on substrate used. The enzyme activity can be examined in terms of the rate of substrate disappearance or the rate of product formation. The simple substrate used for enzyme detection is a non-biological artificial substrate called ortho-nitrophenol-β-galactosidase (ONPG).

ONPG is a common method used to assay β-galactosidase. This method is more simple, rapid, convenient and an inexpensive way than using lactose solution as substrate. Another advantage over lactose solution is many other enzymes presented from using a crude cell extract as a source of β-galactosidase will immediately convert any galactose or glucose formed to other compounds which leads to an error measuring.

The mechanism of action of β-galactosidase on this substrate is same as lactose except the interaction of the active centre with the relatively hydrophobic aglicon moiety (o-

nitrophenol) is stronger than the interaction with the glycosyl part of lactose (Ladero *et al.*, 2002).

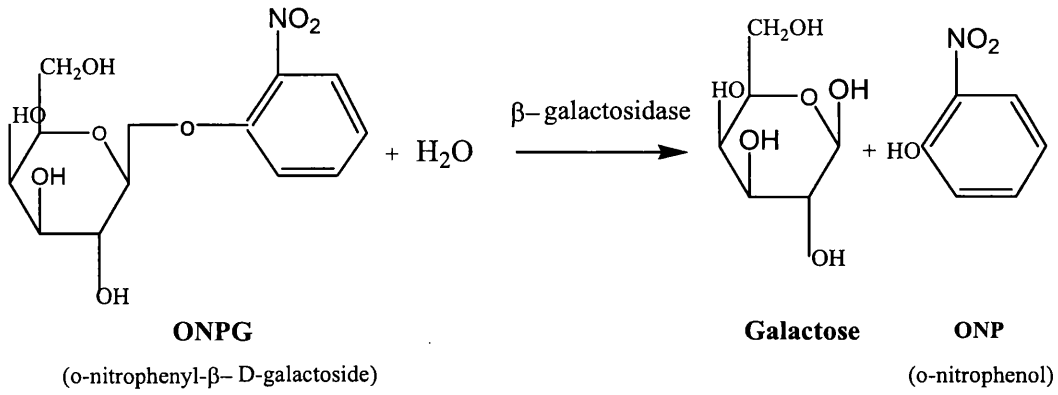


Figure 1.3 *The reaction of β-galactosidase on ONPG.*

ONPG is colorless and composed of galactose bonded to nitrophenol. β-galactosidase will recognize the bond as a substrate and cleave this molecule to produce galactose and ortho-nitrophenol (ONP). ONP is normally bright yellow. The amount of yellow color can be measured in a spectrophotometer at 420 nm and can be used as a measure of the amount of ONP formed in period of time. Therefore, the enzyme activity can be expressed as colour development from the hydrolysis of ONPG to ONP.

When lactose is used as a substrate, the reaction can be directly detected in view of the amount of lactose depletion or products (glucose and galactose) formation. High performance liquid chromatography (HPLC) is one of the methods to monitor the concentrations of all the sugars in both reactants and products. In general detecting the amount of glucose production using enzymatic assay to monitor lactose hydrolysis is more convenient and preferable than measuring the amount of lactose or galactose.

Kreft *et al.* (2001) proposed the cryoscopic method which is using significant linear correlation between the change in freezing point and results from other methods to monitor lactose or glucose level during lactose hydrolysis. The freezing point is measured with an Advanced Cryomatic Milk Cryoscope, model 4C2. A complete hydrolysis of 2.5% lactose with no other reaction influenced would theoretically give a freezing point of 141 milli-degrees Hortvet (m°H) while at 5% lactose provides 281 m°H. The percent hydrolysis can be calculated from the data.

1.3 The importance of β-galactosidase

The β-galactosidase or lactase is a very important enzyme involved in dairy industry. Many studies on the enzyme have been attracted due to its important application in solving the problem of lactose intolerance, food-technology and environmental field from milk product.

1.3.1 Lactose intolerance

Lactose is a disaccharide which composes of two monosaccharides subunits, a galactose and glucose linked together. It is scarcely soluble and low sweetness in comparison with its hydrolysis products. It presented as a natural component of foods only in milk and dairy products (Sieber *et al.*, 1997). Lactose is the only free form sugar making up around 2-8% of the solids in milk. Its solubility is low and can not be absorbed directly from the intestine. To be absorbed, lactose in milk needs to be hydrolyzed by an enzyme β-galactosidase, generally called lactase secreted from human intestinal villi.

It is found that a large proportion of the human population are unable to metabolise lactose due to intestinal enzyme insufficiency. Vesa *et al.* (2000) indicated the prevalence of primary lactose maldigestion in Europe varies from around 3% in Scandinavia to about 70% in Italy. Some countries in Africa and Asia are reaching almost 100%. In the United States the prevalence is 15% among American whites, 53% among Mexican-Americans and 80% in American blacks. Australia and New Zealand have prevalence of 6% and 9% respectively.

In general, that about three quarters of the world adult population is lactose intolerant (Suarez *et al.*, 1995). In addition people that normally are lactose tolerant lose lactase activity capacity later in life (Vesa *et al.*, 2000) which occurs mostly at about 40-50 years. The prevalence therefore varies depending on race and age.

Although milk products provide the principal source of lactose for 50 grams per 1 litre of whole milk (Tamm, 1994). There are also valuable sources of calcium, protein, riboflavin and vitamin B12. The decrement of consuming dairy foods can lead to insufficient intake of calcium which can in turn contribute to the genesis of osteoporosos (Corazza *et al.*, 1995). It is not easy to receive adequate calcium without dairy foods.

Hence it is necessary for lactose intolerant people to consume milk products with low-lactose or lactose-free food in order to obtain appropriate nutrients at the meantime reduction or prevention severe tissue dehydration, cramps, stomach ache and diarrhoea from consuming. The approaches for dealing with lactose intolerance have been proposed for example consuming yogurt instead of milk products, adding some special products such as lactose-hydrolyzed dairy food and lactase additive to reduce the lactose content of products. The hydrolysis of lactose forms glucose and galactose, are then easily absorbed in the intestine and metabolised.

1.3.2 Food – technology

Some food industries use lactose as raw material for making products such as sweets, confectionary, bread and sausage. Its physiological properties give good texture and binds water and colour.

However, the unsatisfied aspect of lactose is relatively low sweetness intensity. It is only about one third as sweet as saccharose and less than half as sweet as glucose (Vesa *et al.*, 2000). Lactose hydrolyzed by β-galactosidase is then introduced to increase the sweetness and decrease grittiness in many dairy products (Gekas and Lopez Leiva, 1985; Zadow, 1992). Thereby there is no requirement for the addition of sugar in the manufacture of milk and its products.

In addition to low sweetness of lactose, its low solubility results in crystal formation at concentrations above 11% (w/v). This provides an unpleasant sandy texture and readily prone to microbiological spoilage. The use of concentrated whey syrups is then unfavourable in many food processes.

The examples used of β-galactosidase when added to milk or liquid whey are obviously shown in ice cream, yogurt and frozen desserts. It is employed to improve scoopability and creaminess, sweetness and digestibility and to reduce sandiness because of crystallization of lactose in concentrated preparation.

Another advantage of lactose hydrolysis by β-galactosidase is forming of some galactosyl–oligosaccharides during hydrolysis reaction. These galactosyl–oligosaccharides are non-digestible carbohydrate consisted of 3-10 molecules of galactose and glucose, water

soluble, mildly sweet in comparison with the commonly used mono and disaccharide, acting as dietary fibre and can stimulate the growth of bifidobacteria in the intestine with a consequent decrease in the concentration of putrefactive bacteria (Cruz *et al.*, 1999) leading to healthy effect in the intestine and the liver (Mahoney, 1998). Oligosaccharides are used as food additives with activity to improve physicochemical characteristics such as in beverage, infant milk powders, yogurts, chewing gums and other dairy products (Karasova *et al.*, 2002).

1.3.3 Environmental problems

Another lactose problem is the effluent produced in cheese and casein manufacture. A large quantities of whey is produced as a by product which is difficult to dispose of. The whey effluent which is a highly polluting product normally contains protein, lactic acid, various salts and lactose. To reduce waste and decrease the cost of cheese-making process, it is necessary recovering and reusing these compounds. The protein fraction can be recovered or removed by ultrafiltraton whereas lactose permeates the ultrafiltration membrane. Low solubility and crystallise out of solution of lactose make problems especially transport by tanker difficult and is the limitation of whey re-utilization. Large amounts are disposed of each year. It is found that 3 million tons per years of lactose is produced from dairy effluents and only a small percentage of this amount is utilised further. If the rest is not properly managed and discharged into water, it can produce high biological oxygen demand which pollutes the environment. Several studies have been proposed to reduce the problem by using β-galactosidase from various microorganisms for example the enzyme from *Kluyveromyces fragilis* (Szczodrak, 2000), *Aspergillus niger* and *Aspergillus oryzae* (Prenosil, 1987).

1.4 The *lac* operon and its use

Jacob and Monod (1961) clarified the molecular and genetic relationship between enzyme induction and repression on lactose metabolism system in *E. coli*. To metabolize lactose, the *lac* operon is required. The *lac* operon consists of a regulatory gene, a promoter, an operator and three structural genes (*lacZ, lacY*, and *lacA*) which require for the uptake and catabolism of lactose. The regulatory gene encodes for the repressor of the *lac* operon. *lacZ* gene encodes for β-galactosidase. *lacY* gene encodes for β-galactoside permease,

which increases permeability of the cell to β-galactosidase. *lacA* gene encodes β-galactoside transacetylase, which transfers an acetyl group from acetyl-CoA to β-galactoside. In the presence of lactose, lactose binds to the repressor causing a conformational change and unable to bind the operator. Thus the RNA polymerase is free to transcribe the *lac* genes. Then the mRNA and protein can be made from these three genes. Lactose can now be metabolized.

The *lac* operon can be used in molecular biology for example the use of *lacZ* fusion technology or using *lacZ* as a reporter gene. Many studies have focused on the fusion of *lacZ* and other genes such as fiu (ferric ion uptake) - *lacZ* fusion in *E. coli* (Newman and Shapiro, 1999), fusion of *lacZ* with *malE* gene encoding the periplasmic maltose binding protein in *E. coli* (Bassford Jr. *et al.,* 1979), fusions of PYK- *lacZ* and PGK- *lacZ* in yeast (Moore *et al.,* 1991). Yocum *et al.* (1984) used the *lacZ* fusion to delimit regulatory elements of the inducible divergent *GAL1-GAL10* promoter in *Saccharomyces cerevisiae.* The fusion of *GAL1*-and *GAL10- lacZ* used to identified a 45 base pair sequence midway between *GAL1* (encodes for galactokinase) and *GAL10* (encodes for uridine diphospho glucose-4-epimerase) which contains information necessary for a normal level of galactose induction toward the *GAL10* side.

Battad-Bernardo *et al.* (2004) inserted *lacZ* from *E. coli* in *Acetobacter xylinus* for cellulose production from whey. A wild-type strain of *A. xylinus* can normally produce large amounts of cellulose in sucrose as a carbon source. After modified the strain and grown in lactose medium, the production of cellulose increased 28-fold and β-galactosidase activity increased 160-fold. Koyoma *et al.* (1990) cloned the genes encoding thermostable α- and β-galactosidase from *Thermus* strain T2 in *E. coli.* After *E. coli* was cultured and grown at 37 °C to single colonies on the enriched agar plates containing X-gal, the plates were then incubated at 70 °C for 2 h. Several colonies on the plates turned blue. The cells died at this temperature but the enzyme produced from thermostable *Thermus* should survive. Then the recombinant plasmids carrying *Thermus* β-galactosidase gene from dead blue *E. coli* colonies were introduced into *T. thermophilus* HB27. *T. thermophilus* HB27 which normally has low enzyme activity expressed thermastable β-galactosidase gene of *Thermus* strain T2.

1.5 Aqueous two phase systems for enzyme purification

An aqueous two phase system (ATPS) can be formed by combining aqueous solutions of two incompatible polymers or from a mixing solution of polymer and salt above critical concentration. Two liquid layers are obtained at equilibrium. The first polymer predominates in one phase and the second polymer or salt predominates in the other phase. The ATPS is an attractive method for purification of biological materials since it constitutes a mild environmental condition containing high water content in each of the liquid phase up to 70-90% and surface tension between the two phases is low, resulting in high mass transfer and decreasing the possibility of denaturation of labile biomolecules (Veide *et al.,* 1983; Walter and Johansson, 1994; Miyuki Minami and Vahan Kilikian, 1998; van Berlo *et al.,* 1998; Balasubramaniam *et al.*, 2003; Rito-Palomares, 2004). Many polymers used in the system have protein-stabilizing properties. In continuous extraction with ATPS is also straightforward and requires relatively simple equipment which is easy to operate (van Berlo *et al.,* 1998). Moreover, the conditions for separation on a large scale do not considerably change from small scale, thus easy in scale-up. However, the limitation of the technique is the lack of knowledge of the molecular mechanism involved in the partitioning process.

The application of ATPS in downstream processing has been focused on the extraction, separation and concentration of various molecules such as recovery of metal ions from aqueous solution (Roger *et al.,* 1996), recovery of food coloring dyes from textile plant waste (Huddleston *et al.,* 1998) and recovery small organic molecules (Roger *et al.,* 1998). ATPS process is also employed in integration of the upstream operation of fermentation and downstream recovery processes. The products formed during fermentation are removed to the other phase which is probably resulting in an increase in the productivity and decrease product inhibition (Rito-Palomares 2004). For example Rito-Palomares *et al.* (2000) removed aroma compounds from the media culture of *Trichoderma harzianum.*

1.5.1 The composition of phase system

The example of polymers used in preparation ATPS are polyethylene glycol (PEG), dextran, polypropylene glycol, polyvinylpyrrolidone, and hydroxypropyldextran. Generally, PEG and dextran are preferably employed in ATPS preparation among polymers because they perform desirable physical properties with non toxicity (Kula *et al.,*

1982) and dextran has a stabilizing effect on microbial cells (Sinha *et al.,* 2000). Zijlstra *et al.* (1996) designed the system containing PEG, dextran and culture medium to support the long-term growth of animal cells. The hybridoma cells were successfully cultured and partitioned to the PEG phase. Johansson and Reczey (1998) used PEG and dextran system to concentrate and purify β-glucosidase from *Aspergillus niger*. They found that too high concentrations of both polymers forced all soluble proteins into the bottom phase (dextran-rich phase) and provided low solubility in concentrated PEG solution. Therefore the separation was not success. The best recoveries of the enzyme was obtained using PEG 12-20% (w/w).The enzyme preferentially partitioned to the bottom phase and recovery in range of 85-95%. It was concentrated up to 700 times and purified 2-3 times.

Although PEG and dextran system is a commonly use, the high cost of fractionated dextran is a limitation for its application in large scale process. Antov (2004) cultivated *Polyporus squamosus* in ATPS which composed of PEG and crude dextran in order to produce endo- and exo-pectinase. The products were enriched in the top phase with partition coefficient about 2.45. Although crude dextran provided similar properties with dextran and less expensive, crude dextran was difficult to handle and remove from the system due to its high molecular weight fractions and high viscosity.

Other alternative inexpensive substitutes of dextran such as derivatives of starch, methylcellulose, cellulose, ethyl hydroxyl ethyl cellulose, agarose, guar gum and polyvinyl alcohol can be used with lower concentration as well. For example, Almeida *et al.* (1998) achieved in purification of cutinase from ATPS composed of PEG and a crude hydroxypropyl starch with presence of sodium chloride or sodium sulphate in the system at pH 4.0. Oliveira *et al.* (2002) studied the partition behaviour of trypsin in PEG and cashew-nut tree gum. The recovery of maximum trypsin was obtained in the cashew-nut tree gum phase with the system consisted of PEG 8000 at pH 7.0 and 1.0 M NaCl. da Silva and Meirelles (2001) used maltodextrin, which is a low-cost starch derivative, to replace dextran in partitioning behaviour of bovine serum albumin, α-lactoalbumin and β-lactoglobulin with polypropylene glycol (PPG). Most proteins partition preferentially to the PPG phase.

The system obtained from polymer and salt are usually preferred for large scale operation since salt is much cheaper than dextran and the phases have a lower viscosity, it is then

easier to handle and a shorter time for phase partitioning is required (van Berlo *et al.,* 1998). Many types of salt, such as potassium dihydrogen phosphate, potassium chloride, sodium dihydrogen phosphate, sodium carbonate, sodium citrate, magnesium sulphate and ammonium sulphate, can be used in forming an ATPS with polymers, especially with PEG (Banik *et al.,* 2003). The most common polymer/salt used is PEG and phosphate salt (generally sodium or potassium phosphate) system due to low cost, widely employ in the past and current application, and suitable range of system pH from 6-9 under which the ATPS are stable (Rito-Palomares, 2004). However, selecting salt also depends on its effect with interesting product because different salts affect the water structure and hydrophobic interactions differently (Gündüz and Korkmaz, 2000). Although the application of polymer-salt system is inexpensive and provides large partition selectivity (Sinha *et al.,* 2000), the system is limited in the presence of high salt concentrations which probably causes protein denature and inhibits cell growth. Furthermore, the recycling of salt from ATPS is poorly investigated. Then, it is difficult to dispose in large amounts without environmental problem.

1.5.2 Partitioning of protein in ATPS process

ATPS process has been widely used for the recovery of macromolecule from fermentation broth and biological products. The biological materials such as cell organelles, protein, enzyme and whole cells are distributed between the two phases based on surface properties of the particles and molecules including size, shape, surface net charge, hydrophobicity and the existence of specific binding sites (Albertsson, 1986 and Brooks *et al.,* 1985). Normally, after phase partitioning the target product biomolecules have to be preferentially partitioned in favor of one phase, whereas the interfering substances and contaminants, such as cells, cell debris, RNA, carbohydrate and lipid, should partition into the other phase. According to phenomenon, the effective separation can be achieved. The partition of molecules between the two phases is described by the partition coefficient (K) which is defined as the ratio of the molecules concentration in the top phase (C_{top}) to that in the bottom phase (C_{bottom}). If the coefficient is higher than 1, the molecules prefer the top phase and if lower than 1 is in the bottom phase.

This process normally emphasizes on the primary purification and recovery of protein especially enzymes from cell debris and other proteins. The examples include separation and purification of amyloglucosidase from *Aspergillus niger* in 7.5 % PEG 6000 and 10%

sodium dihydrogen phosphate system. After equilibration, most enzymes partitioned into the bottom phase with average enzyme activity 3000 U/ml. The percent recovery was 85.2% (Tanuja *et al.*, 1997).

The study of purification of recombinant cutinase from the culture supernatant of *Saccharomyces cerevisiae* was succeeded in ATPS consisted of 20% PEG 1500 and 15% potassium phosphate. The enzyme preferably partitioned to the top phase. Although improving the partition coefficient could be made by optimizing pH, tie-line length and adding salt cation in to the system, these parameters sometimes tend to influence the partitioning of total protein in the system in a similar direction, so the effect is not specific. The basis of incorporating a ligand, which is strongly located in the top phase (less polar polymer), has specificity to the binding sites of the enzyme resulting in pull up the enzyme to the same phase, was applied in this work. It was found that adding 0.5% butylrate in to the system the partition coefficient increased from 17 to 135 and the purification factor was from 10 to 23 (Fernandes *et al.*, 2001).

The alkaline xylanase produced by *Bacillus pumilus* was extracted by partitioning in ATPS and performed the best result in the system composed of 22% PEG 6000, 10% K_2HPO_4 and 12% NaCl. The enzyme preferably partitioned in PEG-rich phase. The purification factor was 33 and a 98% yield of enzyme activity (Bim and Franco, 2000). The partitioning of alkaline protease from *Bacillus* sp. in ATPS performed the most effective in the system consisted of 5% w/v PEG 6000 and 35% w/v K_2HPO_4 (Sinha *et al.*, 1996). The extraction of β-xylosidase from *Trichoderma koningii* fermentation culture was carried out in 25% w/v PEG 1500 and 20-25% w/v sodium dihydrogen phosphate system. The enzyme was recovered with high yield and high concentration in the bottom salt-rich phase (Pan *et al.*, 2001).

The partitioning behaviour of the enzyme between the two phases is uncertain phenomena due to the involvement of many factors in the interactions between the enzyme and phase-forming components such as hydrogen bond, charge interaction, van der Waals' force, hydrophobic interaction and steric effect (Albertsson, 1986; Hachem *et al.*, 1996). The partitioning is also related to its surface properties. The surface of a protein (Figure 1.4) is made of different types of amino acid which generally contains mostly polar amino acid residues and charged side group and probably contains significant hydrophobic regions

(Cantor and Schimmel 1980; Baskir *et al.,* 1989). The various side groups on the protein normally have different pH values, so when the pH of solution changes from acidic to basic values, the protein becomes less positive and more negative charge. Thus partitioning behaviour in a two phase system relate to the net protein charge which is function of the solution.

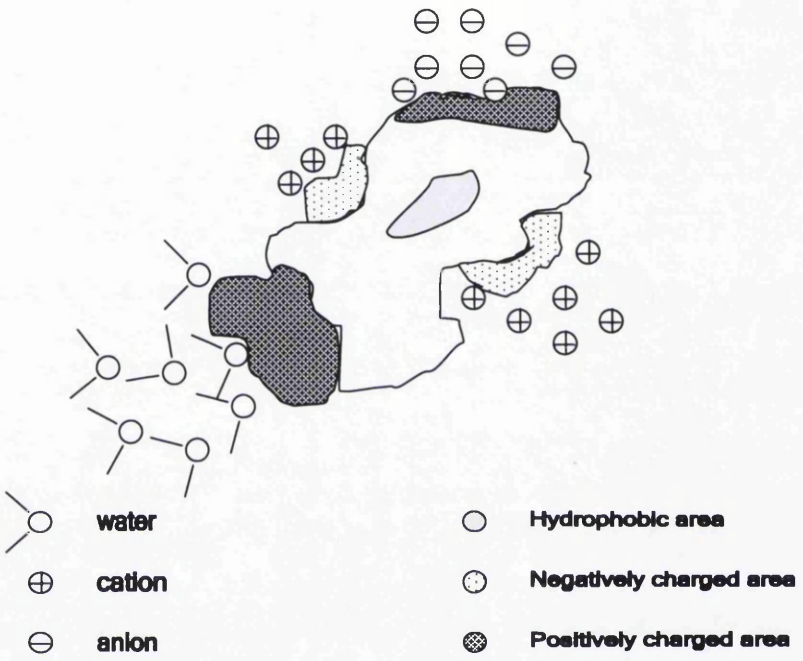


Figure 1.4 *The surface of protein*

In addition to the effect of interaction between enzyme phase forming and surface properties of the enzyme, the physicochemical properties of the two phases also influence partitioning behaviour.

1.5.3 Properties of phase system

The properties of the phase systems rely on many factors such as type and concentration of the polymer (and salt), molecular weight of polymer, viscosity, temperature, interfacial tension and ionic composition.

Normally, an increase in polymer concentrations relate to high density, refractive index and viscosity of the phase. Thus high concentration of polymer provides large difference in properties between the phases. In case of polymer-salt system, lower concentration of salt

is required for ATPS preparation when using the higher the concentration of polymer. The role of molecular weight also concerns with concentration used in forming phase. The higher the molecular weight of the polymer, the lower the concentration required for phase separation. The viscosity of the phase is affected by the molecular weight of polymer. Since the viscosity of a polymer solution mainly depends on the concentration. High viscosity might impact further process. The viscosity of one phase might be decreased by employing a higher molecular weight of the polymer.

The composition of the two phases in the system depends on the temperature. The effect of temperature is very different for different phase system relying on the type of polymer used. For example at high temperature, it is easily to form two phase with small concentration of PEG or salt whereas in case of PEG and dextran system, two phases will easily form at lower temperature (Walter and Johansson, 1994).

The interfacial tension between the two phases of polymers system is very small in comparison to the interfacial tension between an aqueous phase and an organic solvent phase. The interfacial tension is dependent on the polymer composition. The higher the polymer concentration, the larger is the interfacial tension. The ionic composition strongly affects the behaviour of phase system containing polyelectrolytes whereas in the phase diagram of non-ionic polymer-polymer system is little. In general, polymer concentration is required for phase separation to form when the salt concentration is increased (Walter and Johansson, 1994).

1.6 Membrane Technology

A Membrane is defined as a selective permeable barrier and is able to separate components due to differences in physical and chemical properties between membrane and the solutes. Then certain substances can permeate the membrane while other substances are retained. Various methods such as the applications of high pressure, the maintenance of a concentration gradient on the both sides of the membrane and electric potential are enable to transport substances across a membrane. Membrane technology has been an integral part of biotechnological processes such as:

1. Membrane separation process by microfiltration, ultrafiltration, reverse osmosis and nanofiltration;

2. Membrane bioreactor where enzymes, microbial, animal or plant tissue cultures are suspended and retained in a reaction vessel combined with membrane;

3. Membrane chromatography is employed as an option to conventional resin-based chromatography columns. Chromatographic membranes have been formed in a variety of configurations, as staked membranes, hollow fibers, spiral wound membranes and a variety of adsorptive mechanisms, such as ion-exchange, hydrophobic, reversed-phase, and affinity based procedures (Charcosset, 2006);

4. Membrane contactor is defined as the connection of two phases A and B through membrane pores. The membrane contactor involves using a pressure to force a dispersed phase A to permeate through a membrane into a continuous phase B, which flows tangentially to the membrane surface, for the preparation of emulsions and various types of particles, as w/o emulsions, o/w emulsions, and polymeric particles (Charcosset, 2006).

Membrane separation process has been used in industries and become more popular than conventional techniques. This separation process is consistently able to separate a wide variety of emulsion, surfactant, chelating chemistries and various mixtures. It works without the addition of chemicals, with a relatively low energy use, the basic concept is simple to understand, good arranged process conduction and complex instrumentation is not required. Membrane separation can be categorized by mean of pore size within the membrane, the driving force used in separation and transport mechanism (Table 1.2).

Process	Driving force	Separation size	Mechanism	Examples of material Separated
Microfiltration	Pressure gradient 0-1 bar	0.02- 10 μm	Sieving	Large colloids, microbial cells
Ultrafiltration	Pressure gradient 0-10 bar	0.001-0.02μ m	Sieving	Emulsions,colloids, macromolecules, proteins
Reverse osmosis	Pressure gradient 0-100 bar	< 5 nm	Solution-diffusion	Dissolved salts, small organics
Dialysis	Concentration gradient	< 5 nm	Sieving plus diffusivity differences	Treatment of renal failure
Electrodialysis	Electric field gradient	< 5 nm	Ion migration	Dissolved salts

Table 1.2 *Classification of membrane separation processes for liquid systems (Adapted from Coulson and Richardson, 1991).*

Membranes can be operated either through dead-end filtration or through cross-flow filtration (Figure 1.5). Dead-end filtration is the system when solution enters the membrane surface some solids and components will stay behind on the membrane while smaller particles flow through. This depends on the pore size of the membrane. The feed flows perpendicularly through the membrane (James *et al.*, 2003). Cross-flow filtration is the commonly system used with microfiltration and ultrafiltration (Bailey and Meagher, 2000). The solution is recycled in the system. During recirculation, the feed solution flow is parallel or tangential to the membrane surface and permeates pass through the membrane because of a pressure difference. Only small particles are permeable while the larger particles are left the membrane. In addition, the formation of the filter cake is lower than in dead-end mode.

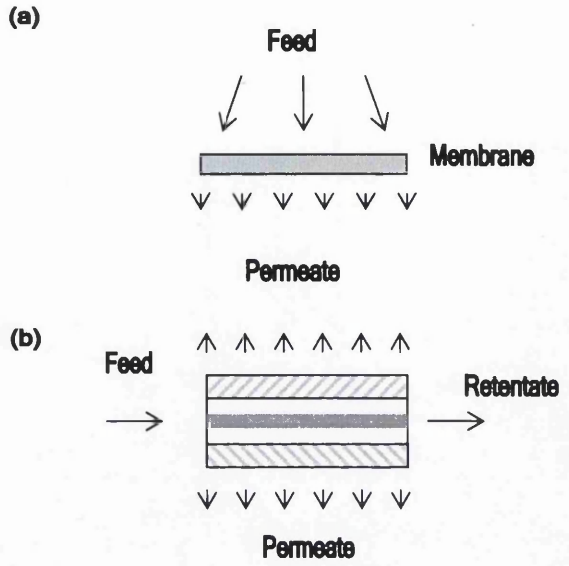


Figure 1.5 *Membrane operational modes: (a) dead-end and (b) cross-flow configurations.*

1.6.1 Protein purification by membrane filtration

1.6.1.1 Microfiltration membrane

Microfiltration (MF) membranes are widely used in pharmaceutical, chemical and semi-conductor manufacturing for separation of enzyme, cells, catalysts and clarifying of extraction fluids. These membranes can also be employed in water and waste water treatment for example removal of suspended particles from textile waste water, clarifying of cleaning oils used in machine construction and prefiltration for ultrafiltration, nanofiltration and reverse osmosis. Microfiltration is considered as a primary solid–liquid separation step. The typical uses in biological products include primary microorganisms removal from fermentation broths, recovery intracellular protein from cell debris and sterile filtration as the final step in protein production (Claudia Sousa *et al.,* 2002). Pore size of MF generally allows particles in the range of 10 and 0.02 μm to pass through. The pressure used is generally lower than that of ultrafiltration process.

The separation of whole cells and cell debris from solution with crossflow microfiltration mode is commonly found in industry rather than dead-end mode since the dead-end filtration is a batch process, so the process must be stopped periodically to remove the accumulated particles on the surface while in case of crossflow mode the deposit layer

does not build up indefinitely and its thickness can be controlled. Therefore, it is possible to remain relatively high fluxes over prolonged time periods (Keskinler *et al.,* 2002). The first step on purification protein produced by fermentation of microorganisms in intracellular or extracellular is the separation of the whole cells from the fermentation broth. In case of intracellular protein, after media separation, cells are needed to re-suspend in buffer or water in smaller volumes than the original volume of fermentation broth in order to ease handling during subsequent purification steps. The intracellular protein is excreted to suspension after the whole cells are disrupted. The separation of suspends and dissolved material in the cells by microfiltration based on their shape and molecular weights (Bailey and Meagher, 2000). Removal of all microbial cells is normally achieved by use of pore diameter 0.2-0.45 μm. The microbial cells will retain on or in the surface layer of the filter. In general, the operation of microfiltration system is performed at constant flux rather than at constant transmembrane pressure in order to enhance product yield (van Reis and Zydney, 2001).

1.6.1.2 Ultrafiltration membrane

Ultrafiltration (UF) is a pressure-modified process that uses semipermeable membranes to separate solution by molecular size, shape and charge without the application of heat or a change of phase. Ultrafiltration membranes have pore diameters range from 1 to 20 nm and are capable of retaining particles in the range of 300-500,000 daltons of molecular weight. In order to assure complete retention when selecting the size of membrane, the molecular weight cut-off (MWCO) of the membrane generally should be 1/3rd to 1/6th of the molecular weight of the retained molecule.

The size of pore is small enough to retain proteins of low molecular mass or some solutes from solvents while smaller molecules such as salts, solvents and water freely pass through the membrane. Removing solvents from solutions results solute concentration or enrichment. Ultrafiltration has been widely used for protein and colloid suspensions concentration rather than chromatography, electrophoresis and affinity purification because this method is easy to scale-up and can yield high throughput of product at low process cost (Ghosh and Cui, 2000). Moreover the method is very gentle, having little adverse effect on bioactivity of the protein molecules and proceesing times are shorter than another methods of concentration (Walsh, 2002). Ultrafiltration process typically performs

feed clarification, concentration of rejected solutes and fractionation of solutes but it is not effective at separating organic solution.

Belfort *et al.* (1994) pointed out the efficiency and productivity during ultrafiltration relied on the physico-chemical properties of the suspension such as pH or salinity. Howell *et al.* (1999) mentioned the factors that influence the fractionation of macromolecules by membrane are non-uniform membrane pore size, concentration polarisation, fouling and solute-solute interactions.

Diafiltration

Diafiltration is a technique that can be employed in combination with any of the other kinds of separation so as to effectively raise either product yield or purity. Buffer is used as diafiltrate and added into the recycle tank while permeate is removed from the membrane unit during diafiltration. When the target product is in the filtrate, diafiltration can be applied by increasing the removal of target product out of the feed component into a collection vessel. For instance, Zhou *et al.* (2006) studied the continuous diafiltration with the recovery of hyaluronic acid from *Streptococcus zooepidemicus* in microfiltration stage. The quantities of hyaluronic acid and protein were removed more from the feed when increasing diavolume. Bailey and Meagher (2000) separated soluble intracellular protein from the debris and inclusion bodies present in a recombinant *Escherichia coli* cell lysate with crossflow microfiltration and diafiltraton. More proteins were removed from cell debris to permeate up to 70-84% after diafiltration with 3 volumes of 150 mM NaCl. In the process where the target product is in the retentate, the diafiltration enhances the purity of product. The undesirable low molecular mass molecules such as salts, ethanol, buffer components and other solvents from a solution, are washed away from product. Cho *et al.* (2003) purified soluble pectin extracted from the mature citrus peel by using crossflow microfiltration with diafiltration. The flavonoids and polyphenols are impurities in pectin products. These impurities in pectin extracts were efficiently removed by the diafiltration. The content of impurities decreased about 34% at two volumes of diafiltration, and finally decreased about 57% at six volumes of diafiltration. Li *et al.* (2006) observed the separation of protease from yellowfin tuna spleen using ultrafiltration. The purity of protease was improved dramatically since the soluble contaminant protein concentration in feed bulk was removed from 11.3 to 0.6 mg/ml after increasing the number of diafiltration volume up to 6.2. The achieved purification factor was 18.

Generally, diafiltration is preceded by an ultrafiltration step in order to initially reduce process volumes and increase further the concentration of retained components. Diafiltration can be performed in terms of continuous mode, discontinuous mode by sequential dilution and discontinuous diafiltration by volume reduction. The technique of continuous mode or known as constant volume diafiltration involves washing out the original low molecular weight solutes in the retentate (feed) by adding volume of diafiltration solvent, having the low molecular mass molecules which are easily to be removed to the feed tank such as water and buffer, at the same rate as filtrate is being generated. Consequently, the total volume of retentate is constant throughout the process. In case of discontinuous mode by sequential dilution implicates first diluting the sample to a predetermined volume by adding water or a new buffer to the retentate the diluted sample is then concentrated back to reach its original volume by ultrafiltration whereas discontinuous mode by volume reduction performs opposite procedure. The sample is first concentrated to a predetermined volume, and then diluted back to reach its original volume with water or replacement buffer. The process is repeated until the unwanted small molecules are removed. Both discontinuous diafiltration by sequential dilution and by volume reduction are also known as batch diafiltration.

Lipnizki *et al.* (2002) compared two process modes of diafiltration, continuous and batch modes. Shorter retention time is required for continuous so it might be safer and decreasing the problem when dealing with non-sterile products. The energy consumption is also low. However, the flexibility of feed volume is not good as batch mode and the design of batch process is commonly more compact leading to smaller space requirement and lower investment costs.

Factors affecting rejection

Generally, the fractionation of molecules from membrane relies on the rejection characteristics of the membrane (Howell *et al.*, 1999). Solute rejection is defined as the ratio of concentration difference across the membrane to the bulk concentration on the permeate side:

$$R = (Ci - Cp)/Ci$$

when R is rejection (or called retention) coefficient, Ci is the solute concentration in inflow and Cp is the solute concentration in permeate

Many factors, such as pore size of membrane, solute-solute interactions, solute-membrane interaction, concentration polarization and fouling, can influence rejection phenomenon.

Pore size of membrane is important within the membrane structure. A membrane will be normally specified to size of the molecule to be separated. Consequently, pore size had better to be smaller. If the pore size is specified as the same size of the molecules, this will cause a rapid blocking of all the membrane pores. The pore size can be measured by optical microscope (good for size around 1 μm), electron microscope (1-2 nm) or atomic force microscope (for size as low as 5 A or 0.5 nm). Keskinler *et al.* (2004) investigated the effect of membrane pore sizes of 0.2 and 0.8 μm with yeast suspension which contained particles between 0.5–1 mm. After the filtration, the pore membrane behavior against yeast particles observed from Scanning Electron Micrographs (SEM) showed some of the yeast particles were seemed to foul or adsorb inside the pores 0.8 μm whereas yeast particles were located only on the surface of the 0.2 μm membrane causing decrease in permeate flux

Concentration polarization takes place on the surface of membrane. Solutes pass through the membrane surface by convective transport of the solvent. Some of them might pass through the membrane while rejected solute accumulates at the membrane surface and exceeds the bulk concentration. Thus a concentration gradient is built up and fairly viscous and gelatinous layers may form (Dina Afonso *et al.,* 2004). This polarized layer alters the resistance of the membrane causing the reduction of permeate flux and solute diffuses away from the membrane surface (James *et al.,* 2003). At the steady state the rate of back diffusion will equal to the rate of removal of solute flux towards the membrane minus the rate of solute leakage through the membrane. The increase in concentration at the membrane surface provides several effects such as it raises the local osmotic pressure, then pumping power must be increased to compensate for the higher effective osmotic pressure and the concentration polarization also increases the solute concentration in the product water.

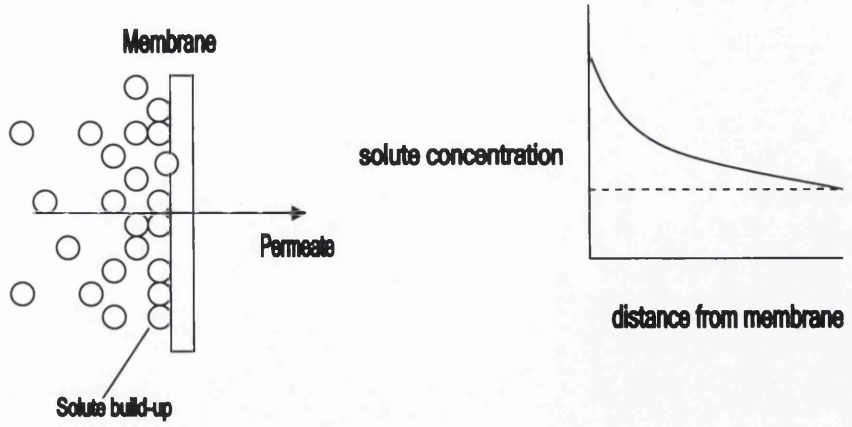


Figure 1.6 *Concentration polarization at a membrane surface (Coulson and Richardson, 1991).*

The effect of solute-solute interactions are negligible at very dilute solutions while in concentrated solutions, it has been found that steric and other repulsive interactions among solutes increase the partition coefficient. For example two solutes, which have different surface charges, probably react each other, then dropping permeate flux. The irregular shape particles, such as carbon enabled the particles to bridge far apart, provide the formation of deposit on the membrane surface easily (Connell *et al.,* 1999). In case of solute-membrane interaction, this effect depends on physicochemical parameters. The flexibility of solute and its shape including surface charge of membrane influence the electrostatic and hydrophobic interaction between solute and membrane. In biological suspensions, different cell sizes and shapes directly affect the specific cake resistance and permeate flux. The effect of cell shape on separation behavior is subtler than that of cell size (Foley *et al.,* 2005). Tanaka *et al.* (1994) said non-spherical cells such as rod-like bacteria have been shown to align parallel to the membrane, especially at high crossflow velocities. This phenomenon results in high compact and resistant filter cakes, leading to decrease in permeate fluxes.

The effect of fouling is the main drawback in membrane operating for example the encountered problems of crossflow microfiltration for recovering soluble proteins from fermentation media and from cell homogenate suspensions which provide low permeate flux rate and poor solute transmission after long operation leading to fouling (Levesley and Hoare, 1999). Fouling phenomena will cause the need of a higher workload so as to keep the filtration capacity at a certain level. The type and amounts of fouling depend on several

factors for example feed water quality, membrane type, membrane material and process design and control.

The membrane fouling mechanism can be categorized into three modes (Figure 1.6) (Belfort *et al.,* 1994): first case takes place when the solute sizes are greater than the pore size of the membrane leading to solute deposition and gel formation on the membrane surface during the dynamic and convective flow condition. Second case is from the solute sizes are much smaller than the pore size of the membrane. In this case, the permeate flux decline mechanism is found to behave according to an adsorption phenomenon rather than gel formation on the membrane surface. The final case results from solute molecules have an approximately equal size to the membrane pore size causing internal blockage or partial blockage of the pore. Then the effective pore size distribution in membrane is decreased and finally causes membrane fouling (Field *et al.*, 1995).

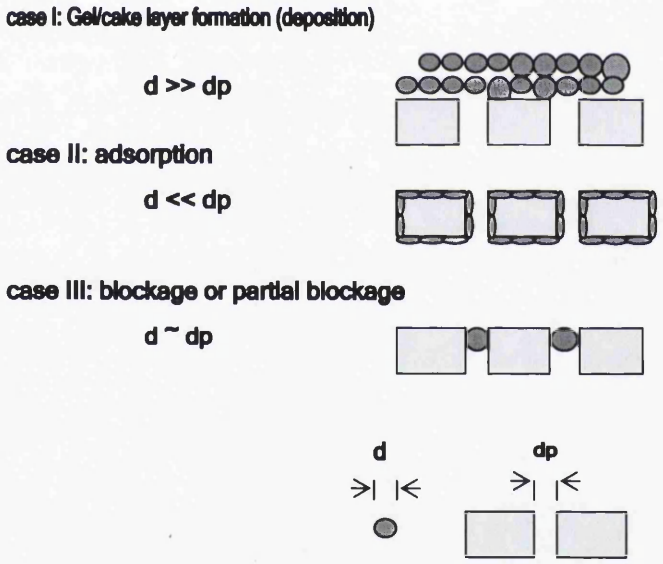


Figure 1.7 *Three cases of membrane fouling (Belfort et al., 1994).*

Howell (1992) indicated the approaches for decreasing the effect of concentration polarisation and fouling phenomena are hydrodynamic by improving mass transfer across the membrane surface, surface modification by changing the surface. Selection suitable hydrodynamic operating parameters such as cross-flow velocity, geometry of the membrane module and adjusting the interactions between solute and membrane as well as between solute and solute are importance (Howell *et al.*, 1999).The operation system at

low and uniform transmembrane pressure, high tangential flow rate or higher operation temperature can also reduce fouling (Belfort *et al.*, 1994).

In addition to selecting suitable hydrodynamic system to avoid fouling, the development of surface modification can improve flux or transmission. Several chemical coating surface membrane have been studied. For example using a radiation-induced grafting of hydrophilic alcoholic hydroxyl groups onto polyethylene membrane reduce non-selective adsorption of proteins (Kim *et al.*, 1994), PVDF membranes with a surface grafting of phosphorylcholine provide less severe flux decline and a 95% reduction in protein adsorption (Dudley *et al.*, 1993).

Effect of operating parameters

It is important to select a proper condition for membrane operating. The performance of crossflow filtration influences from many parameters such as crossflow velocity, transmembrane pressure, temperature and feed characteristics

An increase in crossflow velocity can produce high shear rate and reduce the thickness of the polarized layer on membrane surface as turbulence increases near the surface (Lobo *et al.*, 2006; Song *et al.*, 2006), consequently decreasing the fouled membrane resistance. So a high crossflow velocity will be beneficial to maintain a high permeation flux at steady stage in membrane separation process. However, at excessive shear rate (high crossflow velocity) the biomolecules may easily be denatured and ruptured. Zhou *et al.* (2006) observed the quantities of hyaluronic acid in permeate declined remarkably when crossflow velocity was above 1.25 m/s.

Transmembrane pressure (TMP) is defined as the pressure gradient of the membrane or the average feed pressure minus the permeate pressure. Permeate flux normally increases with an increase in transmembrane pressure, the formation of polarized layer and the resistance to permeation increase as well. However, the flux also depends on the shape of solute. Connell *et al.* (1999) found increasing the pressure had minimum effect on polarized layer compaction and provided high permeate flux of carbon, silt and rough silica whereas the smooth particle surface, glass and aluminium had high deposit on membrane surface at high pressure causing a decrease in permeate flux and finally fouling.

The physical properties of solute might be changed with temperature. For example at high temperature, the viscosity of solute decreases. So the solutes easily pass through the membrane as a result increasing permeate flux. Raising each degree of temperature influences an increase in permeate flux 3-4%. Although high temperature assists the flux, the biomolecules such as protein may risk the denaturation (Dina Afonso *et al.,* 2004).

The concentration, pH value and the ionic strength of feed can affect membrane performance. At steady state, permeate flux decreases with increasing feed concentration. High solute molecules from concentrated feed cause concentration polarization on membrane surface. The pH and the ionic strength strongly influence the charge of molecule, particularly in protein. The proteins are uncharged and have low solubility at their isoelectric point (pI). Muller *et al.* (2003) studied the effect of pH and the ionic strength on Lysozyme (pI =11), the protein performed positively charged at all lower pH values. Thus, it formed a positively charged, layer on top of the membrane resulting in lower transmission due to the electrostatic repulsion effect. When the pH increased, the positive charge on the lysozyme decreased, resulting in a lower repulsion effect through the layer, hence higher transmission and flux. At pH 11 (pI), the lysozyme tended to be the most compact, which results in a higher packing density of the protein layer. This dense layer leads to a decrease of transmission and a slight flux decrease. The ionic strength from addition of NaCl at low concentrations provided low shielding of the positive charge of Lysozym. The interactions between the positively charged protein and negatively charged membrane led to lower fluxes and transmissions. The proteins were much more adsorbed on the membrane surface, resulting in an increase of fouling whereas at higher salt concentrations the shielding effect was increased so that the adsorption effect was weakened, consequently achieving higher fluxes and transmissions.

1.6.2 Membrane bioreactors

Membrane bioreactor (MBR) can be defined as a system integrating biological reactor with a semipermeable membrane (Thomas and Murrer, 2001). The system provides two functions which are bioconversion process and membrane separation. The basic principle of membrane bioreactor is taking the advantage of the size difference between the biocatalyst, substrates and their products. The permeable substrates and products can be removed from the reaction mixture by a specific driving force across the membrane (Cheryan and Mehia, 1986, Lopez *et al.,* 2002). In suitable membrane, the biocatalyst is

entrapped on one side when the substrate transported across the membrane, it will contact with the biocatalyst resulting product. The product molecule must be small enough to permeate the membrane while the biocatalyst and unhydrolyzed or partially hydrolyzed substrates are recycled for further reaction.

Membrane bioreactor is recently an attractive technology and broadly found in various fields while the use of traditional batch reactor in present has been decreasing. The undesirable aspects of the traditional reactor are low efficiency operating, high labour costs, variation of batch to batch, high cost of separation product at the end of process and sometimes producing hazardous and environmental impacts. The biocatalyst used in membrane bioreactor can be retained in system and reused many times. This advantage provides high productivity and improvement in yield. The system does not require additives and is able to function at moderate temperature and pressure (Giorno and Drioli, 2000), then it is easy to control the process, separate product and produces low waste from the process. Moreover, there is no effect from inhibitory end products in the reaction because during conversion process these can be continuously removed.

Kargupta *et al.* (1998) mentioned the capability of *Sacchromyces cerevisiae* in batch ethanol fermentation was completely inhibited when the ethanol concentration in the fermentation broth was over 110 g/l and the inhibition was negligible if the concentration was below 30 g/l whereas using continuous membrane bioreactor assisted an increase in productivity due to continuous removal of ethanol from the process. In addition the overall process cost was effective as the cells were recycled in the system. The hydrolysis of macromolecules, such as starch and cellulose, using enzyme in typical batch reactor were affected by products (e.g. glucose) inhibition as well. Therefore this problem can be avoided by using membrane bioreactor, so the small inhibitory product molecules were able to easily pass through the membrane and be removed continuously from the system, while the large molecules such as enzyme and substrate were retained by the membrane (Belafi-Bako *et al.*, 2007). Hicke *et al.* (1999) studied the synthesis of a polysaccharide from sucrose using immobilized enzyme with porous membrane instead of batch enzyme in order to reduce product inhibition effect and the difficulty on separation between enzyme and product due to their similarity of high molecular weight.

However, the difficulty of sterilization and maintaining aseptic or even sanitary condition in membrane modules are limited. It is found that most of polymeric membrane module

can not be repeatedly sterilized by steam. Some manufacturers use ultraviolet radiation to pre-sterilize it. Chemical sterilization has also been applied but it is not popular because the removal of the chemical sterilant needs lot of sterile water and if it is not properly washed, this might inhibit cells in a microbial process (Cheryan and Mehaia, 1986).

Fouling in membrane bioreactor during filtration is another problem. It occurs from suspended solid, colloid or solute block pore of membrane resulting in low throughput and finally membrane clogging. It is then necessary to control the concentration polarization. Some substrates need to do a pre-treatment before brought to the system and controlling the operating parameters such as temperature, transmembrane pressure and recirculating rate should reduce fouling. Bouhabila *et al.* (2001) said using air bubbles in submerged membrane bioreactor can decrease the deposit forming on the membrane surface. Mercier-Bonin *et al.* (2001) suggested some modern equipment based on fluid instability for example turbulence promoters, generation of Taylor vortices by rotating cylindrical membrane or generation of Dean vortices by flowing in a curved channel can reduce fouling.

The biocatalysts such as enzymes and microbial cells can be used with membrane in form of suspension and immobilization within membrane (Figure 1.8). The first form, biocatalysts are freely suspended in solution in a reaction vessel (i.e. stirred tank reactor) which combines with membrane unit. The membrane only serves as a separation unit which retains biocatalysts and large size of molecules in the system. The other form happens when biocatalysts are immobilized within the membrane, so the membrane acts as a support for the biocatalysts and as a separation unit simultaneously. The biocatalysts can be entrapped inside the porous layer of membrane, bounded on the surface or gelification which occurs when the rejected biocatalyst molecules accumulate on the membrane surface and form a gel layer. The biocatalysts can be bound by ionic binding, covalent binding and adsorption through van der Waals interaction (Giorno and Drioli, 2000). The reaction with immobilized biocatalysts will take place when the substrates pass the membrane to the biocatalysts, then the products can be collected from the other site of reaction.

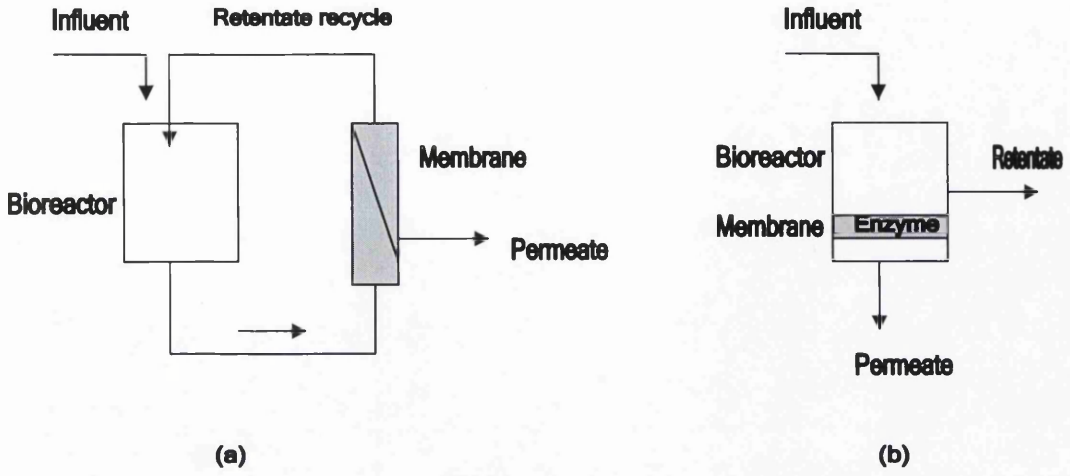


Figure 1.8 *Types of membrane bioreactors: (a) a bioreactor combined with a membrane operation unit with retentate recycled back to bioreactor, (b) a bioreactor integrated with the membrane active as a catalytic and separation unit (adapted from Giorno and Drioli, 2000).*

Although the immobilized biocatalysts provides many advantages including an increase in reactor stability and productivity, improving product purity and quality, reduction in waste (Giorno and Drioli, 2000), less expensive and increase in biocatalyst stability and resistance towards organic solvent (Rios *et al.,* 2004), this technique has to be carefully considered if the initial denaturation step during binding process between biocatalysts and materials of membrane used probably influence on the stability and activity of biocatalysts (Tsai and Shaw, 1998). Bodalo *et al.* (2004) mentioned about some advantages of continuous operation with suspended enzyme over immobilized form. For example the enzyme can be homogeneously distributed in the system without transport limitation, nearly all the native activity can be employed, reduce the cost of carriers for immobilization and time consuming.

1.6.3 Application of membrane reactor

The outstanding advantages have encouraged the increasing trend of applying membrane reactor. Many studies have shown the preparation of biocatalyst with membrane reactor such as in the field of food processing, pharmaceutical, biomedical and wastewater treatment.

The attractive membrane reactor has been considerably used for food processing such as starch hydrolysis, fruit juice clarification, oil and fat hydrolysis and alcohol fermentation. In starch hydrolysis, several oligosaccharides can be produced. The production of maltose syrup after starch hydrolysis can be used in food industry for example bakery, confectionery and brewery and also used in pharmaceutical industry for the production of antibiotic, vaccines etc. due to its attractive properties such as low hygroscopic, low sweetness, low viscosity, non-crystallization, reduced browning capacity and good stability at high temperature. Gaouar *et al.* (1998) studied the bioconversion of cassava starch to maltose syrup. They used the enzyme maltogenase and promozyme to saccharify and debranch starch with different molecular weight cut-off of ultrafiltration at 10, 50 and 150 kDa. The results in different permeate flux and effect of enzyme concentrations were observed as well. Grzeskowiak-Przywecka and Slominska (2005) examined potato starch hydrolysis by a fungal α-amylase in a membrane reactor with ultrafiltration. During the process, the products were continuously separated with simultaneous recycling of unhydrolyzed substrate and enzyme between membrane and reactor vessel. The volume in reactor was kept constant by compensating the permeate flux with continuous feeding substrate. This process provided less effect on product inhibition, high efficiency and low operation costs. Furthermore, they found maltose content from ceramic membrane with tubular mode was about 63% while the content from polymeric membrane with hollow-fibre mode was less than 40%. Maltose content in permeate also depended on enzyme concentration used, type of membrane and its molecular weight cut-off (Grzeskowiak-Przywecka and Slominska, 2007). In addition to maltose, cyclodextrins can be obtained from starch hydrolysis. Slominska *et al.* (2002) used cyclodextrin glucosyltransferase with ultrafiltration membrane reactor to produce cyclodextrins. The product concentration relied on substrate concentration while enzyme concentration showed no effect. The results also indicated ultrafiltration reactor had higher efficiency than batch reactor.

In fruit juice manufacturing, the problem of juice turbidity is mainly from polysaccharides (pectins, cellulose, hemicellulose and starch), proteins and tannins, metals and micro-organisms (Alvarez *et al.,* 1998). It is importance to improve liquefaction and clarification. The application of membrane separation with different modules such as tubular (Alvarez *et al.,* 1998; de Barros, 2003), plate-and-frame (Sheu *et al.,* 1987) and hollow fiber (Jiraratananon and Chanachai, 1996; de Barros, 2003), to clarification has been studied. In addition to assist clarification, fruit juice is pasteurized simultaneously by the membrane

and it can be operated at low temperature, so the flavor of juice is not changed as a result of heat processing. However, the factor affecting membrane operation is mainly due to fouling from polysaccharides such as pectin, cellulose and hemicelluloses particularly when the pectin interacts with sugar causes high turbidity and viscosity. To enhance filtration performance, fruit juices are usually treated with pectolytic enzymes before filtration. Belafi-Bako *et al.* (2007) investigated pectin hydrolysis by polygalacturonase enzyme from *Aspergillus niger* with flat-sheet membrane reactor and compared the result with hydrolysis in shaking flask. They found employing continuous membrane reactor reduced the effect of product inhibition and showed more than 40% higher productivity than in the shaking flask.

Hilal *et al.* (2006) studied three behaviours of lipase-immobilized membranes, which made of regenerated cellulose and polyethersulphone, for synthesis of butyloleate through esterification of oleic acid with *n*-butanol in isooctane. These three forms were (i) lipase adsorption on membranes, (ii) loading of membranes with enzyme by filtration of lipase solution through active or support membrane layers and (iii) covalent attachment of lipase to activated membrane. The lipase inclusion in the wide porous support membrane layer gave highly active membranes able to convert 70–72% of oleic acid in 8 h of reaction time whereas at the same conditions, the immobilized membranes with adsorbed or covalently bounded lipase performed the substrate were able to convert 22–28% and 18–21%, respectively. The low efficiency of covalently bounded lipase was probably due to conformational changes in tertiary structure of lipase during the covalent attachment to the membrane, which results in a partial loss of the lipase activity

The membrane bioreactor has also been utilized in pharmaceutical field. Bryjak *et al.* (1996) designed membrane bioreactor for the continuous hydrolysis of penicillin G with penicillin acylase enzyme to produce 6-aminopenicillanic acid and phenylacetic acid. The penicillin acylase during operating in membrane reactor was more stable with the addition of polyethyleimine and seemed to be effective for industry applications than native enzyme form. Wenten and Widiasa (2002) investigated hydrolysis of penicillin G by hollow fiber membrane bioreactor where penicillin acylase entrapped within membrane pores and found low flux rate was an important factor to avoid gel formation or enzyme retaining in the system and to maximize the degree of conversion. In general, the problems in the production of amino acid, arylpropionic acids, amines and carboxylic acid occur

with the use of expensive cofactors, the low solubility of the substrates in water and the difficult separation of the products from complex solutions. Thereby the membrane reactors have been introduced in field of pharmaceutical to solve these problems. For example synthetic of tyrosine from phenol, pyruvate and ammonia by entrapment in cellulose triacetate membrane, the production of peptides for medical use by hydrolysis of whey protein in polysulfone ultrafiltration membrane, the entrapment of *Curvularia lunata* and *Candida simplex* in polyacrylamide gel in membrane reactor for conversion of cortexolone to hydrocortisone in order to produce steroids (Giorno and Drioli, 2000).

The recent research on the development of artificial organs in membrane reactor is significant attention. For example Catapano *et al.* (1990) studied an alternative approach on membrane bioartificial pancreas for whole organ transplantation in patients with insulin-dependent diabetes. Langer and Vacanti (1993) found the segregating isolated islets which produces insulin and regulate the patient blood-glucose levels can be segregated in the shell of hollow fibre membrane while the blood flows along the other side of membrane. Another example is hepatocytes which is function as a bioartificial liver for the temporary treatment of patients with acute liver failure can be segregated in a hollow fibre membrane (Nyberg *et al.*, 1993). The membrane acts as a support device for cell adhesion and as an immunological barrier between patient's blood and the hepatocytes.

The membrane reactor has also been investigated for treatment of wastewater. Barrios-Martinez *et al.* (2006) employed a membrane bioreactor using activated sludge for treatment of a synthetic effluent containing a large amount of phenol. The system performed a good result on phenol degradation and no phenol was detected in permeate. The treatment of pharmaceutically active compounds (clofibric acid, diclofenac, ibuprofen, ketoprofen, mefenamic acid, naproxen and dichloprop) was studied by combining a bioreactor with hollow fibre microfiltration and compared the result from conventional activated sludge process. The membrane bioreactor system exhibited much better removal for some compounds and the efficient removal depended on their molecular structure (Kimura *et al.*, 2005). Scholz and Fuchs (2000) reported treatment of oil contaminated wastewater in membrane bioreactor. The activated sludge was retained in bioreactor which was connected to a cross flow ultrafiltration. About 99.99% of fuel oil and as well as lubricating oil was successfully removed at retention time of 13.3 h.

1.7 β-galactosidase processing

The application of β-galactosidase to the hydrolysis of lactose in dairy process has been greatly attended. Many technologies concerning enzymatic hydrolysis have been studied. For example Becerra *et al.* (2001) introduced the permeabilized immobilized *Kluyveromyces lactis* cells for milk-whey lactose hydrolysis in packed bed bioreactor. Firstly, the whole cells were encapsulated in calcium alginate beads and then permeabilized with 70% ethanol for 10 min and washed with sterile distilled water. Finally, 1.2 g wet weight of particles were packed into the tubular-vertical bioreactor (0.5 cm internal diameter x 7 cm height). About 99.5% of milk whey lactose was hydrolyzed after 30 h at 30 °C.

Zhou *et al.* (2003) investigated lactose hydrolysis with the immobilized β-galactosidase of *Kluyveromyces lactis* (Maxilact LX 5000) on cotton fabric using glutaraldehyde as a cross-linking agent. The cotton cloth was uniformly packed in a cylindrical reactor so that the substrate solution was passed continuously along the axis of the reactor in a plug flow pattern at a flow rate of 2.8 ml/min at 37 °C and the range of lactose concentration used was 3-12.5% (w/v). The enzyme activity used in the process was about 600-1,200 U/ml. The lactose conversions from the process were up to 90%.

Serio *et al.* (2003) immobilized the Maxilact 5000, which is a highly purified liquid preparation of β-galactosidase derived from *Kluyveromyces marxianus lactis*, on silica sphere of 0.25-0.50 mm. The lactose hydrolysis was carried out in a continuous packed bed reactor and gave about 80% on conversion when lactose concentration was prepared at $2.8x10^2$ mol/dm^3 and operating condition was at 37 °C, flow rate 4 cm^2/min.

Although the β-galactosidase immobilization technique has been widely applied with lactose hydrolysis, the problem on high cost of immobilization, hygienic problem from fat and protein content in milk (Novalin *et al.*, 2005) and loss of enzyme activity during immobilized step need to be considered before selecting this technique.

Apart from immobilization technique, the suspended free enzyme has also been investigated. Panesar *et al.* (2007) used the ethanol-permeabilized yeast cells (*Kluyveromyces marxianus* NCIM 3465) for the lactose hydrolysis in 10% (w/v) skimmed milk at shake flask level. The maximum lactose hydrolysis after 3 h of incubation period

with 120 mg yeast biomass was about 89% under shaking condition 80 rpm at 30 °C. Although the use of permeabilization technology can overcome the problem of enzyme extraction, cost of purification and poor permeability of the cell membrane to lactose, the suitable ethanol concentration on cell permeabilization has to be considered since at high concentration, the enzyme activity decreases, which might be attributed to the leakage of the enzyme from the cells or cell lysis while at low concentration, the enzyme activity may be lower due to an insufficient amount of the agent for effective permeabilization, then non-permeabilized cells were able to ferment products (glucose and galactose) to ethanol.

Novalin *et al.* (2005) studied lactose hydrolysis in skimmed milk using a process called membrane-diffusion reactor. The UV irradiation module and a sterile filtration unit were combined to the system to control the microbiological growth during operation. This technical process directly hydrolyzed lactose in skimmed milk without any ultrafiltration step before enzymatic conversion. The enzyme activity could be used longer in comparison to a batch process and the enzyme would not become product components after the process. The lactose in skimmed milk at concentration 141.5 mM was pumped through the hollow fiber module which contained 120 U/ml Maxilact from *Kluyvermyces lactis*. The conversion rate was achieved at 78.11% at flow rate 165 ml/min and temperature 23±2 °C.

1.8 Aims of the project

The enzyme β-galactosidase can be extracted from yeasts, molds and bacterial cultures. The cost of enzyme production and extensive purification in enzyme preparation for lactose hydrolysis process can be reduced when the extracted enzyme is from dairy bacteria culture especially using feed material derived from the dairy and applying a similar technology of starter culture for β-galactosidase production. As a result, *Lactobacillus delbrueckii* ssp *bulgaricus* which produces relatively high levels of intracellular β-galactosidase in comparison to lactic acid bacteria was chosen as the source of enzyme β-galactosidase for this project.

The aims of this work are to produce and separate β-galactosidase from *L. delbrueckii* by suitable method, to characterize the properties of β-galactosidase, to investigate of β-galactosidase performance and lactose transformation in bioreactor and membrane bioreactor for large-scale production and to determine the process viability by numerical

analysis simulation of the process. The following objectives were considered in order to achieve these aims;

1. The growth of *L. delbrueckii* on suitable medium and pH condition were determined to achieve high cell density. The growth medium with different concentrations of nutrient compositions was investigated in 30-ml pressure tube without pH control. The suitable nutrient composition was chosen on the basis of providing high optical density and specific growth rate. Then the ranges of pH growth were tested in 5-l batch fermenter. The appropriate pH growth with suitable medium was employed for cultivating the bacteria in pilot scale. The cells were harvested during stationary phase and the medium was removed by washing the cell with buffer in microfiltration system. These results are described in Chapter 3.

2. The extraction intracellular enzyme from cells by treatment in a high pressure seems to be an appropriate method for large scale disruption. The relation between operating pressures and enzyme release was studied. Then the separation of β-galactosidase from disrupted cells using two different methods, aqueous two phase system and microfiltration with dialfiltration and concentrated the enzyme by ultrafiltration were determined. The purification factor was employed as a key term to indicate the capable removal of the enzyme and protein from cell debris.

The properties of β-galactosidase from crude extract and partially purified enzyme from membrane system were characterised at different pH and temperatures including their stability which represented in terms of half life. Different substrate concentrations, for both ONPG and lactose solution were used to analyze the enzyme kinetic. The effect of products, glucose and galactose, and the effect of cation ions were determined on the enzyme activity. These results are described in Chapter 4.

3. The first performance of the enzyme on hydrolysis reaction was conducted with lactose in synthetic solution before applying with lactose as skimmed milk. The investigation of crude β-galactosidase on lactose hydrolysis in small scale bioreactor and small stirred cell membrane reactor with different lactose and crude enzyme concentrations was determined. The characteristics and the stability of the enzyme in membrane bioreactor were tested. The effect of lactose concentrations, enzyme concentrations and flow rates during operating the system on glucose production were determined. The mathematical modelling

for describing the aspect of reaction occurring in the system during lactose hydrolysis in both bioreactor and membrane bioreactor was analyzed with the experimental data. These results are described in Chapter 5.

4. The preparation of lactose solution from skimmed milk was carried out by filtering skimmed milk solution with microfiltration and ultrafiltration membranes. The investigation on this solution hydrolysis with crude enzyme and partially purified enzyme in bioreactor was determined in conditions surrounding with different lactose concentrations, enzyme concentrations and temperatures. The hydrolysis reaction in membrane bioreactor was also evaluated. The results of bioconversion from both bioreactor and membrane bioreactor were compared with the results from lactose synthetic solution. These results are described in Chapter 6.

Chapter 2

Materials and Methods

2.1 Materials

The microbial strain of *Lactobacillus delbrueckii* ssp *bulgaricus* NCTC 11778 used for the source of β-galactosidase in this study was purchased from NCIMB LTD (23 St. Machar Drive, Aberdeen, U.K.).

All the chemical compounds were analytical grade and obtained from four companies; Oxoid Limited (Wade Road, Basingstoke, Hampshire, RG24 8PW, UK), BDH Chemicals Ltd.UK, Sigma-Aldrich Chemicals (The Old Brickyard, New Road, Gillingham, Dorset, SP9 4XT, UK) and Fisher Scientifics (Bishop Meadow Road, Loughborough, Leichestershire, LE11 5RG, UK). Except soy-peptone was purchased from IDG (U.K) Ltd (Torey House, 52 Wash Lane, Bury, England, BL9 6AU).

2.2 Preparation of *L. delbrueckii* ssp *bulgaricus*

2.2.1 Cells recovery and preservation

Freezing cells, *Lactobacillus delbrueckii* ssp *bulgaricus* NCTC 11778, were revived into 50 ml serum vials containing fresh medium. The components of medium were 10 g/l bacteriological peptone, 10 g/l soy peptone, 10 g/l yeast extract, 20 g/l glucose, 5 g/l KH_2PO_4, 5g/l sodium acetate, 2 g/l triammonium citrate, 1 ml/l tween80, 0.2 g/l magnesium sulphate, 0.05 g/l manganous sulphate and distilled water. To avoid caramelization reaction, glucose solution was prepared separately under aseptic conditions and introduced to medium after autoclave. The pH medium was adjusted at 6.2±0.1. The

stock culture was grown under anaerobic condition for 12 to 18 hours at 37 °C.

Stock cultures were preserved by mixing culture with an equal volume (50% v/v) of sterile 10% glycerol solution, used as a cryoprotective agent, under aseptic conditions. Then they were preserved into deep freezing (-70 °C) (Heraus Ultra-freezer, UK).

2.2.2 Preparation of a working culture

The stock cultures were transferred at least twice to fresh medium using sterilized disposable syringe and incubated anaerobically at 37± 2 °C for 12 to 18 hours before using as working culture. The working culture was stored at 25 °C and was employed as the starting culture for all experiments up to 14 days. Every two weeks, the culture was regularly re-inoculated into 50 ml serum vials containing fresh medium.

2.3 Preliminary development of growth medium

The composition of the media used in preliminary experiment were soy peptone, yeast extract, lactose, KH_2PO_4, sodium acetate, triammonium citrate, tween80, magnesium sulphate, manganous sulphate. Each ingredient, except magnesium sulphate and manganous sulphate, was tested at various concentrations in medium used. The experiments were designed to change one ingredient in various concentrations at a time while all other ingredients were kept fixed at a specific set of conditions.

All medium components used were dissolved in distilled water and pH adjusted to 6.5 using 1M NaOH. The indicator, Resazurin Dye ($C_{12}H_6NO_4Na$), was added into the medium. It functioned as an anaerobiosis indicator (negative redox potential) changing its color from red to colorless. The medium used was heated until it boiled to eliminate dissolved oxygen. Then 9 ml of medium tested was dispensed into 30-ml pressure tube and

flushed the air out with nitrogen gas for a few minutes. All pressure tubes were sealed with butyl rubber stoppers and aluminium crimp caps, then sterilized in the steam autoclave (Priorclave: Tactrol 2, RSC/E, UK) at 121 °C for 15 min.

The single ingredients to be tested were prepared and autoclaved separately from fixed medium. Different amounts of the single ingredient tested were aseptically introduced into the medium after autoclaving via a sterilized disposable syringe. The pressure tubes were then mixed gently into a vortex. The culture with 10% inoculum size was added and then incubated at 37 °C. Each differential concentration was conducted in triplicated. During the cultivation, the pH was not controlled. The growth of cell was monitored every hour using a spectrophotometer (PU 8625 UV/VIS Philips, France) at the optical density 660 nm.

2.4 Optimum pH on growth condition

The growth of *L. delbrueckii* ssp *bulgaricus* cultured in various pH values was studied in a 5.0 l stirred tank reactor. The pH values studied in the system were at 4.5, 5.0, 5.5, 6.0, 6.5, 7.0 and 7.5. The cells growth was monitored by the measurement of the optical density at 660 nm using a spectrophotometer (Unicam UV/VIS 300) and also indirectly interpreted from the amount of 4M NaOH added to maintain at its pH set point.

2.4.1 Medium preparation in STR

The optimum medium was made up for 3.6 l and adjusted the pH for starting point with 5 M NaOH. The medium consisted of 10 g/l soy peptone, 10 g/l yeast extract, 20 g/l lactose, 2.5 g/l KH_2PO_4, 5g/l sodium acetate, 2 g/l triammonium citrate, 1 ml/l tween80, 0.2 g/l magnesium sulphate and 0.05 g/l manganous sulphate. The medium was then transferred to reactor with a magnetic stir. The head plate was placed on the top of reactor and sealed properly. The calibrated pH probe was fitted on the top and all the ports were sealed with

metallic clips and alumina foil, except the vent line was loosely covered with cotton wood before sterilization.

After sterilization for 15 min, the reactor was removed from autoclave and directly connected to nitrogen gas cylinder. The gas flowed into the medium at low rate. When the medium cooled down, the reactor was joined with water bath and placed on a magnetic stirrer (SM1, Stuart Scientific, UK). The stirring velocity was set at 150-200 rpm. The reason of stirring was to prevent the settling down of the growing cells and disperse heat and gas completely into the medium. The pH probe was joined to the pH controller.

2.4.2 Inoculum preparation for STR

The inoculum size used was 10% v/v and condition growth was at 37 °C. The culture was scaled up after reaching late exponential growth phase. The culture (4 ml) from pressure tube was inoculated to 40 ml growth medium in a 50 ml serum vial and finally up to 400 ml in a 1 l culture bottle using sterilized disposable syringes. The culture bottle has two tubes port, first tube was long down to the bottom of bottle and the other was at headspace and equipped with the air filter. The inoculum from culture bottle was transferred to 5 l fermenter by connecting nitrogen gas to the tube equipped with the air filter of culture bottle. The inoculum was then pressurized and pushed through the first tube into the inoculating port of 5 l fermenter.

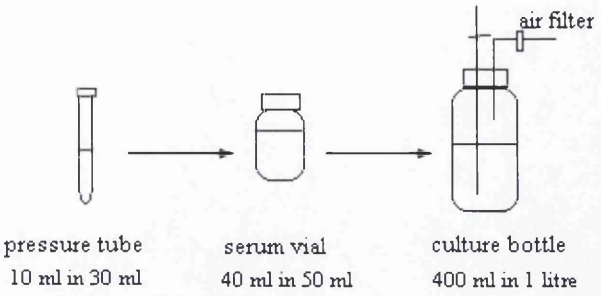


Figure 2.1 *Scale up culture from pressure tube to serum vial and culture bottle before used in 5 l fermenter.*

2.4.3 Stirred tank reactor (STR)

A 5-l glass culture vessel was chosen in this investigation (Figure 2.2). The fermenter head plate was connected with ports for sampling and inoculating addition line, nitrogen gas addition line, alkaline feeding line for pH control, pH probe (Fischer Scientific Ltd., UK), temperature measuring, heat exchanger coil and gases exit. All connecting lines were made of silicone except nitrogen gas line was from butyl in order to protect the exposure to the outside oxygen.

The nitrogen gas line was equipped with a filter (Polyvent filter, 0.2μm, Whatman Filters, UK). The filter was connected with nitrogen gas cylinder via butyl tube and the gas flow was controlled by pressure value and feed in rate of 0.167 vvm/l.

Before sterilized pH probe, the new probe was linked with a pH controller apparatus (Electrolab FerMac 260, UK) using a connector. There were two factors of the slope adjustment (pH 4) and the zero point adjustment (pH 7) for probe calibration on the apparatus. Firstly, the probe was put into pH 4 standard buffer solution. The difference between displayed pH and pH 4 standard buffer solution was adjusted with the slope

adjustment. Then, the probe was washed with distilled water before put into pH 7 standard buffer solution and adjusted by zero point adjustment. Once the probe was autoclaved, it was calibrated with only zero point adjustment just before autoclaving. The accuracy of the probe was also re-checked by measuring the same sample and comparing the reading pH value with another electronic pH meter (Metler Toledo, Seven Gasy, Switzerland).

The pH controller has two peristaltic pumps, pump with alkaline and with acid solution. The alkaline feed port was connected with a plastic bottle containing 4M NaOH solution, which was placed on a digital balance (Ohaus portable advanced, Switzerland), by silicone tube via pH controller. So the amount of 4M NaOH added to maintain pH in system was recorded during cultivation.

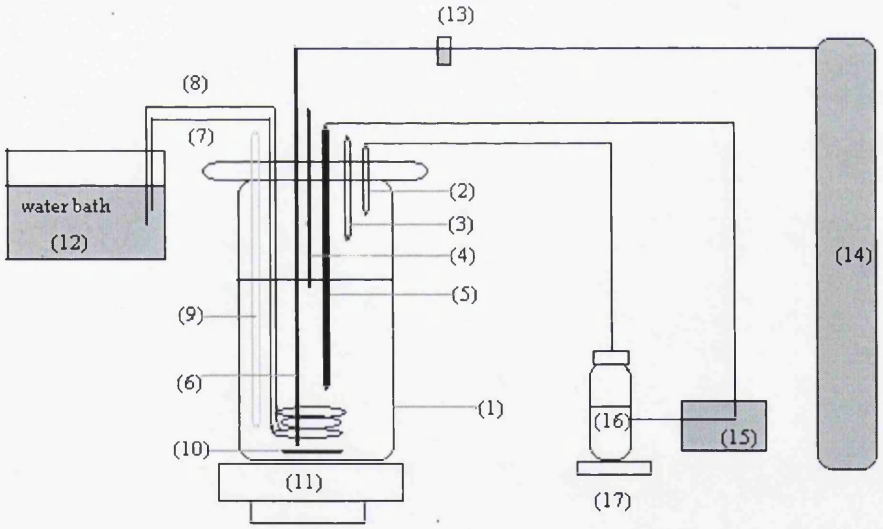


Figure 2.2 *A 5 l stirred tank reactor (STR) system for optimum pH growth experiment. (1) 5 l glass culture vessel, (2) alkaline feed port, (3) vent line, (4) sampling and inoculating port, (5) pH probe, (6) nitrogen addition line, (7) water in, (8) water out, (9) thermometer, (10) magnetic bar, (11) magnetic stirrer, (12) water bath, (13) a filter, (14) nitrogen gas cylinder, (15) pH controller, (16) plastic bottle containing alkaline solution, (17) digital balance.*

Incubation temperature was controlled by heat exchanger. The heat exchanger coil was made of spiral-type of glass tube. The ends of glass tube were connected with a water bath (Grant Water bath, UK) and a pump (Watson Marlow Digital, 505S, UK). The water from water bath circulated in spiral part which was placed into the reactor. The temperature for cultivation system was set at 37 °C.

2.5 Growth of cells in a pilot batch fermenter

To produce enough amount of enzyme for study in this project, the cells were cultivated in 140 l fermenter (ML-4100 New Brunswick Scientific Co., New Brunswick, NJ, USA) (Figure 2.3). The fermenter has a pH probe, thermo sensor, dissolved oxygen probe and antifoam probe which are regulated by a programmable-logic controller (PLC). The system was also equipped with a pump for automatic addition of a neutralizer for pH control. The scheme of 140 l fermenter is shown in Figure 2.4.

To prepare culture medium, 80 l of distilled water was flowed into the fermenter via the filling port which located on the top of the fermenter. The certain amounts of medium components (refer to section 2.4.1) were added through the filling port. Then the mixture was stirred by using the impeller. After all components were completely dissolved, the medium was sampling to measure the pH value and adjusted to be 5.5. The medium was then sterilised in situ with steam. All ports were closed and the steam supply was switched on. The temperature of the medium was increased to 125 °C for 5 min. After completing the sterilisation process, the steam supply was switched off and cooling water was introduced to cooling jacket until the temperature of medium decreased to operating temperature. The condition for growth such as dissolved oxygen level was set at zero, pH was maintained at 5.5± 0.2 and the agitation was controlled at 60 rpm.

Figure 2.3 *Photograph of 140 l fermenter.*

The inoculum was scaled up to 20 l in culture bottle at 37 °C. After it reached late exponential growth phase, the culture was then aseptically transferred by using manually controlled peristaltic pump into 80 l pasturized medium placed into a 140 l steam-jacketed fermenter. During cultivation, 10 M NaOH was fed from alkaline tank through peristaltic pump under PLC control into the fermenter when the pH decreased below the control level. The consumption rate of NaOH used was proportional to cells growth and it was determined by digital balance. The temperature was controlled at 37±1 °C by thermo sensor. When the heat was generated from pumps and the actively growing cells, the coolant water was allowed to flow through the cooling jacket by solenoid valve linked with sensor.

Three samples of liquid culture were collected every hour from the sampling port. The steam was used to sterile the sampling port before and after withdrawing the sample. The cell growth was determined with Unicam UV/VIS 300 Spectrophotometer at optical density 660 nm and the amount of NaOH used during the time was recorded. After 10

hours the fermentation was terminated and the culture was quickly harvested.

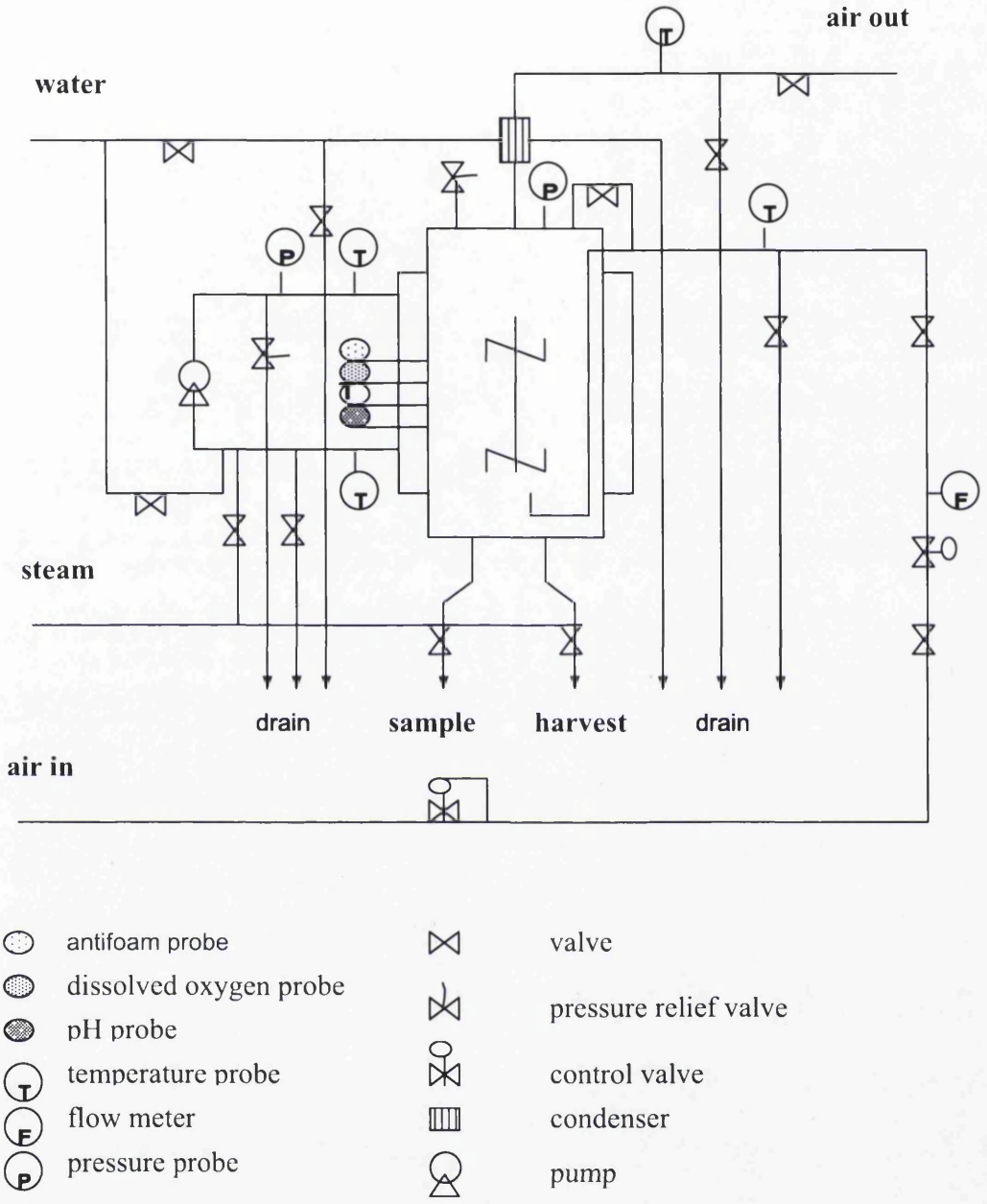


Figure 2.4 *Scheme of 140 l fermenter for cells cultivation.*

2.6 Cell harvesting by microfiltration membrane

The tubular crossflow ceramic microfiltration membrane with nominal pore sizes of 0.2μm and surface areas of 1.8 m^2 (Model CFP-2-G-55, Serial: 19681058450, A/G Technology

Co., Needham, MA) was used for cell harvesting. The photograph of membrane apparatus is shown in Figure 2.5 and diagram in Figure 2.6. All rigid piping in the system was constructed of 1″ stainless steel with an inside diameter of 22 mm. The connection of stainless steel pipe sections including membrane, valves, heat exchanger and pumps were conducted by clamp fittings which provided easily change the design of the pipe work system to suit alternative operational configurations.

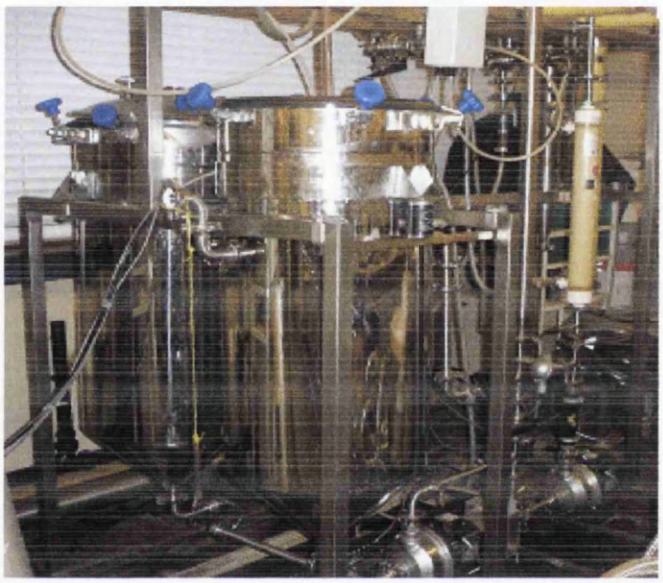


Figure 2.5 *Photograph of microfiltration membrane apparatus for cell harvesting.*

After reaching cultivation time, 100 l of culture was transferred to stainless steel feed tank of microfiltration system. The cell suspension was drawn down from the tank and into the stainless steel piping by feed pump (Lowara, type CEA 210/2, Serial: J 23035/12/93, 0.75 KW, 2800 rpm) and the air in the piping was driven out by the cell suspension. The suspension was then pumped up to microfiltration membrane at a high flow rate up to 70 l/min by the recirculation pump (Lowara, type CEA 210/2, Serial: J 23035/12/93, 0.75 KW, 2800 rpm). The inlet membrane pressure was controlled at 0.8 bar and permeate pressure was 0.6 bar. The permeate fermentation medium passed out through the membrane and collected in permeate vessel while the retentate cells passed the membrane and reached the

top of membrane piping, then passed down through the heat exchanger, back to the feed tank and returned to the loop.

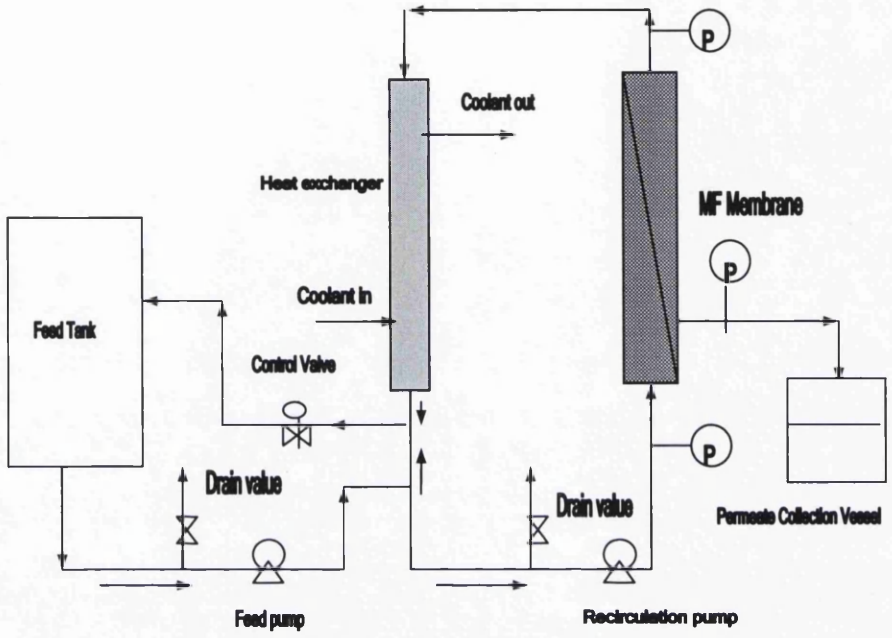


Figure 2.6 *Scheme of microfiltration membrane apparatus for cell harvesting.*

The filtration was operated at about 20 $\pm$2 °C controlled by heat exchanger. The heat exchanger was connected with water supply and the flow rate of water supply manually controlled according to the temperature maintained in the system. The process solution flowed through the inner tube heat exchanger and the coolant water went through the outer shell.

To remove all fermentation medium from the cells, 10 mM phosphate buffer pH 6.5 was added to the tank during the operation and washed the cells for three times. After the end of operation, the final retentate solution was removed from the system and collected in vessel via a drain valve which was situated at the base of the stainless steel loop. The retentate was sampling to measure cells density using spectrometer (Unicam UV/VIS 300) at 660 nm. The cells were kept in freezer at -18 °C for further experiments.

2.7 Cell disruption

The cell disruption was carried out by using mechanical disrupter (Constant Systems Ltd, Daventry, UK, Cell disrupture equipment, B series, model: UPCD40) (Figure 2.7). This disrupter is a single head system with all its internal drillings and external fittings designed for full containment. The machine is equipped with water pipe for cooling system, pressure control valve and control system (internal PLC). A hydraulic pump and accumulator, which placed below the high pressure head, generate the high pressure fluid for the piston to drive liquid through the homogeniser at flow rate up to 200 ml/min. The head of disrupter and jet orifice are shown in Figure 2.8.

***Figure 2.7** Photograph of mechanical cell disrupter.*

The disruption was initiated by the cell suspension in 10 mM phosphate buffer pH 6.5 was introduced into the high pressure cylinder past the inlet valve as the high pressure piston descended. The piston then forced the suspension through the jet orifice at high speed. The rapid transfer of the suspension from a region of high pressure to the atmospheric pressure in the disruption chamber (low pressure) causes cell disruption. The disruption stroke was rapidly driven and governed by a hydraulic operating system which adjusted the discharge

rate to give the set pressure in the high pressure cylinder. Some disruption probably took place on impact with the target, so the stream of disrupted cells was spread radially across the cooled heat exchange surface and then axially down over the inner surface of the disruption chamber for collection. However, impact with the target seemed not to be a main cause of disruption because the geometry of the target had little effect on the amount of disruption obtained. The force for driven the suspension through the jet was proportional to the pressure and the velocity of the piston. The disrupter cycle was continued after collecting disrupted suspension from chamber.

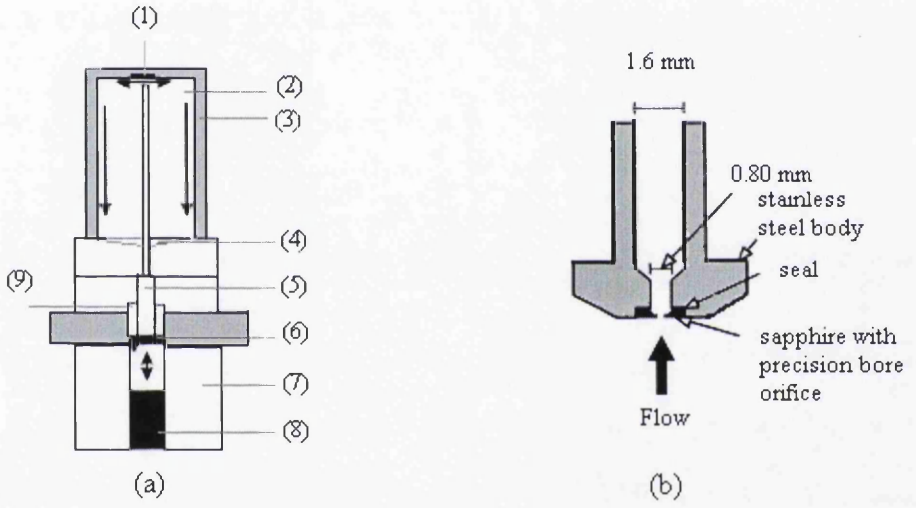


Figure 2.8 *The disrupter head (a) and the orifice assembly (b). (1) target, (2) disruption chamber, (3) cooling jacket, (4) outlet, (5) jet assembly, (6) inlet valve, (7) high pressure cylinder, (8) high pressure piston, (9) inlet (Lovitt et al., 2000).*

The suspension between each passage was controlled at 20±1 °C by the outer cooling jacket to reduce the denaturation of enzyme. The pressure used for disruption varies from 5, 10, 15, 20, 25, 30, 35 and 40 kpsi. The samples from each pressure used were collected and diluted with 10 mM phosphate buffer pH 6.5 to measure the optical density at 660 nm using spectrophotometer.

The effective measurement of disrupter used was also estimated by protein concentration and enzyme β-galactosidase activity. Two types of protein measurement were achieved. Firstly, total protein in the cell suspension was detected by incubating the cells in 0.1 M sodium hydroxide at 90 °C for 10 min. After hydrolyzing the cells suspension in alkaline, the solubilised material were then diluted with distilled water prior to assay. The other measure was of the free protein released after disruption. The cell suspension was centrifuged at 10000 x g for 4 min. The supernatant was then determined soluble protein by Folin colourometric assay (see section 2.13.5.1) and enzyme β-galactosidase activity at 420 nm (see section 2.13.2.1).

$$\text{protein released (\%)} = \frac{\text{protein released after disruption x 100\%}}{\text{total protein in the cell suspension}}$$

Equation 2.1

2.8 Separation of β-galactosidase from disrupted cells by membrane filtration

2.8.1 Membrane filtration apparatus

The tubular crossflow microfiltration membrane with nominal pore sizes of 0.2 μm and surface areas of 3.75 sq. ft (3500 cm^2) (Model CFP-2-E-35A, Serial: 22293NO3, A/G Technology Co., Needham, MA) was employed. All rigid piping in the system was constructed of 1″ stainless steel with an inside diameter of 22 mm. The membrane apparatus is shown in Figure 2.9 and the diagram in Figure 2.10.

Figure 2.9 *Photograph of microfiltration membrane apparatus.*

The connection of stainless steel pipe sections including valve, crossflow membrane and pump were conducted by clamp fittings which provided easily change the design of the pipe work system to suit alternative operational configurations. The stainless steel cooling coil (ID = 0.65 cm, surface area= 4.58×10^{-2} m^2) was put in 8 l feed tank and connected with the water supply in order to counteract the heat generated by the feed pump during operation. The temperature in the system was manually controlled by flow rate of water supply to ensure that the temperature of the process solution with the membrane system was maintained constant. The solution was drawn down through the feed tank and into the stainless steel pipe by the feed pump (Stuart Turner Ltd, 125 Watts) and the air in the piping was driven out by the solution. The pump pressurized the membrane system. The apparatus was operated at constant pressure. The solution went through the membrane with high flow rate and was separated there. The permeate solution passed through the membrane at the flow rate governed by the pump and collected into permeate vessel while the retentate solution passed the membrane and returned to feed tank.

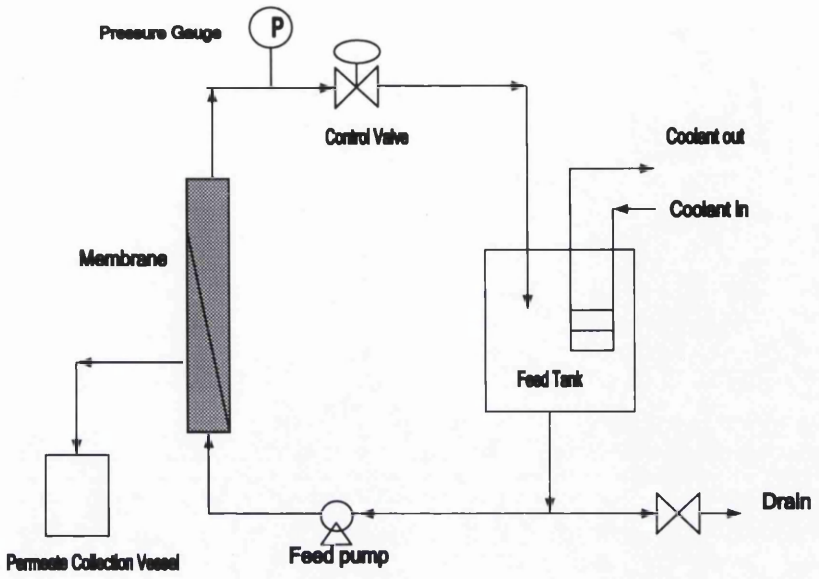


Figure 2.10 *Scheme of microfiltration membrane apparatus.*

2.8.2 Operation of crossflow microfiltration system for enzyme extraction

The operation of microfiltration system was set up with sequential dilution of discontinuous diafiltration. The recirculation rate in the system was controlled by pump at pressure 10 psi.

In the system, 1000 ml of disrupted cells was thawed and diluted with 1000 ml of 10 mM KH_2PO_4 adjusted to pH 6.5 with KOH in 8 l retentate tank. The diluted cell suspensions was then concentrated back to its original volume (1000 ml) by 0.2 μm microfiltration. The first permeate diafiltration was collected. The protein concentration and enzyme activity were then determined by the off-line measurement in both permeate and residual retentate. The fresh 10 mM KH_2PO_4 was replaced to the tank at the same volume. The process was repeated until the enzyme was removed. The temperatures during running system were maintained at 20 $\pm$2 °C. One diafiltration volume was defined as the initial volume of crude enzyme extract.

The performance of membrance filtrate was represented in terms of % transmission and % removed.

$$\text{transmission (\%)} = \frac{\text{permeate protein content at } v=x \text{ x } 100\%}{\text{retentate protein content at } v=x}$$

Equation 2.2

where samples taken at a particular time for the membrane filtration and a particular diafiltration volume (V=x) were compared.

$$\text{removed (\%)} = \frac{\text{retentate protein content at } v=x \text{ X } 100\%}{\text{retentate protein content at } v=0}$$

Equation 2.3

where samples taken at a particular diafiltration volume (V=x) were compared with the samples taken at the starting of the diafiltration (V=0).

The percentage of β-galactosidase recovery from cells was calculated by the following equation:

$$\text{recovery (\%)} = \frac{\text{total activity after filtration x } 100\%}{\text{total activity from crude extract}}$$

Equation 2.4

Purification factor of β-galactosidase during filtration was calculated by the following equation:

$$\text{purification factor} = \frac{\text{specific activity of every diafiltration}}{\text{specific activity of crude enzyme}}$$

Equation 2.5

2.8.3 Hollow fibre ultrafiltation membrane apparatus

The Hollow Fibre Cartridge of 50 kDa molecular weight cut-off (MWCO) (Koch Romicon Hollow Fibre Cartridge 1″ HF 2.0-20-PM50) was applied in the experiment. The length of ultrafilter module is 420 mm, a diameter of about 23 mm and surface area of 0.186 m^2. There are 282 hollow fibers with an internal diameter of 0.5 mm. The fiber and the sleeve are made of polysulphone and the cartridge has epoxy as potting material. This membrane is slightly negative charge. The pH used in range 1.5 to 10 and maximum temperature is about 60 °C.

Figure 2.11 shows the scheme of ultrafiltration membrane apparatus. The feed tank for the system was about 10 l. The ultrafiltration operated in a crossflow mode. All rigid piping in the system was constructed of 1″ stainless steel with an inside diameter of 22 mm. The connection of stainless steel pipe sections including valve, heat exchanger and pump were conducted by clamp fittings which provided easily change the design of the pipe work system to suit alternative operational configurations. The pressure and tangential flow rate were produced by the pump (Stuart Turner Ltd, Type 254, 2800 rev/min) and regulated by the control valve. The solution was drawn down through the feed tank and into the stainless steel pipe. The air in the piping was driven out by the solution. The heat exchanger was connected with water supply and the flow rate of water supply manually controlled according to the temperature maintained in the system. The process solution flowed through the inner tube heat exchanger and the coolant water went through the outer shell. The separation process occurred when the solution was pumped up through the membrane. The permeate solution passed through the membrane at the flow rate governed by the pump and collected into permeate vessel while the retentate solution passed the membrane and returned to feed tank.

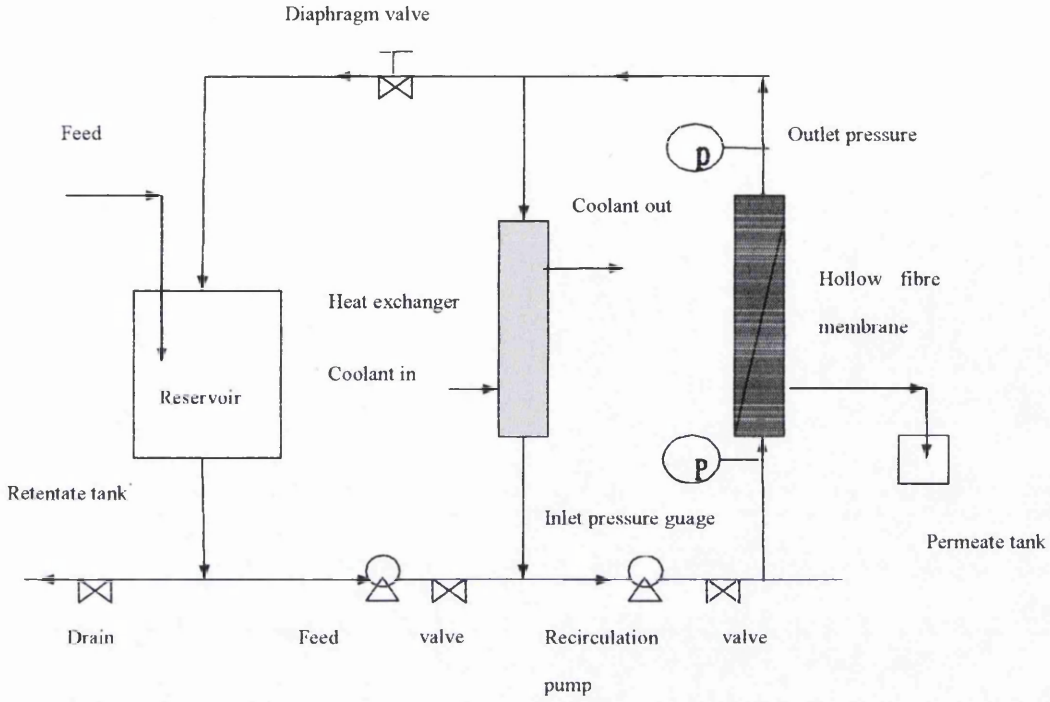


Figure 2.11 *Scheme of ultrafiltration membrane apparatus.*

Figure 2.12 *Photograph of ultrafiltration membrane apparatus.*

2.8.4 Concentration by ultrafiltration

In the experiment, the operating temperature was maintained at 20 $\pm$2 °C and the inlet pressure was 10 psi. The clear diluted solution from the microfiltration was concentrated

by the ultrafiltration apparatus (Figure 2.12). The protein concentrations and enzyme activities from permeate and retentate were regularly determined during concentration every 1 litre volume reduction. A final litre from retentate was collected from the tank and defined as the membrane-purified enzyme.

2.9 Enzyme preparation for bioreactor and membrane bioreactor

After the freezing cells in buffer was thawed and disrupted by mechanical disrupter machine, the suspension was equally divided into 50 ml plastic conical tubes (Fisherbrand, UK). The tubes were then placed into the controlling temperature centrifuge (Biofuge Stratos Sorall, Kendro Products, Germany) with the speed 5000 rpm, at temperature 15 °C for 10 min. Then clear supernatant was separated from cell debris and used as the crude enzyme in bioreactor.

In case of high amount of enzyme required in membrane bioreactor, the enzyme was separated from cells debris by using a 1000 ml vacuum driven bottle top filter with 0.22 μm membrane (Millipore Corporation, Billerica, Massachusetts, U.S.A) connected with vessel. The enzyme was collected as the permeate solution while the cell debris was left on the surface of membrane.

Different concentrations of enzyme used in the experiments were prepared by diluting with 10 mM phosphate buffer at pH. 6.5.

2.10 Substrate preparation

The hydrolytic activity of β-galactosidase on two sources of lactose, from lactose solution and from skimmed milk, were determined in this study.

In case of lactose solution, the certain amount of lactose powder was dissolved in 10 mM

potassium phosphate buffer and adjusted pH to be at 6.5 with KOH. The solution was just prepared before use.

Skimmed milk powder purchased from Tesco Stores Ltd., (UK), containing 36 g/l protein, 52 g/l carbohydrate, 6 g/l fat and 0.6 g/l sodium, was used in this experiment. 10% w/v of the milk powder was prepared with distilled water. The powder suspension in distilled water was continuously stirred until completely dissolving. Then the skimmed milk solution was put into the feed tank of 0.2 μm microfiltration membrane apparatus (as shown in section 2.8.1). The solution was drawn down through the feed tank and into the stainless steel pipe by the feed pump (Stuart Turner Ltd, 125 Watts) and the air in the piping was driven out by the solution. The pump pressurized the membrane system. The solution went through the membrane with high flow rate and was separated there. The clear permeate solution passed through the membrane at the flow rate governed by the pump and collected into permeate vessel while the retentate solution passed the membrane and returned to feed tank.

Then all permeate solution was put into the feed tank of 10 kDa MW cut-off ultrafiltration membrane apparatus (as shown in section 2.8.3). The separation process occurred when the solution was pumped up through the membrane. The permeate solution passed through the membrane at the flow rate governed by the pump and collected into permeate vessel while the retentate solution passed the membrane and returned to feed tank. The crossflow filtration in both systems was carried out with constant pressure. The permeate solution from ultrafiltration membrane was used as substrate. The retentate and permeate from microfiltration and ultrafiltration were collected and measured lactose and protein concentration.

2.11 Enzyme bioreactor

An enzyme bioreactor applied in the experiment was a 100 ml maximum process volume glass bottle with a plastic cap on the top. The temperature of bioreactor was maintained by placing it into controlling incubator (LMS, Incubator, UK). The bioreactor contained a magnetic bar and it was placed on a magnetic stirring plate (SM1, Stuart Scientific, UK). All the experiments in bioreactor were carried out with stirrer speed setting at 200 rpm.

The effects of substrate concentration, enzyme concentration and temperature were investigated in the bioreactor with batch mode. The reaction was started by loading substrate solution into reactor containing the enzyme solution. The samples were collected during the time interval for glucose measurement.

In case of addition substrate solution in batch reactor experiment, three sets of 20 ml substrate solutions were prepared. The enzyme was first loaded in the bioreactor. The reaction was initiated after the addition of first 20 ml lactose solution. The hydrolysis reaction was left for 3 h. Then another 20 ml of substrate solution was added into the reactor. The samples were collected for glucose measurement in every hour.

For glucose measurement, the collected samples from bioreactor were immediately stopped the reaction with 0.1 M HCl. The concentration of glucose production was determined by using GOPOD reagent. All results from the experiments represent the average of duplicate.

2.12 Membrane bioreactor

2.12.1 Stirred-cell membrane reactor

The reactor system composed of an ultrafiltration stirred cell of 50 ml maximum process

volume, a magnetic stirrer and an effective membrane area of 13.4 cm^2 (Figure 2.13). The stirred cell was an Amicon 8050 and able to withstand under operating condition at maximum pressure 75 psi (~5.17 bar), maximum temperature 85 °C and pH in range 2-10.

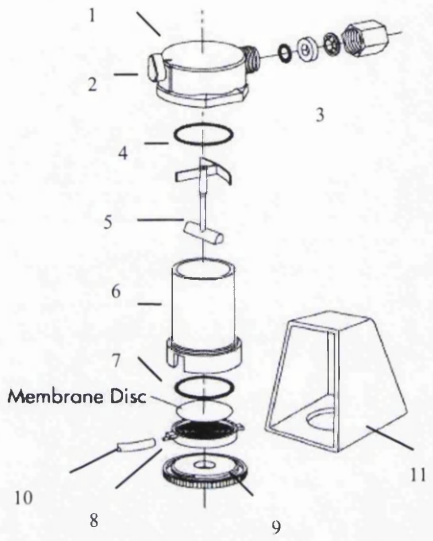


Figure 2.13 *Schematic diagram of the stirred cell (Amicon cell). (1) cap, (2) pressure relief valve, (3) pressure tube fitting assembly, (4) top o-ring, provides seal to maintain pressure in the unit, (5) magnetic impeller, provides cross flow conditions, (6) main body of the stirred cell, (7) bottom o-ring provides seal to maintain pressure in the unit and prevent loss of sample, (8) base with permeate outlet, (9) screw in bottom to secure base in the main body, (10) permeate line and (11) retaining stand prevents displacement of cap when pressure is used in the unit.*

The stirrer speed was set at 400 rpm through the experiment. The molecular weight cut-off (MWCO) of ultrafiltration disc polysulphone membrane was 30 kDa. The pressure in the system was controlled by a pressure adjustment valve fitted with a pressure gauge. The cell was pressurized by constant compressed nitrogen at 30 psi. The operating temperature was controlled at 25 °C. The stirred-cell was operated in a batch dead-end mode.

In the study of rejection of the enzyme from ultrafiltration membrane, 10 ml of the enzyme was loaded into the system. Then 10 ml of phosphate buffer was added to reactor. The

permeate flux was controlled by pressure from compressed nitrogen gas. The system was operated in permeate recycling. The permeate and retentate from the cell were sampled to check the enzyme activity with ONPG every hour.

2.12.2 Membrane bioreactor system

The photograph and scheme of the membrane reactor system are shown in Figure 2.14 and Figure 2.15 respectively. The system was equipped with hollow fibre membrane, reaction vessel, feed pump, recirculation pump, permeate pump, diaphragm valve, pressure gauge, substrate reservoir, heat exchanger and connected with nitrogen gas tank.

The Hollow Fibre Cartridge of 50 kDa molecular weight cut-off (MWCO) (Koch Romicon Hollow Fibre Cartridge 1″ HF 2.0-20-PM50) was connected with reactor system. The length of ultrafilter module is 420 mm, a diameter of about 23 mm and surface area of 0.186 m^2. There are 282 hollow fibres with an internal diameter of 0.5 mm. The fibre and the sleeve are made of polysulphone and the cartridge has epoxy as potting material. The membrane filtration operated in crossflow mode.

Figure 2.14 *Photograph of membrane reactor system.*

The reaction vessel was constructed with 5.5 l glass vessel with head plate. The head plate was connected with level indicator, feeding line, nitrogen gas addition line, cooling coil and recycling-in line. The substrate was feed to the vessel with electrical signal which was from the level of fluid in vessel was transmitted by fluid conductivity, so the electric signals from level indicator controlled pinch valve. The recycling-out line (or draining line) was constructed in the bottom of the bioreactor.

All rigid piping in the system was constructed of 1″ stainless steel with an inside diameter of 22 mm, with the exception of the pipes connecting feed pump (Brook Hansen from Michael Smith Engineers Ltd., type PD63SFH, 0.12KW, 2870 rev/min) to the main stainless steel loop and the base of 5.5 l glass reaction vessel. The connection of stainless steel pipe sections including valve, crossflow membrane and pumps were conducted by clamp fittings which provided easily change the design of the pipe work system to suit alternative operational configurations. The system was controlled at constant pressure and constant flow rate of permeate. The digital control permeate pump (Watson Marlow Ltd., Model 505 DU) was set up at outlet permeate line to control flow rate.

The operation of reactor system was started by feeding the substrate which kept in feed reservoir to retentate tank and the enzyme was then added. The enzyme usually was rejected from membrane and recycled back to the system while low molecular weigh products and nonreacted substrate freely permeated the membrane. Therefore the volume of liquid inside the system is remained constant by continuous feeding with fresh substrate solution via electrovalve connected to level sensor which set up in retentate tank. Mixing the liquid in system was achieved by mean of recirculation of the reactor volume.

After the enzyme and substrate solution was filled to reach the volume in the glass retentate, the liquid was drawn down through the tank and into the stainless steel pipes by

feed pump. The membrane system was pressurized with this pump. When the solution was flown through the system, the air in the piping was driven out. The recirculation pump (Brook Hansen from Michael Smith Engineers Ltd., type ED 80 BD, 0.37 KW, 2820 rev/min) generated the pressure, tangential flow rate and pumped the solution up through the hollow fibre membrane, so the separation took place there. The pressure inlet of the membrane was controlled by the diaphragm valve. The permeate solution passed out through the membrane at the flow rate governed by the permeate pump while the fluid passed through the membrane and reached the top of membrane piping was divided into two paths. Some of it was returned through the diaphragm valve and the rest pass down through the heat exchanger and back around the stainless steel loop. The diaphragm valve governed the proportions of the solution taking each route and the feed flow rate generated by feed pump.

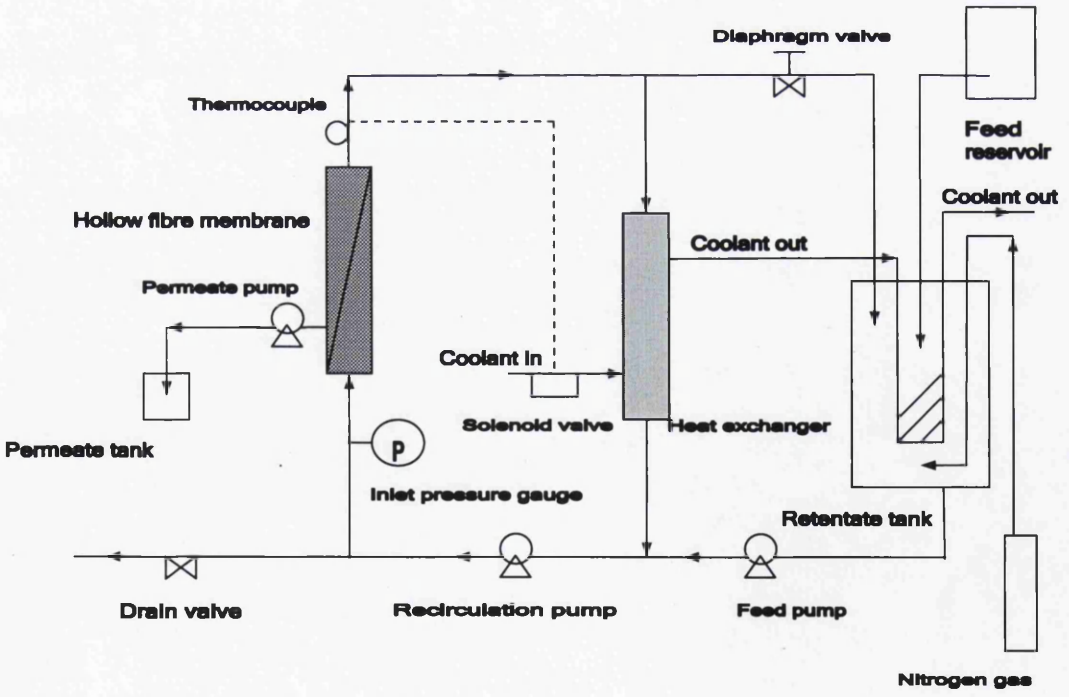


Figure 2.15 Scheme of membrane reactor system

The heat generated in the system from two pumps was controlled by heat exchangers. The temperature of the process solution can be normally increased to about 50 °C in 20 minutes when operating system without temperature control. To maintain the system at specific temperature and enzyme activity for the reaction, it was essential to cool the solution with heat exchangers. Two heat exchangers, a single pass tube exchanger and stainless steel cooling coil located in retentate tank, were connected in the system. The dimension of outer shell in single pass tube exchanger are 435 mm by 50 mm, with the inside diameter of the inner tube being 22 mm. The inner-diameter of stainless steel cooling coil is 0.65 cm and surface area is 2.5×10^{-2} m^2. Flow of the coolant water to the shell of single pass tube exchanger was controlled by the solenoid valve linked to a thermocouple to ensure that the temperature of the process solution with the membrane system was remained constant. The process solution passed through the inner tube and the coolant through the outer shell. The solenoid valve was automatically open or shut depending on the temperature of the solution within the process, which was measured by a thermocouple attached to the stainless steel pipe work. The coolant water out from outer shell exchanger was passed to the cooling coil to maintain the temperature of liquid in retentate tank. The nitrogen gas tank was connected to the bioreactor using gas line. The gas flowed into the solution at low rate and controlled by pressure valve in order to stabilize enzyme solution in the system.

Samples from permeate and retentate were taken at time interval during the process and the reaction was stopped by addition the equal volume of 0.1 M HCl prior to measure glucose concentration. In addition, the enzyme activity and protein concentration were directly measured from the solution. The permeate flow rates were determined using a graduated cylinder and a stopwatch.

The retentate solution was removed from the system after the end of operation via a drain valve which was situated at the base of the stainless steel loop.

2.12.3 Lactose hydrolysis in membrane bioreactor

In case of the effect of addition of substrate during hydrolysis reaction, the experiment was carried out in batch mode of membrane bioreactor with permeate recycling in the system. Three sets with the same amount of lactose solution were prepared. First amount of lactose solution was loaded into the reactor tank. After adjusting and controlling the temperature to be 25 °C, the enzyme was added. Mixing substrate and the enzyme was achieved by recirculation of the reactor volume. The reaction was initiated and left for 3 h. Then another lactose solution was added into the reactor. The final amount of lactose solution was added after reaction time for 6 h. The samples were collected for glucose measurement during time interval.

In the continuous mode, lactose solution was loaded into the reactor tank at the required concentration. After adjusting the temperature to be 25 °C, the amount of enzyme was added. Mixing substrate and the enzyme was achieved by recirculation of the reactor volume. The total working volume was fixed at 2500 ml. The substrate was continuously fed into reactor by the rate of feed flow rate adjusted to equal the permeate flow rate in order to remain constant reactor volume. The constant permeate flow rate was maintained by permeate pump. Samples from permeate was taken at intervals during continuous process. Low molecular weight products and non-reacted substrate passed through the membrane while the enzyme was retained in the system. After sampling, the reaction was stopped by 0.1 M HCl before determining glucose concentration. The operating temperature was controlled at 25 °C.

2.12.4 Regeneration of membrane bioreactor

After each operation, the membrane reactor system was regenerated. Firstly distilled water was used to rinse the system for twice. The permeate piping and the diaphragm valve were fully opened. After drained distilled water, 0.1 M NaOH was circulated through the reactor for 30 min. Then distilled water was replaced to rinse the system. After the membrane was neutralized, 0.1 M Citric acid was added and circulated for another 30 min. Finally the cleaning process was ended up by rinsing distilled water until the system was neutrality.

2.13 Analytical methods

2.13.1 Measurement of cell growth

Determination of cell growth was monitored, as an increased in turbidity in terms of Optical Density (OD) at 660 nm. Reading the OD was continued until it reached the stationary phase. The growth curves were obtained by plotting OD against time and the specific growth rates (μ) of bacteria from each composition concentration was calculated from Log plot of OD versus time during the exponential phase. In addition, the relative yields on the effect of each component on the cell growth were obviously shown by plotting the proportion of the maximum OD (OD_{max}) of each concentration and the highest OD_{max} (OD_{max0}) from specific concentration against that component concentration.

The growth of cell from preliminary development of growth medium experiments was monitored by placing the pressure tube into a spectrophotometer at 660 nm (PU 8625 UV/VIS Philips, France) fitted with a test tube holder. The tube had a 1.8-cm light path.

In case of optimum pH and growth in 100 l fermenter experiments, the growth measurement was conducted in plastic cuvette with 1 cm light path fitted into a Unicam

UV/VIS 300 Spectrophotometer. The samples needed to be diluted with distilled water in case of the optical density above 2 before measuring.

2.13.2 Assay of β-galactosidase activity

The activity of β-galactosidase from crude enzyme and partially purified enzyme were determined by either considering the absorbance changed using spectrophotometer (UV 300, Unicam, UK) with ortho-nitrophenol-β-galactosidase (ONPG) or discontinuous assay with lactose and the results were performed in terms of glucose measurement as outlined in the section 2.13.2.1 and 2.13.2.2 respectively.

2.13.2.1 Assay with ONPG as a substrate

The rate of change of absorbance with time was measured after mixing 2 ml of 0.1M phosphate buffer pH 6.5, 1 ml of crude enzyme or partially purified enzyme and 0.5 ml of 45 mM ONPG substrate which had the final concentration available in excess of the reaction. The solution was rapidly and completely mixed in cuvette. The reaction velocities of each condition were then determined during the first few minutes of the assay in absorbance at 420 nm temperature at 20 °C.

All experiments were run in triplicate and were averaged. The activity of the enzyme is expressed as enzyme unit (U). One unit of enzyme is defined as the amount of enzyme required to liberate 1μmol of o-nitrophenol or glucose per min per ml at condition used. Finding the protein content of the solution leaded to calculating the specific activity which is defined as units of β-galactosidase per mg protein. The unit of the enzyme is calculated as following equation (John, 2002):

$$\text{Unit of enzyme activity in total sample} = [(dA/dt)/\varepsilon] \times V_f \times 1000 \times (V_t/V_s)$$

Equation 2.6

where dA/dt is the rate of change of absorbance with time, ε the molar extinction coefficient (see Appendix 1.1), V_f the final volume in the cuvette (ml), V_t the total volume of sample (ml), V_s is the amount of enzyme taken from sample.

2.13.2.2 Assay with lactose as a substrate in terms of glucose measurement

The amount of glucose released during lactose hydrolysis was measured by the method of Trinder (1969) using GOPOD reagent. The principles of reaction were as followed:

$$\text{Glucose} + O_2 + H_2O \xrightarrow{\text{Glucose oxidase}} \text{Gluconate} + H_2O_2$$

$$2H_2O_2 + \text{p-Hydroxybenzoic acid} + 4\ \text{Aminoantipyrine} \xrightarrow{\text{Peroxidase}} \text{Quinoneimine Dye} + 4H_2O$$

The following table shows the reagent compositions and formulation of GOPOD reagent used in the assay (Megazyme Int., Ireland).

Reagent	Composition
Reagent Buffer	1M Potassium dihydrogen orthophosphate, 0.4% Sodium azide and 200 mM p- Hydroxybenzoic acid were dissolved in 1 l distilled water
Glucose Reagent	the reagent consists of 12,000 units of Glucose oxidase, 650 units of Peroxidase and 0.4 mmol 4- Aminoantipyrine

Table 2.1 *The reagents used in glucose measurement*

The preparation of GOPOD reagent was diluting 50 ml of the reagent buffer to 1 l with distilled water, then glucose reagent was dissolved in this buffer and adjusted pH to be 6.0 using KOH.

The assays were initiated by addition 0.1 ml of water and 0.1 ml of glucose sample, which had been stopped the reaction in 0.1 M HCl, in 3 ml GOPOD reagent then mixed thoroughly. The reaction was incubated at 40 °C for 20 min. The absorbance was determined at 510 nm. The amount of liberated glucose of samples was considered with the calibration graph of glucose concentration as a function of the absorbance using glucose as a standard glucose (see Appendix 1.4).

2.13.3 Stability of β-galactosidase activity

The stability of enzymes from both crude extract and partially purified enzyme from membrane separation were performed at temperature 4, 20, 30, 40, 50 and 60 °C. The enzymes were incubated with 0.1M potassium dihydrogen phosphate pH 6.5 at different temperature. Then the sample was withdrawn at regular intervals and checked the activity with ONPG. The stability was considered in terms of the residual activity which defined by the equation below:

$$\text{residual activity} = \frac{\text{the activity of sample at t=t x100 \%}}{\text{the activity of sample at t=0}} \qquad \textit{Equation 2.7}$$

A straight line with declined slope (k_d) from plotting graph between natural logarithmic residual activity versus time was used to calculate half-life ($t_{1/2}$) value of the enzyme in hour as following equation:

$$t_{1/2} = \frac{\ln 2}{k_d} \qquad \textit{Equation 2.8}$$

The half life of the β-galactosidase activity was defined as the reaction time when the activity reduced to half of its initial activity.

2.13.4 Kinetics of β-galactosidase assay

The assays were carried out with two substrates, ONPG and lactose, at room temperature. The wide range concentrations of substrates were prepared. For ONPG as artificial substrate, the rates of the enzymes were measured as mention in enzyme assay. The concentration of ONPG varied from 2 mM to 100 mM. In case of lactose as real substrate, the enzymes were added into different concentrations (5-100 mM) of lactose at ratio 1:1. Aliquots were incubated at room temperature and withdrawn at regular intervals. The reaction was stopped after adding 0.1 M HCl. β-galactosidase activity was measured in form of glucose release using GOPOD reagent with absorbance at 510 nm. The data from absorbance were converted to mM glucose concentration comparing with standard curve. The slope of straight graph from glucose product concentration versus reaction times during first period of measurement provided the initial reaction rates which were then used to derive K_m and V_{max} value.

2.13.5 Protein assay

The procedure for determination of protein concentration was selected depending on sensitivity, convenience and interference by other substances present in the solution. Two methods, Folin and Bradford assays, were applied through this study.

2.13.5.1 Folin colourometric assay for protein

The Folin colourometric assay was performed under alkaline condition. Proteins are complex with copper. When Folin reagent is added, the phosphomolybdic phosphotungstic

in Folin reagent binds to the protein. Bound reagent is slowly reduced and change color from yellow to blue (Lowry *et al.*, 1951).

Before assay the protein concentration in samples, Bovin Serum Albumin (BSA) was used as a standard protein to set the standard line at 750 nm. The calibration line was employed to consider the equivalent protein concentration of the sample. The following table shows the reagent compositions and formulation used in the assay.

Reagent	Composition
Reagent A	20 g of sodium carbonate (Na_2CO_3) were dissolved in 1 l of 0.1M NaOH solution
Reagent B	0.5 g of $CuSO_4$ were dissolved in 100 ml of a 1%(w/v) aqueous solution of sodium tartrate ($[CH(OH)COONa]_2$)
Reagent C	Reagent A was mixed with Reagent B just before use in a ratio of 50 to 1
Reagent D	A diluted Folin reagent was prepared, the supplied reagent was diluted with distilled water in a ratio of 1 to 1 and discarded on the day of use

Table 2.2 *The reagents used in Folin assay*

Distilled water was added to the sample to get a total volume of 0.5 ml and a protein concentration less than 250 μg/l. Then 2.5 ml of reagent C was added and well mixed. The mixture was allowed to stand for at least 10 min at room temperature. Afterwards 0.25 ml of reagent D was combined to the mixture and mixed immediately. After addition to the alkaline, protein-containing solution, the reactivity of this reagent lasts only seconds. After 30 min or longer at room temperature the absorbance of the mixture was determined

against a blank at 750 nm in a spectrophotometer. Triple assays were tested and the results were average with typically less than 5% variation. Then the protein concentrations of samples were considered with calibration graph of the protein concentration as a function of the absorbance using BSA as a standard protein (see Appendix 1.2).

2.13.5.2 Bradford assay for protein

Bradford assay is a simpler, faster, more sensitive and less likely to suffer from interference by many compounds that limit the use of other assays. The assay is based on binding of the dye Coomassie Blue G250 to protein causing a shift in the absorption spectrum of the dye (Bradford, 1976). The dye normally exists in two forms: the red anionic form and it is converted to a blue form when the dye binds to amino groups of proteins. The amount of protein bound is detected at absorbance 595 nm due to the absorption maxima of the various dyes and protein-dye complex.

The Bradford reagents were prepared by dissolving 50 mg of Coomassie brilliant blue G250 in 25 ml of 95% ethanol. Then 50 ml of 85% (w/v) phosphoric acid were added into the solution. The mixture was made up to 500 ml in volumetric flask with distilled water. The solution reagent was finally filtered through Whatman No.1 filter paper before use. The reagent can be stored for four week and must be kept in the dark bottle at 4 °C. Prior to use the dye reagent needed to be filtered again for avoiding the precipitation.

The procedure of the assay was conducted by taking 40 µl of sample into 2 ml Bradford reagent. The solution was gently inverted several times to mix and check the absorbance at 595 nm. The protein concentration of sample was determined by comparing its absorbance to a standard curve obtained from plotting the absorbance of standard BSA solution containing 200-1000 µg/ml protein (see Appendix 1.3).

2.13.6 Determination of lactose concentration

The lactose content was analyzed by isocratic high performance liquid chromatography (HPLC, Varian Model Prostar 210/215) with a CarboPac MA1 column (length 250 mm, diameter 4.0 mm) and guard column MA1(length 50 mm, diameter 4.0 mm) (Dionex Corporation, USA). The analysis cell to measure INT Amperometric was joined to ED 40 pulsed electrochemical detector (Dionex). The results were performed in terms of mVolts-based.

The mobile phase used was 480 mM sodium hydroxide prepared with deionized water. The mobile phase was delivered to the system by pump (Varian Pro-Star Corporation, Canada). The flow rate was maintained at 0.4 ml/min and the pressure was controlled in range from 1500 psi to 1800 psi. The standard lactose and samples were introduced to the equipment by filtering through a syringe filter. Then 20 μl of sample were injected to the injection loop. The total run time of elution was 30 min. The temperature was at 25 °C.

The data obtained from HPLC was analyzed with Prostar Workstation Data analysis software package (Varian Co., Canada).The results based on the area of peak appeared on the chromatogram and compared with standard lactose (see Appendix 1.5). All results data represented the average of triplicate.

Chapter 3

Cultivation of *Lactobacillus delbrueckii* ssp *bulgaricus*

NCTC 11778

3.1 Introduction

The bacterial culture, *Lactobacillus delbrueckii* ssp *bulgaricus*, was selected to be the source of the enzyme β-galactosidase through this project. This strain is extensively exploited in the dairy industry and has been known as probiotic strain due to its efficient and reliable utilization of milk constituents, mainly lactose and caseins, and good resistance to low pH. The reason of choosing was the bacteria considered as safe for consumer because they have been categorized as a non harmful bacterium (GRAS, Generally Recognized as Safe by the FDA) then the extensive purification of enzyme preparation would not be serious leading to an economical process of lactose-reduced milk product. In addition, the enzyme from bacteria showed high hydrolysis ability compared to another lactic acid bacteria (Hickey *et al.*, 1986; Shah and Jelen, 1990 and 1991; Smart *et al.*, 1993 and Kreft *et al.*, 2001).

L. delbrueckii ssp *bulgaricus* is a gram-positive, facultative anaerobic, non-spore forming and homofermentative lactic acid bacterium which converts glucose to lactic acid almost exclusively. They are non-flagellated, polymorphic rods which grow in chains occurring predominantly in pairs or short form. The chain form related to the age of the cells. Short rods predominate in young cultures and longer filamentous forms occur more frequently in older cultures. Their single layer cell walls are mainly structured by peptidoglycan and teichoic acids. The cells have size in diameter range from 0.5 to 0.8 mm and length from

2.0 to 9.0 mm. They are able to ferment a limited number of carbohydrates such as glucose and lactose but incapable of fermenting galactose. This culture synthesizes D-lactic acid as the major metabolic end product from hexose sugars via the Embden-Meyerhof pathway. The optimum growth is in the range of 37-50 °C and can survive up to 55 °C or higher. The cells decrease rapidly at temperature below 20 °C. Then the culture should not be stored less than this point.

The aim of this work was to determine the suitable components medium for the growth of *L. delbrueckii* ssp *bulgaricus* NCTC 11778 at pH 6.5, temperature 37 °C. The experiments were conducted in 30 ml pressure tubes with starting pH at 6.5. After making a decision on optimisation media components, the growth was then determined in 5-l fermenter under pH control to examine the optimum pH. The optimized conditions were applied for scale up the growth in pilot fermenter. The cultures were harvested after reaching stationary phase and washed out medium with phosphate buffer using crossflow microfiltration system. The cells were collected and used for further work on enzyme preparation and utilisation.

3.2 Preliminary experiment on growth

Growth medium should commercially consist of inexpensive ingredients, spending a short time frame for reaching a high cell density in order to reduce production costs and ease of harvest (Heenan *et al.*, 2002). Many studies have been focused on the formulation of a suitable inexpensive medium for enhancement of lactic acid or biomasss production of lactic acid bacteria (Gupta and Gandhi, 1995; Bury *et al.*, 1998; Bury *et al.*, 2000; Heenan *et al.*, 2002). Schiraldi *et al.* (2003) used semidefined medium (SDM), using dextrose as carbon source, to grow *Lactobacillus delbrueckii* in microfiltration bioreactor obtaining high cell density. Chervaux *et al.* (2000) mentioned that using chemically defined medium

(CDM) exhibited the growth rate three times lower than growth rate in rich and complex medium such as milk and MRS. They developed CDM to milieu proche du lait (MPL), which contained 60 components, for 22 strains of *L. delbrueckii*. Although this medium provided good growth rate in range from 0.55 to 1 h^{-1}, the cost of medium components need to be considered.

The typically complex medium for Lactobacilli group is MRS medium (de Man *et al.*, 1960) or Reinforced Clostridial Medium RCM (Hirsch and Grinsted, 1954). These mediums contain meat or milk extracts. Heenan *et al.* (2002) investigated the growth of probiotic bacteria in SPY medium, which composed of soy peptone, yeast extract and glucose, and compared the growth from MRS medium. The result of bacteria growth in SPY was similar or better than in MRS. However, in addition to carbohydrate and nitrogen sources in growth medium, the nature growth of lactic acid bacteria requires numerous growth factors for example minerals and vitamins to be used for enzyme production (Vasiljevic and Jelen, 2001).

Vasiljevic and Jelen (2001) cultivated *L. delbrueckii* ssp *bulgaricus* ATCC 11842 in skim milk, whey and whey permeate basal media, supplemented with whey protein products, yeast extract or MRS medium, at pH 5.6 and 43 °C. They found that whey supplemented with MRS showed the highest specific growth rate and provided two times growth rate comparing with unsupplemented whey while using skim milk as a growth medium gave the highest β–galactosidase activity. The probable explanation was cultivation on the medium containing glucose or glucose plus lactose supported the cell growth but did not encourage the enzyme production. Although these dairy based media show good results in cultivation, the use of an efficient and economic downstream process to recover and isolate the cells from various impurities in the fermentation broth are significant.

Although the MRS medium is commonly used for growing the cells, one of the ingredients in MRS broth is beef extract, unacceptable component in food manufacturing companies in UK. Moreover, there has been a growth consumer vegetarianism increasing around the world (Beardsworth and Keil, 1991). The cells should not be cultured in a growth medium having animal-derived ingredients so as to prevent a carry-over of these ingredients into the product.

In addition to make a growth medium free from animal-derived ingredients, the impact on medium cost of producing cells on a large scale is considerable. Consequently, it is very important to choose optimized growth component medium in order to obtain high biomass. In this study, the developed medium similar to MRS was investigated. The various concentrations of each main ingredient of medium were determined to find the simple and effective medium in β-galactosidase production.

The experiments were performed to observe the growth characteristics of *L. delbrueckii* on different concentrations of yeast extract, soy peptone, lactose, potassium dihydrogen phosphate, sodium acetate, triammonium citrate and tween80 while magnesium sulphate and manganous sulphate were fixed through out the experiments at 0.02 and 0.005% respectively. The growth condition carried out at 37 °C and started pH at 6.5. Each component experiment was conducted in triplicate.

3.2.1 The effect of nitrogen source on growth

The effect of the nitrogen source on growth at different concentrations was examined by the addition of various concentrations yeast extract from 0, 0.2, 0.4, 0.6, 0.8 and 1.0%. The result is presented in growth curve (Figure 3.1). When the cells grew in the medium containing yeast extract, the cells density enhanced when the concentration of yeast extract increased. It was clearly seen when comparing the growth curve between yeast extract 0%

and 1.0%. The final cells density of 1.0% yeast extract was almost two times medium without yeast extract. Several studies mentioned yeast extract contains amino acids, peptides, water soluble vitamins, some minerals and carbohydrate and it is a good nutrient source for many microorganisms (Peppler, 1982; Jackson *et al.*, 1998).

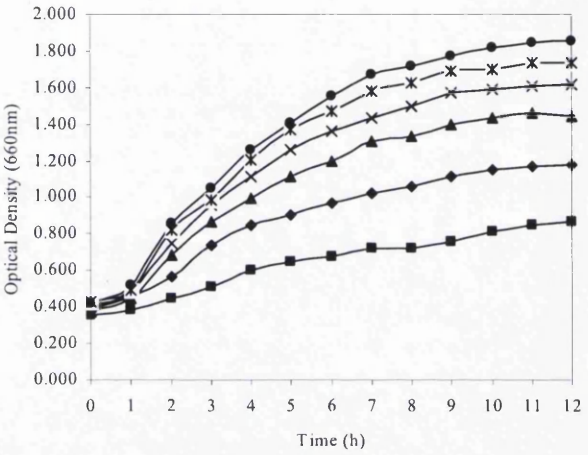


Figure 3.1 *The growth curve of L. delbrueckii grown in pressure tube at different yeast extract concentrations from 0 % (■), 0.2% (◆), 0.4% (▲), 0.6% (×), 0.8% (✳) and 1.0% (●). The growth condition was started at pH 6.5 using KOH and the temperature was controlled at 37 °C. OD was measured with 18 mm light path (pressure tube diameter).*

Figure 3.2 shows the relationship between specific growth rate (μ) and yeast extract concentration. It was found that the specific growth rate of cells grown without yeast extract was only 0.15 h^{-1} while at concentration from 0.4-0.8% were 0.34 h^{-1}. Although the specific growth rates at these concentrations (0.34 h^{-1}) were not great difference comparing with 1.0% yeast extract (0.35 h^{-1}), the cell yield at 1.0% concentration was significant.

The relative yield is shown in graph plotting the ratio of OD_{max}/OD_{max0} (when OD_{max} is defined as maximum cell density obtained from each concentration and OD_{max0} is defined as maximum cell density obtained from the best concentration) versus yeast extract

concentration (Figure 3.2). The increasing ratio of OD_{max}/OD_{max0} was obvious as yeast extract concentration increased. It can be interpreted that yeast extract enhanced the growth of *L. delbrueckii*.

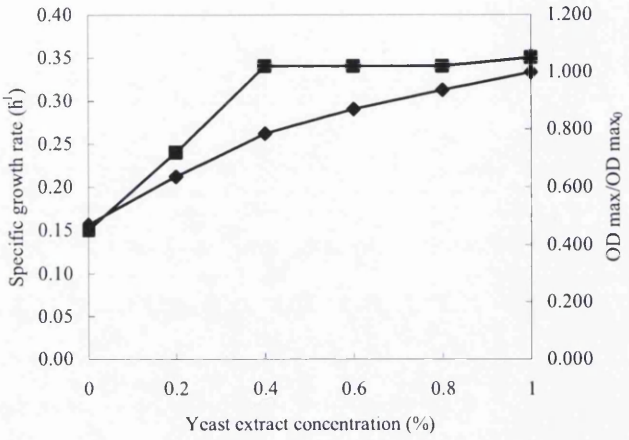


Figure 3.2 *Effect of yeast extract concentration on the specific growth (■) and growth yield ratio OD_{max}/OD_{max0} (◆) of L. delbrueckii. Operating conditions are mentioned in Figure 3.1.*

The other nitrogen source from soy peptone was also examined. Soy peptone contains carbohydrate peptides and amino acids but is low in vitamins or minerals as compared yeast extract. Riviere (1977) mentioned that lactic acid bacteria were incapable of synthesizing some certain amino acids and had a limiting ability on synthesis the nutrients from simpler nitrogenous compounds. Vitamins and amino acids then often need to be added to the medium. Consequently, in addition to yeast extract, soy peptone also serves as nitrogen source which enriched in various amino acids was investigated. The various soy peptone concentrations 0, 0.2, 0.4, 0.6, 0.8 and 1.0% were examined on the effect of growth.

The supplement of soy peptone did not obviously modify the growth curve from 0 to 1.0% soy peptone as can be seen from Figure 3.3.

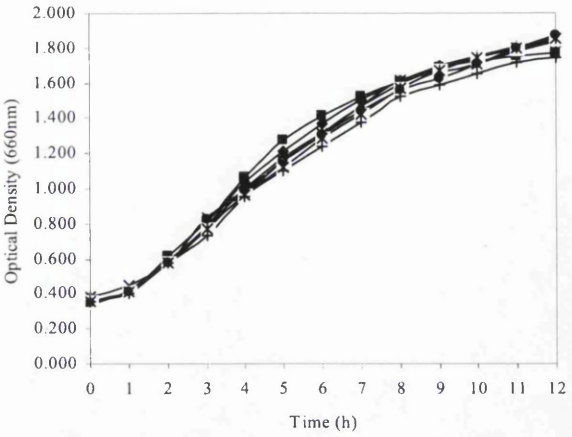


Figure 3.3 *The growth curve of L. delbrueckii grown in pressure tube at different soy peptone concentrations from 0 % (■), 0.2% (◆), 0.4% (▲), 0.6% (×), 0.8% (∗), 1.0% (●) and 1.2% (+). The growth condition was started at pH 6.5 using KOH and the temperature was controlled at 37 °C. OD was measured with 18 mm light path (pressure tube diameter).*

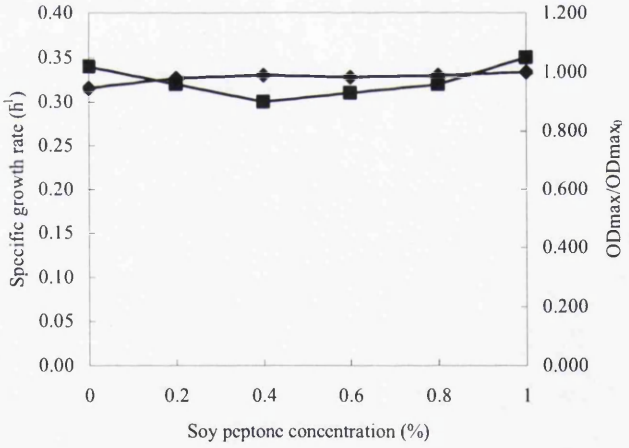


Figure 3.4 *Effect of soy peptone concentration on the specific growth (■) and growth yield ratio OD_{max}/OD_{max0} (◆) of L. delbrueckii. Operating conditions are mentioned in Figure 3.3.*

The trend of yields on various concentrations (Figure 3.4) was relatively stable. No clear change on growth was observed when concentration of peptone was increased up to1.0%. Although soy peptone did not show clearly effect on the growth, it affected the rate of

growth. For example, in Figure 3.4 the specific growth rate at 0.4% soy peptone was 0.30 h^{-1} whereas at 0.8% was 0.32 h^{-1} and the highest was 0.35 h^{-1} at 1.0% soy peptone.

3.2.2 The effect of carbon source on growth

The carbon source is normally a major ingredient of the culture medium and used as primary energy source for bacteria. The carbon source is a major constituent of cellular material. Then the cell biomass is proportional to the initial concentrations of carbon source. Although, carbon source is important for growth, the inhibitory effect from substrate or end-product formation must be considered.

Smart *et al.* (1993) found that when cultivated 19 out of 21 lactobacillus strains containing β-galactosidase on media containing glucose or glucose plus lactose. They showed very low or non-detectable β-galactosidase activity. Hickey *et al.* (1986, cited in Vasiljevic and Jelen, 2001) observed that β-galactosidase activity of several *L. bulgaricus* strains significantly declined when adding small amounts of glucose to a growing culture. It could be indicated that glucose could cause partial repression of the lac operon, resulting in the lower β-galactosidase activity. Chervaux *et al.* (2000) also indicated that the growth rate of *L. bulgaricus* was markedly increased in lactose rather than on glucose, mannose and fructose. The doubling times of *L. bulgaricus* in medium containing lactose was about an hour while with glucose was 3 hours. Better growth in lactose might be due to characteristics of the transporters involved in glucose or lactose uptake (Chervaux *et al.*, 2000).

Thereby lactose was chosen to use as carbon source through this project. The lactose concentrations tested were 0, 0.4, 0.8, 1.2, 1.6 and 2.0%. It can be seen from Figure 3.5 that lactose had significant effect on the cells growth. The growth was the proportional to the lactose concentration. *L. delbrueckii* did not grow in the medium without lactose (0%).

After 7 hours the cells density in 0.2% lactose medium started to drop. This was probably from the limitation of lactose.

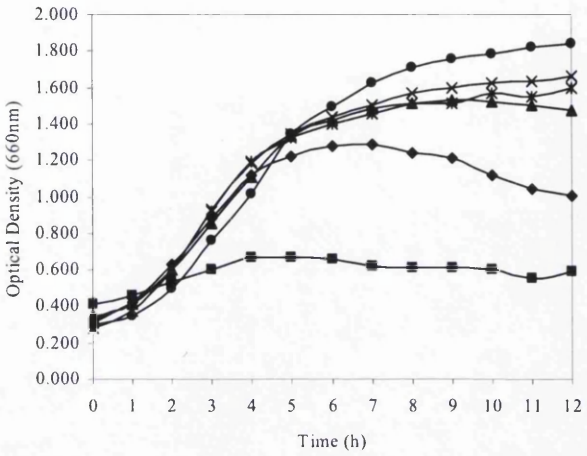


Figure 3.5 *The growth curve of L. delbrueckii grown in pressure tube at different lactose concentrations from 0 % (■), 0.4% (◆), 0.8% (▲), 1.2% (×), 1.6% (✳) and 2.0% (●). The growth condition was started at pH 6.5 using KOH and the temperature was controlled at 37 °C. OD was measured with 18 mm light path (pressure tube diameter).*

The effect of lactose concentration on the specific growth rate (μ) is shown in Figure 3.6. High lactose concentrations stimulated the growth rate. The maximum specific growth rate (μ_{max}) was 0.35 h^{-1} at 2.0% lactose. The specific growth rate at 1.2% and 1.6% were 0.34 h^{-1} which were not much different from 2.0%. Figure 3.6 also represents the OD_{max}/OD_{max0} at different concentrations. The growth ability increased when high concentration of lactose was present. The highest yield ratio (OD_{max}/OD_{max0}) was at 2.0% lactose concentration. Although the specific growth rates from lactose concentration in range of 1.2 and 2.0% were not significantly, the better cell density was achieved at high lactose concentration.

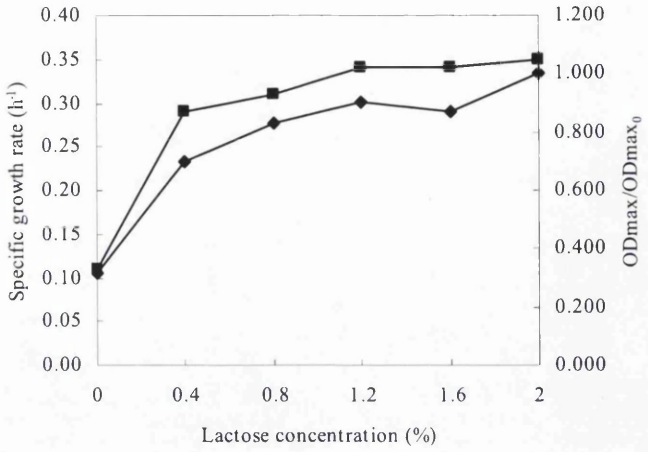


Figure 3.6 *Effect of lactose concentration on the specific growth (■) and growth yield ratio OD_{max}/OD_{max0} (◆) of L. delbrueckii. Operating conditions are mentioned in Figure 3.5.*

3.2.3 The effect of potassium dihydrogen phosphate

The addition of potassium dihydrogen phosphate (KH_2PO_4) in the medium at 0.25% and 0.5% (w/v) did not impact cells density much in comparison to medium without phosphate whereas the growth curve of cells at concentration 0.75% and 1.0% showed lower cells density (Figure 3.7).

The cell yields presented in Figure 3.8 indicates that the growth of *L. delbrueckii* appeared to be inhibited by phosphate. The cell biomass declined when increased KH_2PO_4 concentration. However, phosphate can increase buffering capacity in medium. Phosphate also requires for phage resistance because phosphate binds calcium while phage requires calcium to attach themselves to the bacterial cells (Potter and Nelson, 1953). Slow growth rate was observed when the medium containing no potassium phosphate or above concentration 0.5%. Although the specific growth rates at both 0.25% and 0.5% concentrations were about equal (Figure 3.8), the phosphate concentration at 0.25% provided higher cells density and suited to use through the project.

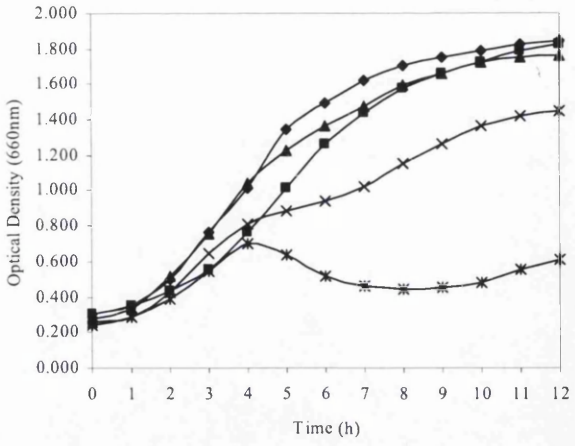


Figure 3.7 *The growth curve of L. delbrueckii grown in pressure tube at different KH_2PO_4 concentrations from 0 % (■), 0.25% (◆), 0.50% (▲), 0.75% (×) and 1.0% (✳). The growth condition was started at pH 6.5 using KOH and the temperature was controlled at 37 °C. OD was measured with 18 mm light path (pressure tube diameter).*

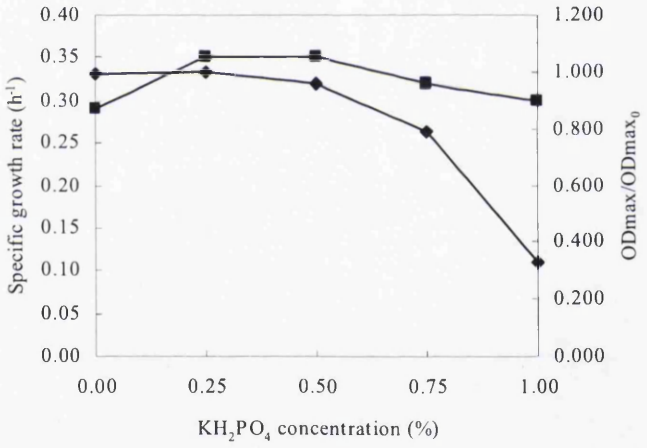


Figure 3.8 *Effect of KH_2PO_4concentration on the specific growth (■) and growth yield ratio OD_{max}/OD_{max0} (◆) of L. delbrueckii. Operating conditions are mentioned in Figure 3.7.*

3.2.4 The effect of sodium acetate

In addition to phosphate buffer, culture medium may be buffered by sodium acetate to facilitate growth without acid inhibition. Chervaux *et al.* (2000) mentioned that increased

buffering capacity probably affected the growth of *L. bulgaricus* CNRZ 397. The growth yield was higher when acetate was increased in medium to 75 mM or addition of another buffer in the presence of 15 mM also increased the yield. Peter and Snell (1954) found sodium acetate was an essential nutrient for *L. delbrueckii* strain 9649. The small amount of acetate added to a phosphate buffer medium greatly increased the growth rate of these bacteria while the growth in the absence of sodium acetate was very slow.

The effect of sodium acetate on the growth of *L. delbrueckii* was tested. A comparison of the cell growth on media with and without sodium acetate addition was found that medium containing sodium acetate enhanced the cells density as shown in Figure 3.9. However, increasing acetate at high concentration did not significantly improve cell growth. The maximum cell growth was obtained at 0.5 %.

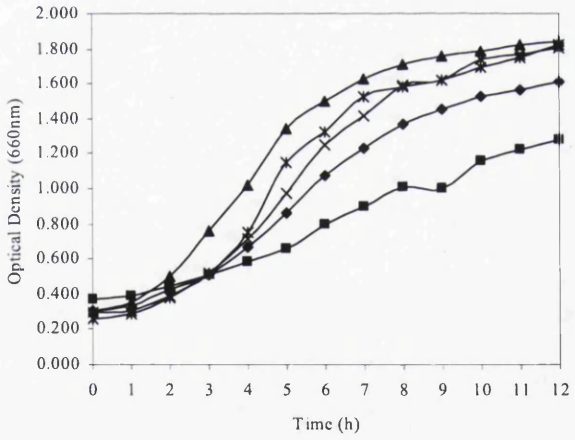


Figure 3.9 *The growth curve of L. delbrueckii grown in pressure tube at different sodium acetate concentrations from 0 % (■), 0.25% (◆), 0.50% (▲), 0.75% (×) and 1.0% (✳). The growth condition was started at pH 6.5 using KOH and the temperature was controlled at 37 °C. OD was measured with 18 mm light path (pressure tube diameter).*

The specific growth rate was affected by sodium acetate concentration (Figure 3.10). The rate increased following the concentration from 0% to 0.5% (0.13 h^{-1} to 0.35 h^{-1}) and it

was slightly decreased at higher concentration. Figure 3.10 also shows that the addition of sodium acetate to medium improved cell yield. The yields increased in proportion to sodium acetate concentration. The concentration varied from 0.5% to 1.0% did not show great different yields. Then the results indicated that 0.5% sodium acetate was satisfied to provide enough nutrients and support the growth of the culture.

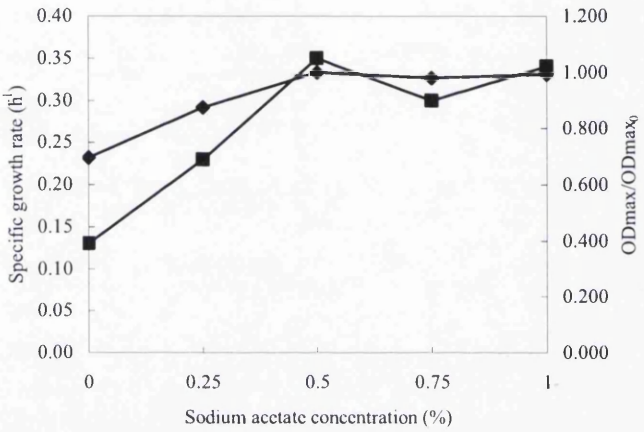


Figure 3.10 *Effect of sodium acetate concentration on the specific growth (■) and growth yield ratio OD_{max}/OD_{max0} (◆) of L. delbrueckii. Operating conditions are mentioned in Figure 3.9*

3.2.5 The effect of triammonium citrate

Five concentrations of triammonium citrate (0, 0.2, 0.4, 0.6, 0.8 and 1.0%) were examined on the effect of growth. It can be seen from Figure 3.11 when using high concentration of triammonium citrate, the growth inclined decrement. The growth was completely inhibited in the present concentration from 0.6% to 1.0%.

The specific growth rate and yield were significantly lower when more concentrations of triammonium citrate were presented (Figure 3.12). Although the presence of triammonium citrate seemed to reduce the growth, it has been known that many microorganisms including some lactic acid bacteria are incapable of growing in the absence of carbon

dioxide. Henriksen *et al.* (2000) said there was no growth of *L. lactic* observed when the culture were sparged with pure nitrogen whereas the culture performed exponential growth in the presence of little amount of carbon dioxide. They also found citrate was partially able to replace carbon dioxide required for growth of *L. lactic.* Drinan *et al.* (1976) indicated the function of citrate in medium can serve as an additional source of carbon and pyruvate. In the absence of citrate, the rate of pyruvate formation is rate limiting in the heterofermentative lactobacilli. Harvey and Collins (1963) found citrate also acts as a source of carbon for synthesis of some essential cell constituent. Thereby 0.2% triammonium citrate was chosen to support the growth for the project.

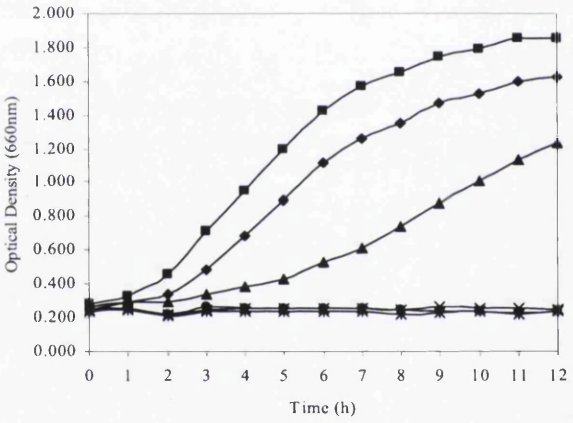


Figure 3.11 *The growth curve of L. delbrueckii grown in pressure tube at different triammonium citrate concentrations from 0 % (■), 0.2% (◆), 0.4% (▲), 0.6% (×), 0.8% (✳) and 1.0% (●). The growth condition was started at pH 6.5 using KOH and the temperature was controlled at 37 °C. OD was measured with 18 mm light path (pressure tube diameter).*

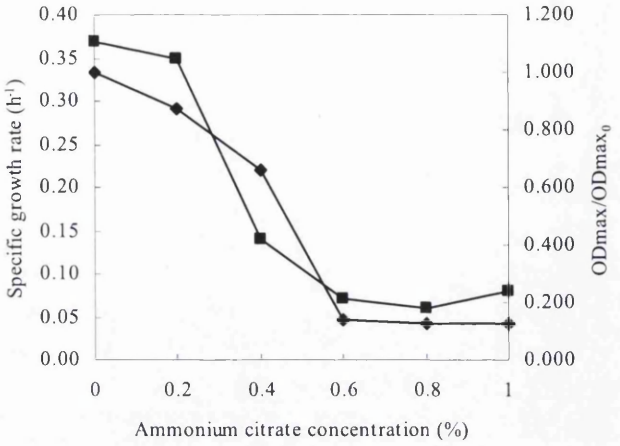


Figure 3.12 *Effect of triammonium citrate concentration on the specific growth (■) and growth yield ratio OD_{max}/OD_{max0} (◆) of L. delbrueckii. Operating conditions are mentioned in Figure 3.11.*

3.2.6 The effect of tween80

Tween80 (Polyoxyethylene sorbitan monooleate) is generally added to the growth media of Lactobacillus because it can enhance the growth of many lactic acid bacteria (Johnsson *et al.*, 1995). Partanen *et al.* (2001) indicated from 6 of 7 *L. delbrueckii* strains needed tween80 as a fatty acid to supplement the growth. Chervaux *et al.* (2000) found that tween 80 was an essential element for efficient growth of *L. bulgaricus* CNRZ 397. It was used as putative lipid source for bacteria and also acted as a detergent in the medium.

The presence of tween80 at concentration 0.1% did not greatly effect growth of *L. delbrueckii* in comparison to 0.01% tween80 and without tween80 (Figure 3.13). However, 0.1% (v/v) tween80 slightly encouraged specific growth rate and cell yield (Figure 3.14).

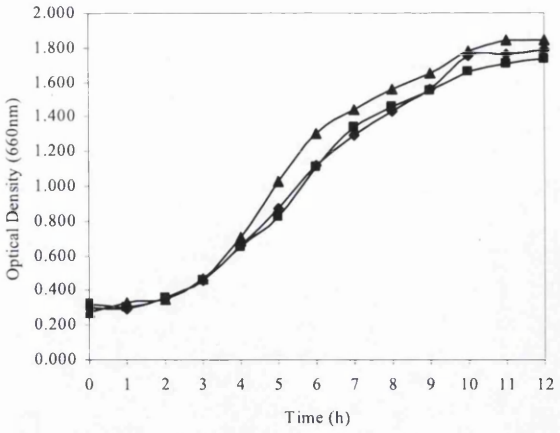


Figure 3.13 *The growth curve of L. delbrueckii grown in pressure tube at different tween80 concentrations from 0% (■), 0.01% (◆) and 0.1% (▲). The growth condition was started at pH 6.5 using KOH and the temperature was controlled at 37 °C. OD was measured with 18 mm light path (pressure tube diameter).*

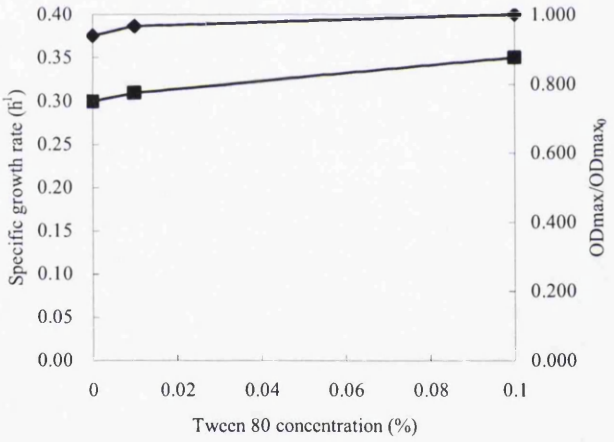


Figure 3.14 *Effect of tween80 concentration on the specific growth (■) and growth yield ratio OD_{max}/OD_{max0} (◆) of L. delbrueckii. Operating conditions are mentioned in Figure 3.13*

3.3 The effect of medium pH on the growth of *Lactobacillus delbrueckii* ssp *bulgaricus*

The influences of pH on culture growth from 4.5, 5.0, 5.5, 6.0, 6.5, 7.0 and 7.5 were determined in 5-l batch fermenter. About 10% v/v inoculum culture was inoculated into 4 l sterile medium. The cells were cultivated for 10 hours at controlling temperature 37 °C. The samples were taken from the fermenter to determine the cell concentration using spectrophotometer at OD 660 nm every hour and the amount of 4M NaOH added in system to maintain at its pH set point was recorded.

The growth curve of *L. delbrueckii* at different pH values is presented in Figure 3.15. The growth of cells was concerned with pH. The cell density increased very slowly at pH 4.5 and 7.0 and the growth was inhibited at pH above 7.5. The culture grew well at pH values from 5.0 to 6.5. Growing cells at pH 6.0 and 6.5 after reaching the stationary phase, the maximum optical densities were lower in comparison to pH 5.0 and 5.5.

Although the cells density between pH 5.0 and 5.5 were not significantly different, the cultivation time for pH 5.5 was shorter. The average time taken for cells cultivated at pH 5.0 to reach stationary phase was about 7 hours while pH at 5.5 was around 5 hours.

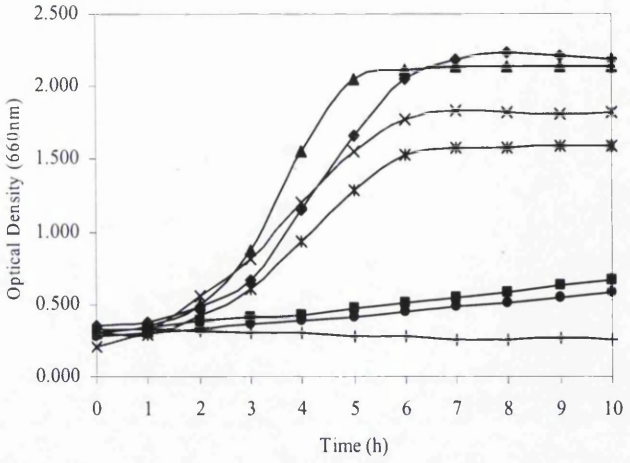


Figure 3.15 *The growth of L. delbrueckii ssp bulgaricus cultivated in various pH medium from 4.5 (■), 5.0 (◆), 5.5 (▲), 6.0 (×), 6.5 (✳), 7.0 (●) and 7.5 (+) in batch fermenter containing 4 l working volume. The inoculum size used was 10 %v/v. The temperature was controlled at 37 °C. The stirring velocity was maintained at 200 rpm.*

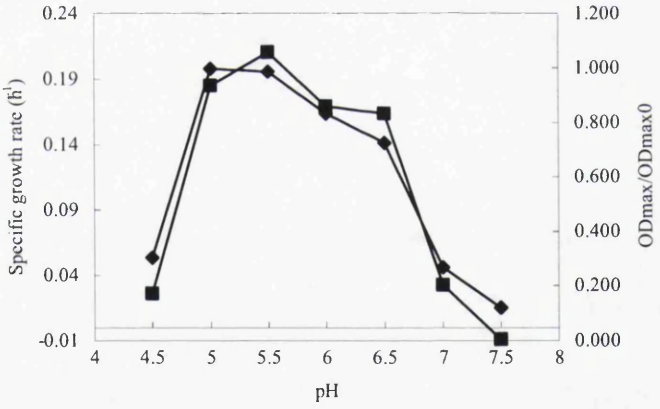


Figure 3.16 *The specific growth rate (■) and growth yield ratio OD_{max}/OD_{max0} (◆) of L. delbrueckii versus different pH conditions. Operating conditions are mentioned in Figure 3.15.*

The effects of pH control during cultivation on the specific growth rate of cells and growth yield ratio are indicated in Figure 3.16. The specific growth rate of pH values from 5.0 to 6.5 were in range 0.16-0.21 for this operating condition. The highest specific growth rate was 0.21 at pH control 5.5 while at pH 4.5 and 7.0 showed very low rate and totally

inhibited the growth at pH 7.5. Good growth yield ratio was obtained at pH value range 5.0-5.5.

The results appeared to agree with Gadgil and Venkatesh (1997). The highest cell concentration obtained from MRS medium was at pH around 5.6 in comparison with pH 4.2 and 5.0. In addition to the effect on cells growth, the medium pH strongly affects the chain length of cells. Rhee and Pack (1980) studied the relationships between a medium pH and the chain length of *L. bulgaricus* cells. The cell chains increased their lengths with an increase in pH from 4.5 to 8.0. At fixed pH 4.5, most of the cells had chain length approximately 8 μm with remaining single or paired form and the length was able to increase to about 100 μm at pH 7.5 whereas at pH above 8.0 the cell chains became folded into clumps and the growth was very poor. They also found that the formation of long chains under alkaline conditions was due to the difficulty in synthesis of the autolytic enzyme for dechain the cells while pH in range 5.0 to 6.5 was an optimum pH for enzyme activity and synthesis.

The growth of *L. delbrueckii* ssp *bulgaricus* was also indirectly interpreted from the amount of NaOH used. In general the pH is dropped when the cells excrete products. The faster the growth, the more rapidly the medium becomes acid and needs more alkaline to keep the pH stable. Consequently, the amount of NaOH, as a neutralizer, used to control pH during cultivation can inform the proportion of growth, as can be seen in Figure 3.17. The cultures cultivated at medium pH 5.5 showed maximum total amount of NaOH used for adjusting pH in comparison to the culture grown in medium others pH.

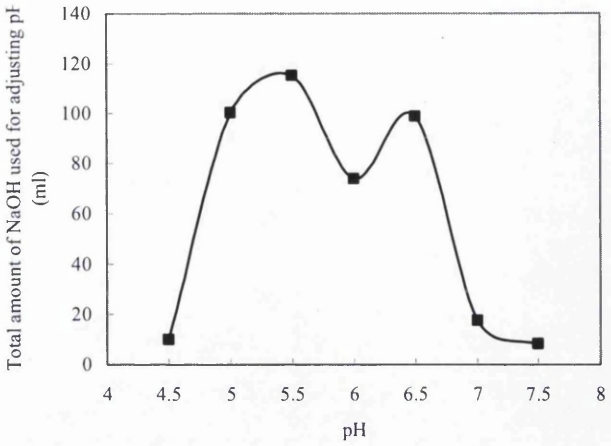


Figure 3.17 *The total amount of NaOH used for adjusting pH for cultivation Lactobacillus delbrueckii ssp bulgaricus in various pH mediums. Operating conditions are mentioned in Figure 3.15.*

3.4 Growth of *L. delbrueckii* in pilot batch fermenter

The medium was prepared according to the results from preliminary development of growth medium (section 3.2) and used in scale-up biomass of *L. delbrueckii*. After sterilization of 80 l growth medium in steam-jacketed fermenter, the anaerobic condition was prepared by flushing with nitrogen gas for at least 30 min into medium. The fermenter system was set at temperature 37 $\pm$ 1 °C, agitation velocity was at 60 rpm. The pH was controlled at 5.5 $\pm$ 0.2 with 10 M NaOH. The 20 l of *L. delbrueckii* inoculum was then transferred into fermenter.

Scale-up cultivation in batch fermenter was conducted in order to produce large volumes of culture for further experiment. The result of cells growth is shown in Figure 3.18. The optical density of cell at 660 nm slowly increased in first 3 hours and grown rapidly during time 3-7 h with the corresponding amount of NaOH used for adjusted pH. After 7 hours the growth reached stationary phase. The approximate optical density during stationary phase was 3.908. The specific growth rate of cells in this system condition was 0.61 h^{-1}.

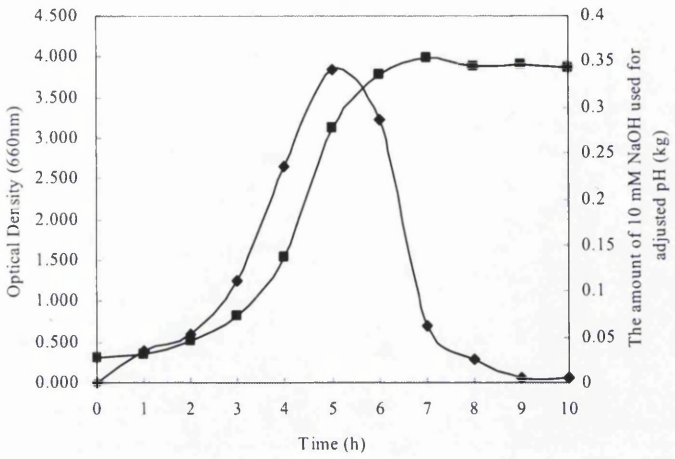


Figure 3.18 *The growth curve (■) and the amount of NaOH used (◆) to control the pH in 100 l batch fermenter system with the cultivation time (h). The pH value was set at 5.5 ± 0.2 with 10 M NaOH. The temperature was controlled at 37 ± 1 °C and agitation velocity was at 60 rpm.*

3.5 Conclusions

Using the optimised medium, good growth could be obtained on pressure tube to the 100 l pilot scale.

1. From the preliminary development of growth medium results, it can be seen that the growth performance of *L. delbrueckii* varied according to the composition of culture medium. A medium containing no meat extract was produced. The nitrogen source, yeast extract, played an important role affecting both the cell growth rate and yield while the other nitrogen source, soy peptone at the concentration present in the experiment did not show obviously effect on optical density and not improve specific growth rate much. Kask *et al.* (1999) indicated that most lactic acid bacteria have limited biosynthetic abilities. Therefore, the addition of soy peptone enriched with amino acid was essential for these organisms incapable of synthesizing some certain amino acids in the cells.

Lactose was used as carbon source since it encouraged the cells to produce enzyme β-galactosidase rather than using glucose (Smart *et al.*, 1993; Vasiljevic and Jelen, 2001). Lactose had significant effect on the cells growth. High lactose concentration (2.0% w/v) gave good results in both cell growth rate and growth yield.

The concentration of KH_2PO_4 in medium upper 0.75% decreased the cell density and specific growth rate while the concentration in range 0.25% to 0.5% did not affect cells density much comparing to medium without KH_2PO_4 and provided slightly higher specific growth rate. In case of sodium acetate, the cell yield increased in proportion to sodium acetate concentration. However, the concentration in range from 0.5% to 1.0% did not show great different yield. Therefore at 0.5% sodium acetate in medium was satisfied for supporting the growth.

Increasing triammonium citrate concentration seemed to inhibit the growth. Low concentration of citrate needs to be added into medium to act as a source of carbon for synthesis of some essential cell constituent. The presence of tween80 provided slightly effect on specific growth rate and cell yield. Although the effects of magnesium and manganous sulphate did not investigate because they are very small amount use and may not perform the big different on growth, these elements can play a role as key.catalyst or stabilizer of tertiary structure on the metalo-enzymes and can act as cofactor for superoxide dismutase (SOD) to neutralize the toxicity by oxygen for anaerobic survival (Shin and Park, 1997; Chang and So, 1998; Ahn *et al.,* 2001). Therefore, only adequate levels of these elements based on used in MRS was directly applied in this medium.

2. The studied results on growth media for *L. delbrueckii* in pressure tube medium with the pH starting point at 6.5 indicated that the differences in the component of culture medium can significantly affect growth performance. The suitable ingredients consisted of yeast

extract 10 g/l, soy peptone 10 g/l, lactose 20 g/l, KH_2PO_4 2.5 g/l, sodium acetate 5 g/l, triammonium citrate 2 g/l, tween80 1 ml/l, magnesium sulphate 0.2 g/l and manganous sulphate 0.05 g/l. This media produced the desirable specific growth rate of the cells at 0.35 $h^{-1}$and used for further growth.

3. The investigation on the effect of pH against the cell growth was conducted in a wide range of pH values from 4.5 to 7.5 at control temperature 37 °C. The pH factor clearly affected the growth of *L. delbrueckii*. The cells grew slowly at pH value 4.5 and upper pH 7.0. The optimum pH was range from 5.0 to 5.5. Although at pH 5.0 provided slightly higher cell density, at pH 5.5 was chosen because growth time to stationary phase was shorter.

When the cells cultivation reached stationary phase, the medium was removed using MF membrane system with phosphate buffer. The cell suspension in buffer was further disrupted to release the enzyme β-galactosidase. The enzyme was separated and investigated its properties in the next chapter.

Chapter 4

Purification and Characterization of β-galactosidase

4.1. Introduction

The aim of the work in this chapter was to purify and characterize the β-galactosidase from *L. delbrueckii* ssp *bulgaricus*. The process of isolation and purification of intracellular enzyme generally require a primary stage of cell disruption and subsequent separation of soluble proteins and enzyme from cell debris. In this experiment, the cells were disrupted with pressure homogenizer. The influence of operating pressure during the cell disruption was determined. After cell disruption, two separation methods, aqueous two phase system and membrane separations were investigated. The removal of enzyme and protein from cells debris were measured and used in calculation of purification factor. The easier method and providing good enzyme recovery will be selected for enzyme properties experiments.

Although the enzyme is widely produced and found in nature in animals, plants and microorganisms, different sources of the enzyme or even environmental conditions of the enzyme production give enzyme of different quantities. Several environmental factors can seriously affect the activity and the stability of the enzyme by changing the specific three dimensional structures or spatial conformation of the protein (Jurado *et al.,* 2004). These can be related to physical conditions such as shaking, pumping, pH, temperature and chemical conditions from inhibitor and activator. It is necessary to determine the properties of the enzyme before using it with membrane reactor system in order to facilitate the effective design of process in hydrolysis of the lactose from milk. Better knowledge of enzyme properties under operating conditions could help optimize product formation in the process and so aid the overall viability of the process.

In this study, the effect of shaking, optimal pH, effect of temperature and stability of β-galactosidase were investigated. The kinetic studies of both o-nitrophenyl-β-D-galactosidase (ONPG) and lactose were carried out. The effects of the end products, galactose, glucose and some mono and divalent cations were also investigated as part of the kinetic characterization.

4.2 The effect of pressure used on cell disruption

Lactobacillus delbrueckii ssp *bulgaricus* produces β-galactosidase as an intracellular enzyme. The enzyme is not released to the outside of cells during fermentation condition. Moreover *L. delbrueckii*, Gram positive bacteria, have a very thick cell wall and are harder to disrupt in comparison to Gram negative bacteria and yeasts (Bury *et al.*, 2001). The potential method of disruption must ensure that the released high enzyme is achieved and not denatured by the process. Many techniques, based on mechanical or chemical methods, are available and satisfactory only at laboratory scale while appropriate proved methods for large scale production are limited. The mechanical methods are usually preferable as most suitable for large scale. One of the common methods used in large scale for cell disruption is forcing the cell suspension through a small orifice at high pressure called as the high pressure homogenizer. The cells are exploded by the extremely high shear forces generated during suspension pass through the system. In general, homogenizer is operated at pressure in range 50 to 120 MPa, then the cell suspension is required to pass the homogenizer several passages to obtain maximum yield. However, the number of passes in the system leads to controlling temperature problem. Bury *et al.* (2001) investigated the release of β-galactosidase from *L. delbrueckii* by using the Rannie high pressure lab homogenizer at 135 MPa and found the temperature was increased 36 °C per pass or 0.26 °C per MPa. The suspension was passed through the system for three times and each passage was cooled to decrease the denaturation of the enzyme by heat except during the second and the third passage the suspension was not cooled. It was found that the activity of the enzyme was not raised between the second and the third pass. The possible reason was some heat occurred and inactivated the enzyme resulting in no net gain or loss, although an extra enzyme may have been released during the passage.

Lovitt *et al.* (2000) investigated a new type of commercial cell disrupter manufactured by Constant Systems Ltd (Daventry, UK) which is capable of operation at very high pressures of up to 275 MPa (40 kpsi). The low pressure chamber of this equipment is design with integral cooling jacket, so the problem of excessive temperature occurred during operation at high pressure is controlled. In addition to this advantage, volumes of a few millilitres or several litres of working cell suspension can be used with ease.

The efficient cell disrupter was chosen in this study to find out an appropriate pressure used for disruption. The results from disruption at various pressures were compared by the optical density at 660 nm using spectrophotometer, release of protein content and release of intracellular β-galactosidase.

Cells were grown as described in section 2.5 of the Materials and Methods. The cells were harvested using microfiltration membrane as describe in section 2.6 and diluted 10 times to measure to optical density of cell suspension. Then 200 ml of cell suspensions with temperature about 15 °C were passed through the disrupter at different pressures ranging from 5 to 40 kpsi. The cell suspension was drawn into the high-pressure chamber and passed the inlet valve. The inlet valve was rapidly closed by the pressure generated in the cylinder and the cell suspension was simultaneously forced through the jet orifice which has diameter 0.18 mm. The cell disrupted is thought to take place when they pass quickly from the area of high pressure to the atmospheric pressure in the disruption chamber. The outer chamber is connected with chilled water to cool and keep temperature for chamber system.

The effect of disruption pressure on the reduction of cell suspension optical density and release of protein are shown in Figure 4.1. The operating pressure was related to the reduction of optical density at 660 nm and this indicative of particle size reduction. The cell optical density decreased rapidly from undisrupted cells at O.D. 14.2 to 2.2 when the operating pressure was increased up to 40 kpsi.

The effect of operating pressure in disruption was also assessed as soluble protein release from cell suspension. The total protein of undisrupted cell suspension was determined by treating the suspension in 0.1 M NaOH at 90 °C for 10 min. This figure was then used for comparison with free protein released from disrupted cells. The percentage of protein released was related to operating pressures. The protein released increased from 42.5% when no pressure used in disrupter to 62.2% protein released at pressure 5 kpsi. It increased slightly during pressure 15-30 kpsi and rose gradually up to 81% at pressure 40 kpsi. It can be seen that increasing operating pressure released more protein into a soluble form. The high initial levels of protein are caused by release of protein due to freeze and thaw disruption.

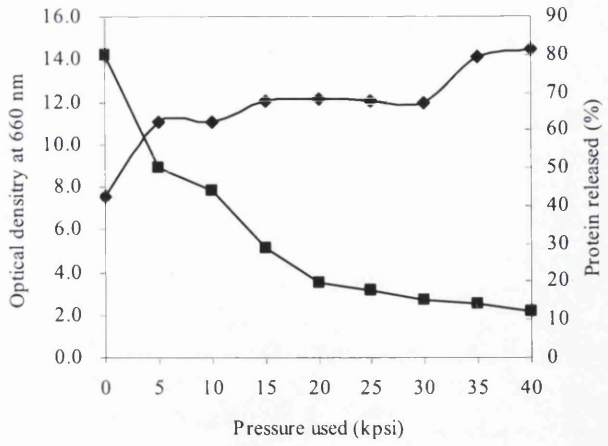


Figure 4.1 *The optical density of disrupted cells (■) and the percentage of protein released (◆) versus various operating pressures of high pressure homoginizer.*

The cell free supernatant from disrupted sample was determined for the intracellular enzyme, β-galactosidase. The correlation of enzyme activity and enzyme specific activity with operating pressure are shown in Figure 4.2. The enzyme activity of samples was slightly increased as operating pressure rose. However, the activity released did not greatly affect when the pressure was used from 25 kpsi to 40 kpsi. The maximum enzyme activity was detected at 35 kpsi with 2.37 μmol/min.

The trend of specific activity was not stable. It slightly rose at pressured used from 5 to 30 kpsi and decreased at pressure after 30 kpsi. The reason of declining was the enzyme levels in solution did not increase but they remained almost constant at high pressure while greater quantities of protein became solubilized at high operating pressure. The highest specific activity was found in range 25 to 30 kpsi with a maximum of 1.54 μmol/min/mg protein.

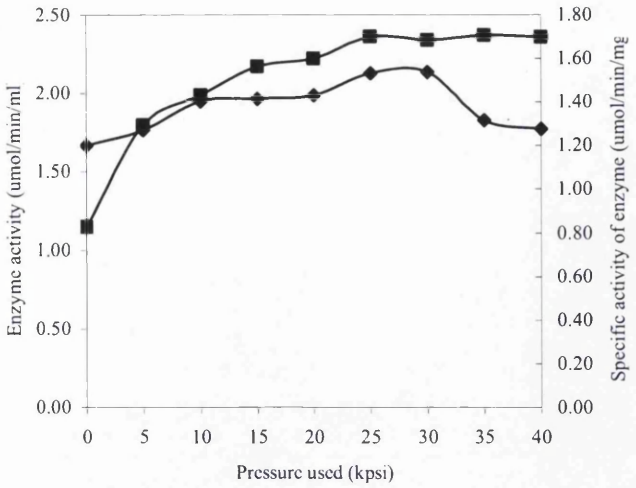


Figure 4.2 *The activity of β-galactosidase (■) and specific activity of β-galactosidase (◆) versus operating pressure.*

Bury *et al.* (2001) studied three methods of cell disruption in *L. delbruekii*, a sonication, a high-speed bead mill and a high-pressure homoginizer. The results were compared by the release of intracellular β-galactosidase and by SEM. The amount of enzyme released from sonication was less than half that released by the bead mill or high-pressure homoginizer and the result from SEM showed that more intact cells were observed after 6 min of sonication in comparison to other two methods. In addition, it was found that the bead milling and high-pressure homoginizer methods were suitable for large scale while sonication was limited to bench scale production.

4.3 Aqueous two phase separation

Aqueous two phase systems are useful for the separation and purification of enzyme because of fewer problems in denaturing fragile molecules, then simplicity, then low cost and relatively easy to scale up. The systems investigated in this study were composed of polymer (PEG) and salt (KH_2PO_4). The partition is occurred by the unvarying distribution of the salt ions in the top and bottom phase and by the different of the electrostatic which causes from inequality of the salt distribution (Han and Lee, 1997). In general, phosphate ion is found in the bottom phase. So the bottom phase enriches with negative charge rather than the top phase. Therefore, the enzymes might prefer to partition to the phase which enriches the opposite but complementary surface charge. The aim of this work was to

extract a high β-galactosidase recovery from cells debris in two-phase system without affecting biochemical properties. Many parameters were considered to obtain satisfactory enzyme purification and product yield. Three types of PEG molecular weight (MW 1500, 3350 and 8000) were investigated on various concentrations. In addition the role of salt concentration, pH and influence of NaCl were also considered.

4.3.1 Phase diagrams of aqueous two phase systems

The first stage of the work was to establish the phase diagram for the system. This was done by mixing the two components until the cloud point (phase separation) was observed. A stock solution containing 45% w/w polyethylene glycol (PEG MW.1500, 3350 and 8000) and a stock solution containing 30% w/w potassium dihydrogen phosphate (KH_2PO_4) were prepared. The salt stock solution was adjusted pH to 6.5 using KOH. Different amounts of PEG stock solution were taken into 20 ml test tubes and diluted with demineralized water until the final total volume in each tube was 5 ml. Then a small amount of salt stock solution was gradually added to each of the tubes. After adding salt solution, each time the tube was shaken with a rotamixer. The salt solution was added until the cloud point shown. This first appearance of turbidity indicated that the system was about to enter the two-phase area. The experiments were carried out at temperature 25 °C and done in triplicate.

Phase diagram of the different PEG molecular weights and potassium dihydrogen phosphate at pH 6.5 performed concentrations of phase components at which separation occured above binodial line. After phase separation was allowed to take place, a high concentration of PEG was found at the upper phase while the lower became a salt rich phase. Figure 4.3 shows that the amount of salt needed for phase separation decreased with increasing the concentration of PEG. As can be seen from curve, the molecular weight of polymer influenced the phase diagram. Lower concentration of polymer was needed for two phase separation when the higher molecular weight was applied.

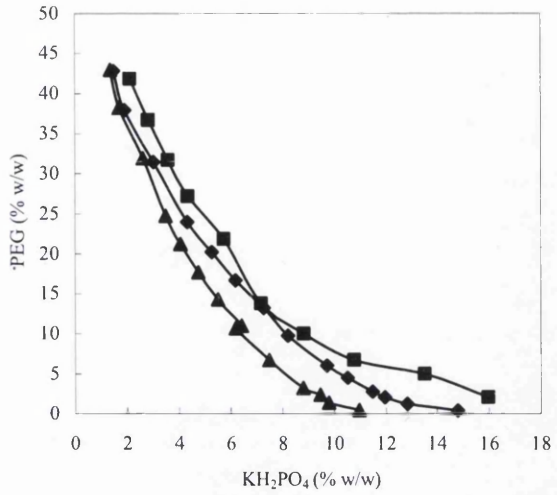


Figure 4.3 *Phase diagram of PEG 1500 (■), 3350 (◆) and 8000 (▲) with KH_2PO_4 two phase system, pH 6.5, temperature at 25 °C.*

4.3.2 Partitioning behaviours of β-galactosidase in different molecular weights and concentrations of polyethylene glycol

After the binodial curves were determined, combinations of polymer and salt were selected for enzyme partitioning test. The partitions of β-galactosidase in the systems composed of three molecular weights of PEG (1500, 3350 and 8000) with different concentrations were studied. In this experiment, the concentration of KH_2PO_4 was kept constant at 8% w/w and pH was set with potassium hydroxide to be 6.5. Then calculated amount of PEG was added to this solution. Finally, a certain amount of debris suspension was weighed out (10 g) and dissolved in the solution and the total mixture was set to a total mass of 30 g by adding demineralised water. The phase system was completely mixed before settling for phase separation. The system was then left to equilibrium at temperature 25 °C for overnight. The phases between upper and bottom were carefully separated using a syringe with a long needle. The volume of phase, enzyme and protein partitioning in each phase were determined.

The partition coefficient, K is defined as the ratio of total protein concentration or enzyme activity in the top phase to in the bottom phase.

$$K_P = \frac{P_T}{P_B} \qquad \textit{Equation 4.1}$$

$$K_E = \frac{E_T}{E_B} \qquad \textit{Equation 4.2}$$

where P_T and P_B are total concentration of protein (mg/ml) of top and bottom phase respectively. E_T and E_B are the enzyme activity in the top and bottom phase respectively.

The ratio of volume in top phase to bottom phase (R_V) is defined as the volume of the top phase (V_T) to the bottom phase (V_B), yield recovery from top phase (R_T) and from bottom phase (R_B) were calculated in order to evaluate the purification ratio (PR) from aqueous two phase system, according to given equations:

$$R_V = \frac{V_T}{V_B} \qquad \textit{Equation 4.3}$$

$$R_T = \frac{100}{1 + \frac{1}{R_V K_{(P \text{ or } E)}}} \qquad \textit{Equation 4.4}$$

$$R_B = \frac{100}{1 + R_V K_{(P \text{ or } E)}} \qquad \textit{Equation 4.5}$$

$$PR = \frac{\text{Recovery of Enzyme from phase}}{\text{Recovery of Protein from phase}} \qquad \textit{Equation 4.6}$$

The effect of PEG concentrations on enzyme and protein recovery in the top phase are shown in Table 4.1. It was found that increasing the molecular weight of PEG in comparison to the same concentration showed a tendency of the β-galactosidase recovery to be concentrated in the bottom salt-rich phase. The enzyme preferred partitioning in salt-rich phase rather than in the top PEG-rich phase. So partition selectivity was low at high molecular weight PEG since the separation of enzyme from cell debris and contaminating proteins was poor.

Obviously, the concentration of PEG 8000 from 8.5% to 13.5% w/w showed enzyme recovery in top phase decreased from 81.33% to 0.39% respectively and adding PEG

Table 4.1 *Partitioning behaviours of β-galactosidase in two phase systems with different molecular weights and concentrations of polyethylene glycol when the concentration of KH_2PO_4 was fixed at 8% w/w. The pH value in the system was at 6.5 and temperature was at 25 °C.*

molecular weight	system composition (%w/w)		Volume	K_{enzyme}	enzyme recovery		$K_{protein}$	protein recovery		purification factor	
of PEG	PEG	KH_2PO_4	(top/bottom)		Top phase	Bottom phase		Top phase	Bottom phase	Top phase	Bottom phase
1500	12.5	8	3.78	1.795	87.15	12.85	2.274	89.57	10.43	0.97	1.23
	13.5	8	2.73	1.554	80.93	19.07	3.636	90.85	9.15	0.89	2.08
	14.5	8	2.26	1.357	75.41	24.59	1.022	69.79	30.21	1.08	0.81
	15.5	8	2.44	1.408	77.45	22.55	1.424	77.66	22.34	1.00	1.01
	16.5	8	1.91	1.076	67.24	32.76	1.200	69.59	30.41	0.97	1.08
3350	10.5	8	2.13	1.284	73.19	26.81	1.346	74.10	25.90	0.99	1.04
	11.5	8	2.57	1.457	78.93	21.07	2.153	84.70	15.30	0.93	1.38
	12.5	8	3.21	1.651	84.14	15.86	1.990	86.48	13.52	0.97	1.17
	13.5	8	6.14	2.265	93.30	6.70	3.046	94.93	5.07	0.98	1.32
	14.5	8	2.85	0.020	5.41	94.59	0.192	35.39	64.61	0.15	1.46
8000	8.5	8	2.85	1.531	81.33	18.67	2.052	85.38	14.62	0.95	1.28
	9.5	8	6.65	0.666	81.60	18.40	1.320	89.78	10.22	0.91	1.80
	10.5	8	1.35	0.005	0.62	99.38	0.065	8.13	91.87	0.08	1.08
	11.5	8	1.46	0.006	0.83	99.17	0.053	7.16	92.84	0.12	1.07
	12.5	8	1.34	0.005	0.66	99.34	0.030	3.85	96.15	0.17	1.03
	13.5	8	1.32	0.003	0.39	99.61	0.040	5.02	94.98	0.08	1.05

above 10.5% w/w, influenced the enzyme favoured in bottom phase more than 99%. Increasing the concentration from 12.5% to 16.5% w/w in PEG 1500 reduced the enzyme recovery in top phase from 87.15% to 67.24% and protein recovery from 89.57% to 69.59%. The results showed that the partition of the enzyme was similar to that contaminant proteins and an increase in PEG concentration did not improve purification. In case of PEG 3350, increasing the concentrations from 10.5% to 13.5% w/w did not show a big difference for enzyme and protein recovery in the top phase and the best recovery seemed to be at PEG 13.5% w/w. However, increasing the concentration of PEG 3350 in the system up to 14.5% w/w, it was found that more than 90% of the enzyme partitioned to bottom phase. As can be seen from the results, good enzyme recovery in the top phase was obtained at low concentration of PEG when low PEG molecular weight was replaced by higher one.

The partition coefficients of enzyme (K_{enz}) and protein ($K_{protein}$) (see Table 4.1) of the lowest studied molecular weight of PEG (1500) were higher than high molecular weights at the same concentration of PEG (3350 and 8000). It indicated that some of the enzyme removed to the PEG rich phase when low molecular weight of PEG was applied as seen from the change in β-galactosidase partitioning moved from the bottom phase to the top phase when PEG 8000 was replaced by PEG 3350 and 1500 respectively. The differential partitioning of enzyme may influence by the interfacial tension between the phases (Bamberger *et al.*, 1984; Srinivas *et al.*, 2002). Decreasing the molecular weight of phase forming polymers also decreases interfacial tension (Wu *et al.*, 1996).

The β-galactosidase recovery in top phase was good when the system used was close to the critical point. Away from the critical point β-galactosidase tended to favor in the bottom phase. Tanuja *et al.* (1997) mentioned that in general, partitioning was quite even at the two phase system was set near the critical point of binodal curve because the compositions of the two phase were almost equal. Farther from the critical point, each of the phases was rich in one of the polymer and reduced in the other which leaded to uneven partitioning. The observed purification factor results of β-galactosidase in the top phase, even in good enzyme recovery system, were poor because most of the contaminants were removed with the enzyme to the top phase.

4.3.3 Partitioning behaviours of β-galactosidase in different concentrations of KH_2PO_4

The effect on the partition coefficient of β-galactosidase as function of KH_2PO_4 concentrations varied from 7% to 10% w/w was examined with constant PEG concentrations 13.5% w/w for PEG 1500 and 3350 while PEG 8000 was 8.5% w/w. The pH of phosphate salt solution was adjusted at 6.5 using KOH. The calculated amount of PEG was then added to salt solution. The 10 g debris suspension was then added to the solution and the total mixture was set to a total mass of 30 g by adding demineralised water. The two phase system was completely mixed before settling for phase separation. The system was then left to equilibrium at temperature 25 °C for overnight. The phases between upper and bottom were carefully separated using a syringe with a long needle for phase removal. The volume of phase, enzyme and protein partitioning in each phase were then determined.

The influence of KH_2PO_4 concentrations on partition coefficient of β-galactosidase is shown in Figure 4.4. The enzyme partition coefficient in PEG 1500 was significantly changed by increasing KH_2PO_4 concentrations with a fall and then a slight rise. When PEG 3350 and 8000 were used, the partition coefficient was reduced quickly when the KH_2PO_4 concentration was increased from 8.5% to 9% w/w. Han and Lee (1997) noted that increasing potassium phosphate concentration made the bottom phase became more negatively charge while the top PEG-rich phase became more positively charge. Thus most proteins which have positively charge will partition to the bottom phase. For example, neutral protease from *Bacillus subtilis*, had the isoelectric point at pH about 8.95, partitioning to the bottom phase increased as the potassium phosphate concentration increased. The result was similar to partition behaviour of Lysozyme, whose surface charge is positive, in PEG and phosphate system.

The yield of the enzyme in the top phase at various concentrations of KH_2PO_4 with different molecular weights of PEG is shown in Figure 4.5. The tendency of enzyme recovery in the phase seemed to be following the enzyme partitioning coefficient.

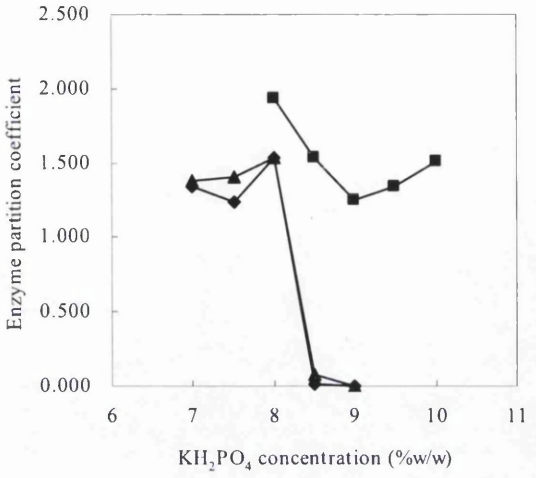


Figure 4.4 *Influence of KH_2PO_4 concentrations on partition coefficient of β-galactosidase in 13.5% w/w for PEG 1500 (■), 13.5% w/w for PEG 3350 (◆) and 8.5% w/w for PEG 8000 (▲) systems. The pH value in the system was at 6.5 and temperature was at 25 °C. The enzyme partition coefficient is calculated from equation 4.2.*

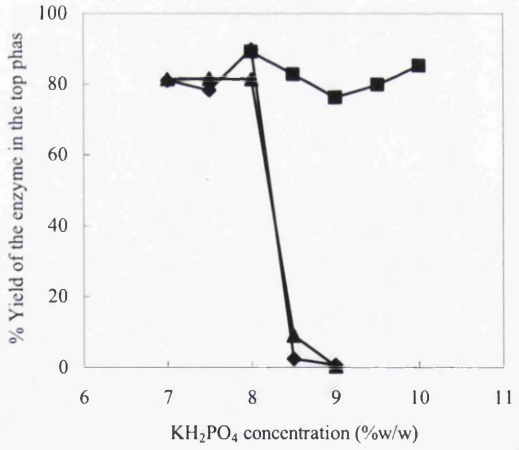


Figure 4.5 *Influence of KH_2PO_4 concentrations on yield of β-galactosidase in bottom phase in system consisted of 13.5% w/w for PEG 1500 (■), 13.5% w/w for PEG 3350 (◆) and 8.5% w/w for PEG 8000 (▲) systems. The pH value in the system was at 6.5 and temperature was at 25 °C. The yield of the enzyme in the top phase is calculated from equation 4.4.*

4.3.4 Effect of NaCl concentration on partitioning

The aqueous two phase system was set up for measuring the influence of NaCl on enzyme partitioning in different concentrations from 0 to 5% w/w. A fixed condition used in the system for PEG 1500 and 3350 were 13.5% w/w PEG and 8% w/w KH_2PO_4 and for PEG 8000 was 8.5% w/w with 8% w/w KH_2PO_4. The pH value through this experiment was set at 6.5 and temperature was at 25 °C.

An increase in NaCl concentration changes the partitioning of proteins between two phases. Gündüz and Korkmaz (2000) found BSA favourably partitioned to the bottom phase with increasing NaCl concentration up to 0.2 M as has been observed with Ipomoea peroxidase (Srinivas *et al.,* 2002) while increasing the recovery of amylase from the top phase was succeeded by using NaCl at 1-10% w/w concentration to induce the enzyme partition in the PEG-rich phase (Schmidt *et al.,* 1994).

The ion of NaCl distributed unequally between the phases causes an electrostatic potential difference between the phases and leads to the change of partition coefficient of specific proteins according their charge (Albertsson, 1986; Albertsson *et al.*, 1990 Engel *et al.*, 2000, Shang *et al.,* 2004).

Figure 4.6 shows the β-galactosidase yield recovery in different molecular weights of PEG with various concentrations of NaCl (%w/w). The addition of NaCl contributed to the increase of the β-galactosidase recovery in the bottom phase rather than top phase because in general salt can change the electrostatic charge of the system and causes the molecules with positive charge to prefer the salt-rich phase. As can be seen, the percent yield recovery in the PEG 3350 and 8000 decreased from about 80% in the system without the salt to almost 0% when the system containing NaCl from 1 to 5% w/w. In case of the PEG 1500 system, the best yield recovery in the top phase was about 85-90% in the system containing NaCl range 0-3% w/w.

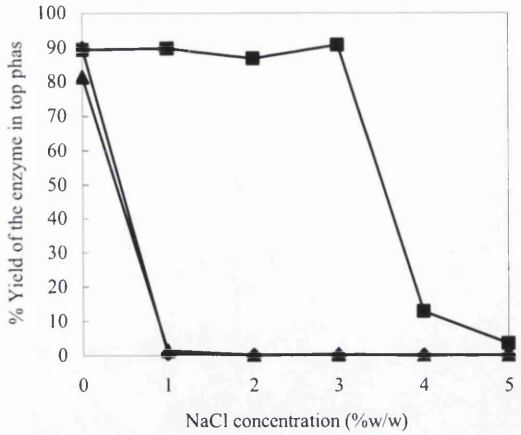


Figure 4.6 *Influence of NaCl concentrations on yield of β-galactosidase in bottom phase; phase composition 13.5% w/w PEG 1500 with 8% w/w KH_2PO_4 (■), 13.5% w/w PEG 3350 with 8% w/w KH_2PO_4 (◆) and 8.5 %w/w PEG 8000 with 8% w/w KH_2PO_4 (▲) systems. The experiments were conducted at pH 6.5, temperature 25 °C. The yield of the enzyme in the top phase is calculated from equation 4.4.*

4.3.5 Effect of pH on partitioning

The role of pH on the aqueous two phase system has been investigated with many proteins. It was found that the negatively charged protein should prefer the PEG rich phase while the positively charged protein distributes in salt phase (Schmidt *et al.*, 1994; Silva *et al.*, 2002). So when the pH increases above the isoelectric point of the proteins, their charge become negative and will strongly interact with PEG-rich phase, then the partition coefficient increases (Engel *et al.*, 2000).

The pH can also change the charge of chemical groups in the side chain of protein leading to a net charge modification of overall macromolecule then changing its partitioning properties (Asenjo, 1990). Shang *et al.* (2004) observed distribution of four amino acids in PEG/salt system with changing pH value. When the pH of the system increased, the concentration of OH^- increased, the lysine cations were partially neutralized causing reduction of electrostatic interaction between the molecules and phase forming system while the increase of the HPO_4^{2-} caused an increase in repulsion with phenylalanine, methionine and cysteine anions.

The effect of various pH values on enzyme partitioning coefficient was investigated in the system containing 13.5% w/w PEG (MW. 1500 and 3350) with 8% w/w KH_2PO_4 and 8.5% w/w PEG 8000 with 8% w/w KH_2PO_4. The temperature in this experiment was set at 25 °C. The various pH values were adjusted using KOH. The cell disrupted was then added.

It can be seen from Figure 4.7 that different pH values influenced enzyme partitioning coefficient. The best partition coefficients in PEG 3350 and 8000 were obtained at pH value 6.5 while in PEG 1500 was pH values range from 5.5 to 6.5. At pH value higher than 6.5, the partition coefficient started to decrease. It was probably because pH modified the net charge of protein to be more positive. The enzyme extraction performed with PEG 1500 showed higher partition coefficients at the same pH values as compared to PEG 3350 and 8000.

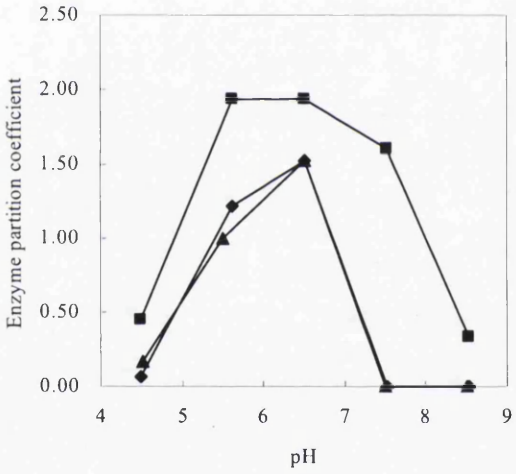


Figure 4.7 *Influence of pH on partition coefficient of β-galactosidase in phase composition 13.5% w/w PEG 1500 with 8% w/w KH_2PO_4 (■), 13.5% w/w PEG 3350 with 8% w/w KH_2PO_4 (◆) and 8.5 %w/w PEG 8000 with 8% w/w KH_2PO_4 (▲) systems. The experiments were conducted at temperature 25 °C. The enzyme partition coefficient is calculated from equation 4.2.*

The effect of pH on β-galactosidase purification ratio in top phase is displayed in Figure 4.8. The highest purification ratio in PEG1500 system was 1.02 at pH 7.5 while the maximum in PEG 3350 and 8000 system was 1.01 and 0.95 respectively at pH 6.5.

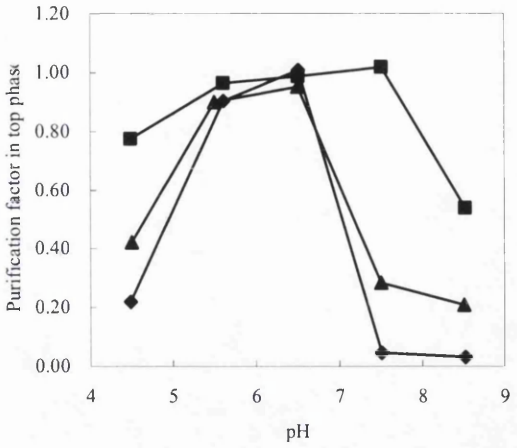


Figure 4.8 *Influence of pH on purification ratio of β-galactosidase in bottom phase; phase composition 13.5% w/w PEG 1500 with 8% w/w KH_2PO_4 (■), 13.5% w/w PEG 3350 with 8% w/w KH_2PO_4 (◆) and 8.5% w/w PEG 8000 with 8% w/w KH_2PO_4 (▲) systems. The experiments were conducted at temperature 25 °C. The purification factor is calculated from equation 4.6.*

4.4 Membrane separation

An alternative approach to large scale ATPS separation is the use of membrane. The advantage being that this is a purely physical process with no substantial addition of chemicals. The combination of microfiltration and ultrafiltration were applied to improve separation and purification of the enzyme from cell debris. The cross-flow microfiltration membrane is becoming the technology of choice for cell separation due to providing a cheap and effective method of purification and maintaining biological activity in product. The cells and debris are retained in the MF system while allowing the enzyme and some protein to pass through and be recovered in the permeate solution. To increase the passage of enzyme through the membrane, the discontinuous diafiltration technique was involved. The number of volume diafiltration related to enzyme removal was determined. After all volumes of permeate from microfiltration were collected, the enzyme was concentrated and purified further ultrafiltration membrane which retained the β-galactosidase but allowed peptides and salts to pass through the system.

4.4.1 Diafiltration of microfiltration

The first stage was to remove the enzyme by diafiltration of the cells disrupted. Discontinuous diafiltration was used with 0.2 μm microfiltration system to effectively remove more permeable soluble proteins (enzyme) from disrupted cells. This is described in detail in section 2.8.2 of Materials and Methods. A buffer was prepared from 10 mM KH_2PO_4 at pH 6.5 and employed as diafiltrate. The disrupted cells were diluted with an equal volume phosphate buffer. The diluted solution was then concentrated back to its original volume. The fresh equal volume phosphate was added to feed tank. The process of diafiltration was repeated for 5 times. During the dialfiltration, the enzyme and protein removal and transmission were determined with off-line measurement (see section 2.13.2.1 and 2.13.5.1).

The percentage of transmission and removal for both enzyme and protein versus number of volume exchanges for a diafiltration are shown in Figure 4.9. The enzyme and protein were washed out from the feed and collected in the permeate solution. The percentage of enzyme removal obviously increased from 27% to 82% and protein removal gradually raised from 9% to 30% with an increase in volume diafiltration. The percent transmission of protein tended to slowly decrease from the beginning of diafiltration and in each diafiltration was not great different while in case of enzyme gradually declined after the second diafiltration. The initial percent transmission of enzyme was 42% and increased to 58% in second diafiltration before steadily reduced to 29% in final diafiltration.

The content of the enzyme in retentate declined quickly, so the percent residual enzyme activity decreased to about 65% at first diafiltration and continuously dropped to approximately 23% after 5 times diafiltration. The content of protein gradually declined. The residual protein in retentate after final diafiltration was around 47% as shown in Figure 4.10. Purification factor of β-galactosidase was in range 2.3-3.0 fold during each diafiltration run.

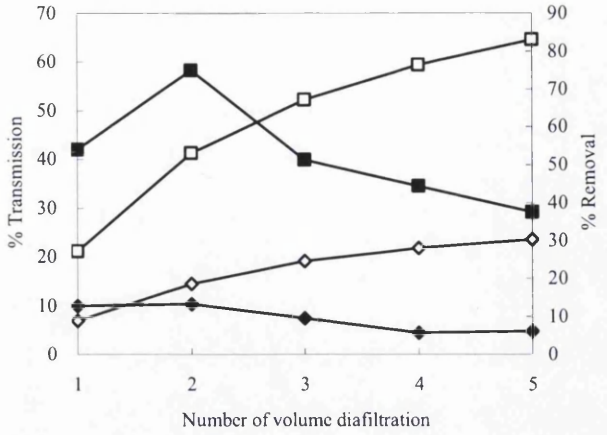


Figure 4.9 *Plots of percent transmission of enzyme (■), protein (◆) and percent removal of enzyme (□), protein (◇) versus number of volume diafiltration. Experiments were carried out in the constant volume diafiltration mode with a litre of cell suspension. 1 diafiltration volume = initial volume (1l). The percentages of tramsmission and removal are calculated from equation 2.2 and 2.3 respectively (see Materials and Methods).*

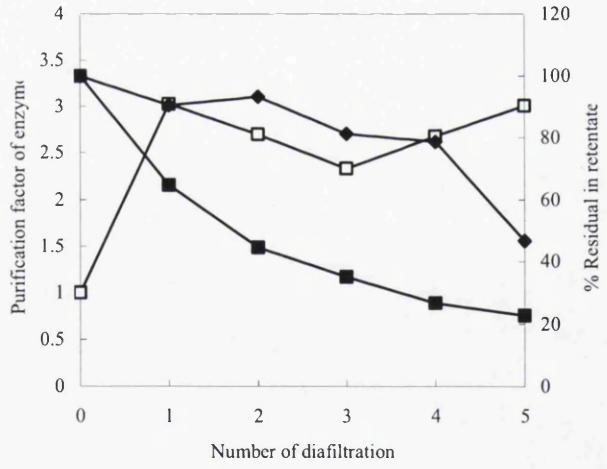


Figure 4.10 *Purification factor of the enzyme (□) and percent residual enzyme (■) and protein (◆) in retentate versus number of volume diafiltration. Operating conditions are the same as result in Figure 4.9. The percentages of residual in retentate and purification factor are calculated from equation 2.4 and 2.5 respectively (see Materials and Methods).*

4.4.2 Concentration of β-galactosidase by ultrafiltration

All collected permeate from microfiltration system (5 l) was put in 8 l feed tank. The enzyme concentrate process was operated with 50 kDa molecular weight cut-off crossflow ultrafiltration. During concentration to volume reduction every 1 litre by ultrafiltration, the samples from permeate and retentate were withdrawn regularly to determine the activity of enzyme and protein concentration (Figure 4.11). The β-galactosidase and protein were retained in the retentate while small molecules such as water and phosphate buffer passed through the membrane. The activity of β-galactosidase and protein concentration increased gradually from 0.33 μmol/min/ml and 0.16 mg/ml to 1.23 μmol/min/ml and 0.48 mg/ml respectively when the volume reduction was from 0 to 4 l.

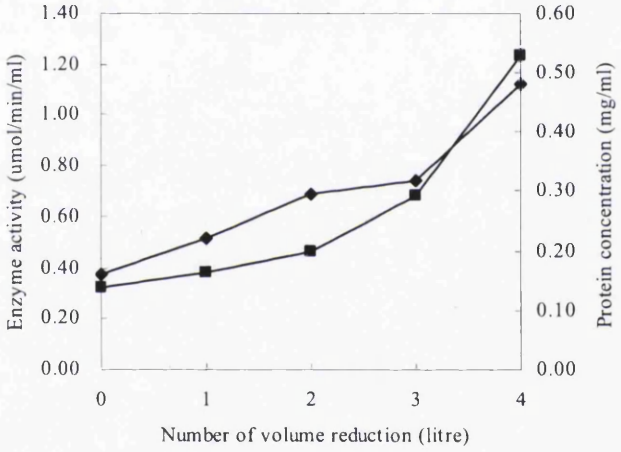


Figure 4.11 *The activity of β-galactosidase (■) and protein concentration (◆) as a function of number of volume concentration in ultrafiltration system. The operating pressure was maintained at 10 psi and temperature was at 25 °C.*

After extraction the enzyme from disrupted cells with the diafiltration in microfiltration membrane system, all permeate was collected at a final volume of 5 l and then concentrated by ultrafiltration. The β-galactosidase activity and protein concentration of retentate and permeate from both microfiltration and ultrafltration were measured (see section 2.13.2.1 and 2.13.5.1 of Materials and Methods) and are summarized in Table 4.2.

Sample	Total activity (μmol/min)	Total protein (mg)	Specific activity (μmol/min/mg)	Recovery (%)	Purification (fold)
Crude enzyme	2256.44	2555.00	0.88	100.00	1.00
Microfiltration					
Retentate	510.53	1192.00	0.43	22.63	0.49
Permeate	1623.15	802.86	2.03	71.93	2.31
Ultrafiltration					
Retentate	1234.11	482.00	2.56	54.69	2.91
Permeate	0.00	0.00	0.00	0.00	0.00

Table4.2 *Summary of membrane-purified β-galactosidase by microfiltration and ultrafiltration membrane system from L. delbrueckii* ssp *bulgaricus.*

Total activity is defined as μmol of ONP /min.

Protein was determined according to the Lowry method, using BSA as a standard.

Specific activity is defined as *μmol of ONP / min/ mg protein.*

The total activity of the enzyme from all volume of diafiltration microfiltration was about 71.93% able to pass through membrane while the rest was in the retentate and lose during running system. The protein concentration in permeate was 31.42%. The purification of enzyme was 2.31 when the specific activity of permeate was compared with crude enzyme.

The concentrated operation in ultrafiltration system performed that most of the enzyme and protein were kept in the retentate. About 54.69% enzyme activity was achieved recovery from the whole system. The loss amount of protein and enzyme during filtration might be from the adsorption on membrane surface. However, the specific activity was up to 2.56 which was 2.91 fold of purification with respect to the crude enzyme.

As this membrane preparation is purely a physical separation, this material as compared to that in the ATPS system is relatively simple and gave a slight higher purity. This membrane preparation was compared to crude extract.

4.5 Characterization of β-galactosidase

4.5.1 Effect of physical forces on enzyme activity

The structure of protein can be easily affected by physical forces such as stirring, shaking, pumping and filtration. The shear forces from vigorous shaking or stirring in certain instances can destroy biological activity. These can expose protein to air/liquid and liquid/solid boundaries which provide hydrophobic interface and lead to breaking conformational bonds, denaturation and aggregation through exposure of hydrophobic core residues (Tatford *et al.,* 2004). Along with denaturation when vortexed the solution, protein tends to uncontrollably foam and might provide a side effect in oxygenation which can result in oxidation of the protein. Then oxygenated environment can influence structure and biological activity.

The stability of the β-galactosidase enzyme activity in the shaking and static conditions was studied for 24 hours. The 20 ml of enzyme solution (0.88 μmol/min/mg protein) was mixed with 40 ml of phosphate buffer pH 6.5 in 250 ml Erlenmeyer flasks. The suspension was set into two conditions, static and shaking conditions at speed 200 rpm. The experiments were carried out at temperature 25 °C. At interval time, the suspension was sampling and tested with 45 mM ONPG to measure the residual activity (see section 2.13.2.1 of Materials and Methods).

The results showed (Figure 4.12) that the activities of enzyme during the time being in both shaking and static conditions provided similar trend. Shaking the solution did not have a significant effect on the β-galactosidase activity as shown after incubation for 24 hours. The residual activity of enzyme in static condition was 98.7% while 95.4% was left in shaking condition.

In addition to the effect of shaking, the stability of enzyme during ultrafiltration was also tested. 5 l of enzyme solution was added into feed tank. The solution was run through the close system with 50 kDa molecular weight cut-off membrane at temperature 20 °C and inlet pressure was 10 psi and samples were taken to determine the activity every 5 min.

Figure 4.13 shows the result of this work. The filtration process provided an effect on enzyme activity with a residual activity reduced by 10% after running for 5 mins. During

further operating time of the process, the activity gradually decreased. The residual activity after 60 minutes was 82.1% of the original.

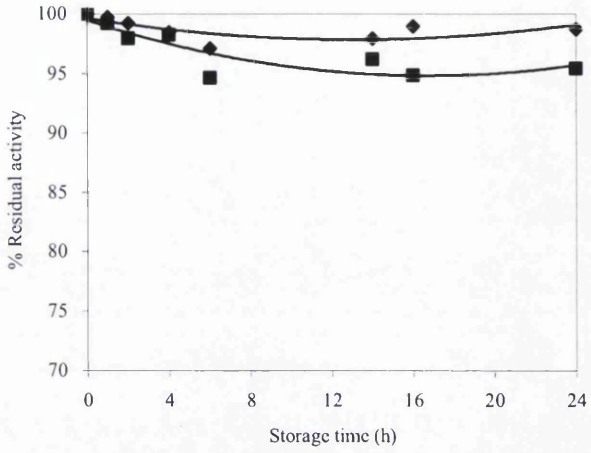


Figure 4.12 *Residual activity (%) of β-galactosidase in static (◆) and shaking (■) conditions when 45 mM ONPG was used as substrate. Incubation temperature was 25 °C and the speed of shaking was 200 rpm. The residual activity (%) is calculated from equation 2.7 (see Materials and Methods).*

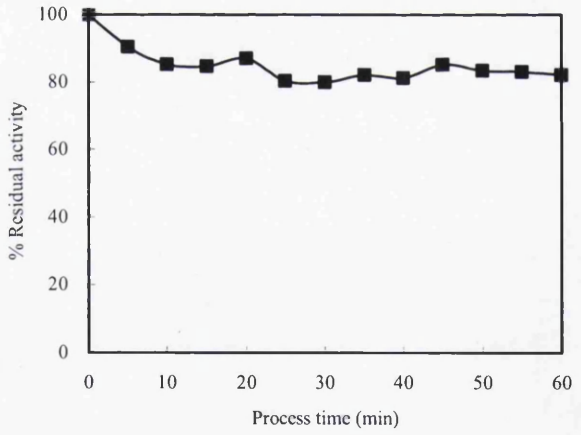


Figure 4.13 *Residual activity (%) of β-galactosidase during ultrafiltration process in a close system. The operated temperature was 20 °C and pressure was 10 psi. The residual activity (%) is calculated from equation 2.7 (see Materials and Methods).*

4.5.2 pH optimum of β-galactosidase activity

The hydrogen ion concentrations of a solution can affect the tertiary structure and in so doing how the substrate binds and interacts with the active site. The pH provides an effect of the state of ionization of acidic or basic amino acids in protein. The changes in the state of ionization of amino acids in an enzyme lead to an alternation of ionic bonds within tertiary structure of the protein. This can then lead to altered protein recognition or an enzyme might become inactive.

The activity of crude and partial purified β-galactosidase were tested at several pH values at the range 4.5-8.0 in order to determine the influence of pH on β-galactosidase performance. Potassium dihydrogen phosphate at 100mM adjusted pH by using KOH was applied as buffer in this experiment. ONPG and lactose were used as substrates.

The profiles of pH on the relative activity enzyme are shown in Figure 4.14 using ONPG as substrate. The enzymes performed good relative activity more than 80% at pH 6.0-7.0 range. The optimum pH for action was in the neutral range, with a peak at pH 6.5 for both enzyme preparations. The enzyme activity decreased sharply less than 30% when pH dropped below 4.5 and above pH 8.0.

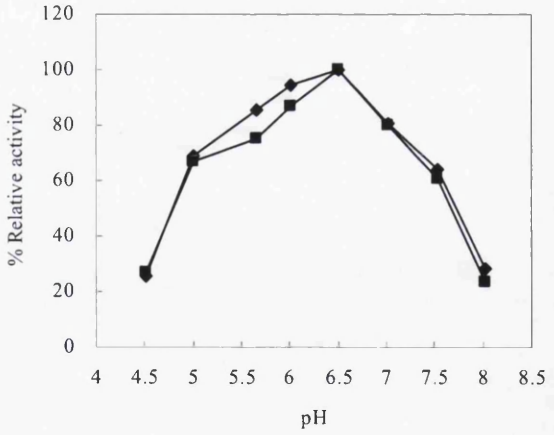


Figure 4.14 *The effect of pH on the activity of β-galactosidase from crude enzyme (■) and partially purified enzyme (◆) at temperature 25 °C, using 45 mM ONPG as substrate. Relative activity is defined as the ratio of reaction rate obtained from considering condition and maximum reaction rate obtained from the best condition.*

The crude β-galactosidase produced from four strains of lactic acid bacteria, *L. delbrueckii* subsp *bulgaricus* B-5b, *L. helveticus* LH-17, *L. delbrueckii* subsp *lactis* SBT-2080 and *L. acidophilus* SBT-2068, exhibited the optimal pH condition for the enzyme activity in neutral range at pH 6.0-7.0 (Wang and Sakakibara, 1997). The peak activity of enzyme from *Bacillus coagulans* was also in the same range (Batra *et al.,* 2002). However, the optimum pH from different sources is various. The β-galactosidase from *Penicillium chrysogenum* had 95% activity in a broad pH optimum of 4-5 and it decreased sharply to 15% activity at pH 3 while higher pH range, the decrease of activity was not so sharp. More than 30% of enzyme activity was retained at pH 7(Nagy *et al.,* 2001). The maximum activity of the enzyme from *Penicillium simplicissimum* was at pH 4.0-4.6. The fungal β-galactosidase produced from *Aspergillus oryzae* and *Aspergillus niger* also performed maximum activity in pH 4.0 to 4.6 (Cruz *et al.,* 1999). Consequently, the behaviour of the enzyme activity from bacteria mostly performs well at neutral pH while the enzyme from fungal prefers acid range.

In addition to source of enzyme, the optimum pH enzyme activity may shift due to the change of enzyme form. Zhou and Chen (2001) found free β-galactosidase from *Kluyveromyces lactis* had very low activity at the pH around 5.5 and performed optimal pH in the region of 6.6 to 7.0 while the optimum pH of immobilized enzyme was risen up to a more alkaline value of 7.7. Moreover, Ohmiya *et al.* (1977) presented the optimal pH of immobilized *E. coli* was at 8.0 while from free enzyme was about 7.5. The results appeared to agree with Ates and Mehmetoglu (1997) which found the optimal of immobilized enzyme from these fungi was changed to an acidic pH value compared to the free enzyme.

Ladero *et al.* (2002) reported that the β-galactosidase from *Kluyveromyces fragilis* was most active over ONPG and lactose at pH 6.5-6.8. The enzyme from *Thermotoga maritima* had an optimum pH at the same value 6.5 for both substrates (Kim *et al.*, 2004) while the enzyme from *Thermus* sp strain T2 showed the most active pH over ONPG and lactose in different. The slightly acid pH 5.0 was optimum over ONPG while neutral pH 6.5 was over lactose. The enzyme from thermophilic fungus *Talaromyces thermophilus* had maximum activity on ONPG at pH range 6.0-6.5 whereas with lactose was slightly lower in range 5.5-6.0 (Nakkharat and Haltrich, 2006). In addition to using ONPG as substrate

for optimum pH experiment, it is better to investigate the interaction activity on lactose substrate to obtain the appropriate condition in hydrolysis process.

Figure 4.15 shows the relative activity (%) of both crude and partially purified enzymes at pH values from 4.5 to 8.0 when lactose was used as substrate. This assay used glucose production rate as the measurement of hydrolysis (see section 2.13.2.2 for detail).The equal volume of enzyme solution was added into 100 mM lactose dissolved in different pH phosphate buffer. The samples were taken at different times to measure glucose concentrations. Numerical differentiation of glucose concentration versus reaction times was used to determine the initial reaction rates.

A broader range of optimum pH (6.0-7.5) was obviously shown with purified enzyme while for crude enzyme was from 6.0 to 6.5. However, the most active pH value for crude and partially purified enzymes, using lactose as substrate, was at 6.5 and was similar to ONPG assay. This optimum pH value of the enzyme is also appropriate to hydrolyse lactose in neutral dairy products.

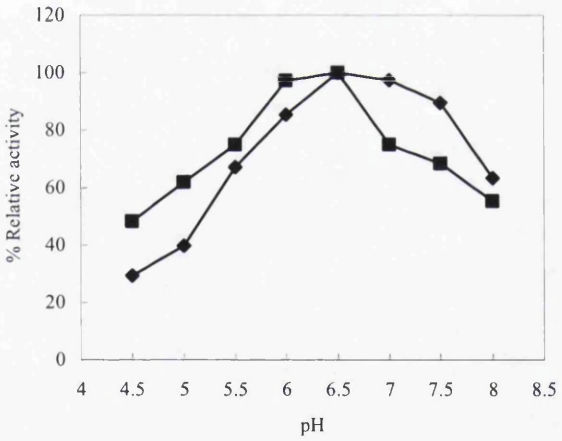


Figure 4.15 *The effect of pH on the activity of β-galactosidase from crude enzyme (■) and partially purified enzyme (◆) at temperature 25 °C, using 100 mM lactose as substrate. Relative activity is defined in Figure 4.14.*

4.5.3 Effect of temperature on kinetics and stability

Increases in temperature generally lead to increases in the rate of reactions. As more energetic collisions between enzyme and substrate occur with rising temperature and activation energy, a change in chemical state will result. However, at high temperature also causes an increase internal energy in the enzyme making it more unstable, leading to a thermal denaturation and inactive of the enzyme.

The effect of temperature on enzyme activity was assayed by treating 1ml of enzyme sample in 2 ml of 0.1M phosphate buffer pH 6.5 at different temperatures from 20 to 65 °C. After adding 0.5 ml of 45 mM ONPG, the reaction velocity was measured under control temperature spectrophotometer.

The results of the experiment are shown in Figure 4.16. The activity of both crude and partially purified enzyme increased with the rising temperature from 20 to 65 °C. From the temperature in range 20 to 50, partially purified enzyme showed higher activity than crude enzyme at the same temperature. After 55 °C, crude enzyme showed a slightly higher activity at 60-65 °C while at 60 °C partially purified enzyme performed 85.9% residual activity.

Ohmiya *et al.* (1977) reported the immobilized β-galactosidase from *L. bulgaricus* and *E. coli* had the highest activity at near 55 °C and 50 °C respectively at pH 6.5 in 0.7 M phosphate buffer and the optimum temperature from *K. lactis* was only 37 °C. The optimal temperature for β-galactosidase activity from *Thermotoga maritima* with ONPG was at 85 °C and 80 °C with lactose and above these temperatures the activity dropped quickly (Kim *et al.*, 2004). In general, thermostable enzymes are desirable in industrial processes due to they can accelerate the reactions and prevent microbial contamination during operation (Cruz *et al.*, 1999). However, it is very important to consider the stability of the enzyme at high temperature.

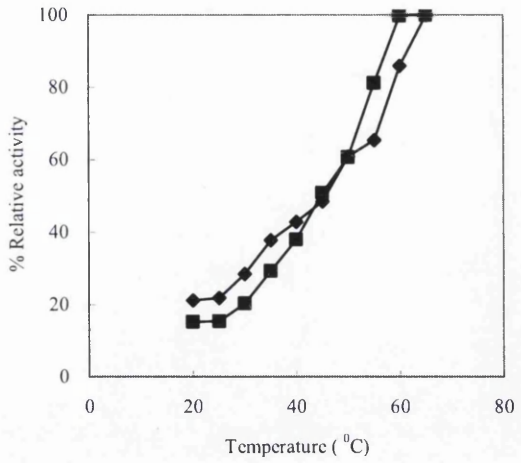


Figure 4.16 *The effect of temperature on the activity of β-galactosidase from crude enzyme (■) and partially purified enzyme (◆) in 0.1M phosphate buffer pH 6.5. 45 mM ONPG was used as substrate. Relative activity is defined in Figure 4.14.*

The data from measuring the reaction rate at different temperatures were used in activation energy (Ea) calculation. A slope of straight line that correlates logarithm (Log) activity of the enzyme with the inverse of the absolute temperature, 1/T (Figure 4.17) is –Ea/2.3R according to Arrhenius equation:

$$k = A\, e^{-Ea/RT} \qquad \textbf{\textit{Equation 4.7}}$$

The Ea values from crude and partially purified enzymes under this condition were 38.41 kJ/mole and 35.46 kJ/mole respectively. Vasiljevic and Jelen (2002) determined the activation energy of crude β-galactosidase from *L. bulgaricus* 11842 with three different neutralizers, NaOH, KOH and NH_4OH, during the lactose hydrolysis in skim milk. The calculated data from Arrhenius plot were 42.8, 43.5 and 42.7 kJ/mole respectively which proved that there was no significant difference among neutralizers.

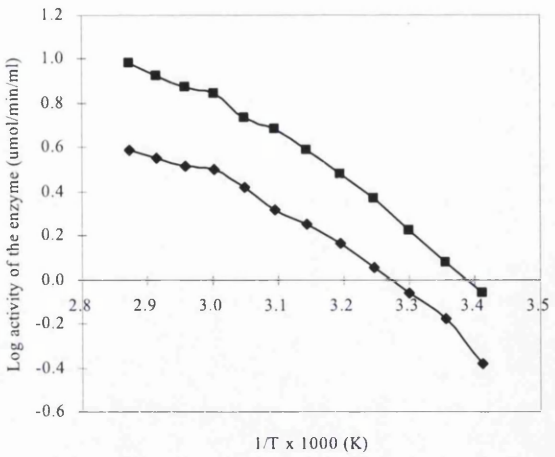


Figure 4.17 *The relationship between Log activity of the enzyme and inverse of absolute temperature from crude enzyme (■) and partially purified enzyme (◆). The operating conditions are mentioned in Figure4.16.*

It is necessary to assay the activity of the enzyme at regular intervals. The stability of many enzymes depends on condition used especially they are unstable and some might precipitate at more extreme pH and temperature (Tipton, 2002). Although at high temperature can increase the reaction rate of enzyme, the stability of the enzyme may decrease rapidly due to thermal degradation.

The thermal stability of β-galactosidase was measured by incubating the enzyme in 0.1M phosphate buffer pH 6.5 at different temperatures (see Materials and Methods in section 2.13.3). The samples were withdrawn periodically to check the residual activity.

The relation between natural logarithm of residual β-galactosidase activities versus exposure times (h) for crude and partially purified enzymes are performed in Figure 4.18 and 4.19 respectively. It can be observed that both enzymes were inactivated in accordance with first order kinetics. The residual activity of both enzymes obviously decreased from temperature at 50 °C in crude enzyme and 40 °C for partially purified enzyme.

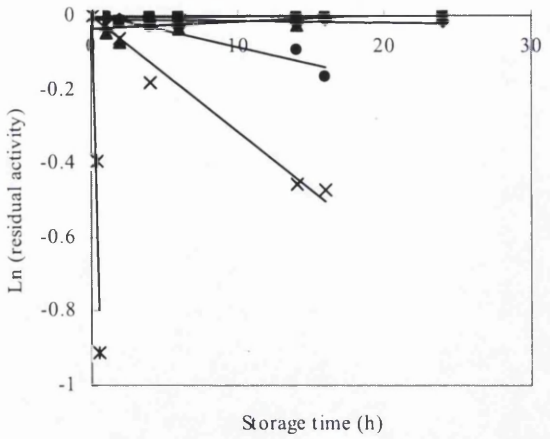


Figure 4.18 *Effect of temperature and exposure time on the stability of β-galactosidase activity from crude extract in 0.1M phosphate buffer pH 6.5 and 45 mM ONPG was used as substrate. Temperatures tested were at 4 (■), 20 (◆), 30 (▲), 40 (●), 50 (×) and 60 (✳) °C. Ln residual activity is defined as the natural logarithm of the ratio between the activity of the enzyme at considering time and the activity of the enzyme at starting time.*

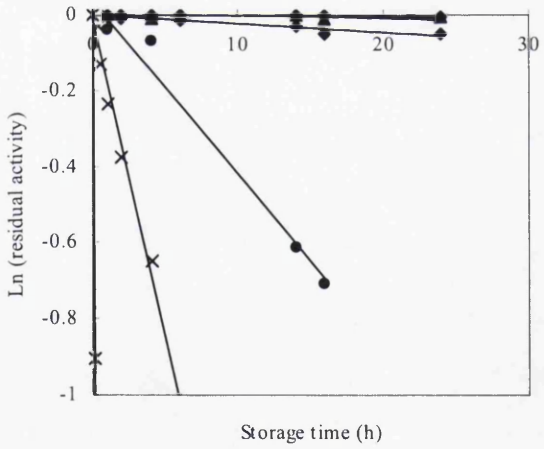


Figure 4.19 *Effect of temperature and exposure time on the stability of partially purified β-galactosidase activity in 0.1M phosphate buffer pH 6.5. 45 mM ONPG was used as substrate. Temperatures tested were at 4 (■), 20 (◆), 30 (▲), 40 (●), 50 (×) and 60 (✳) °C. Ln residual activity is defined as the natural logarithm of the ratio between the activity of the enzyme at considering time and the activity of the enzyme at starting time.*

The values of β-galactosidase half-life ($t_{1/2}$) for first order decay were calculated from declined slope (k_d) of data from Figure 4.18 and 4.19. Therefore, half-life represents stability as following:

$$t_{1/2} = \frac{\ln 2}{k_d} \qquad \textit{Equation 4.8}$$

Figure 4.20 indicates half-life times of crude and purified enzyme versus temperatures. The half-life times of crude enzyme were 3465, 1733, 347, 77, 22 and 0.37 h while the half-life time of partially purified enzyme were 3465, 630, 301, 15, 4 and 0.07 h at 4, 20, 30, 40, 50 and 60 °C respectively. The half-life times for both enzymes were decreased rapidly at high temperature especially when exposure at 60 °C and reduced about 98% compared with exposure at 50 °C. The enzyme from crude extract provided greater storage activity than the one from membrane-purified source.

Kreft and Jelen (2000) sonicated *L. bulgaricus* strain 11842 culture and held in potassium and sodium buffers. The presence of potassium resulted in higher stability than presence in sodium and the half-life time of the enzyme at 61 °C pH 6.0 in potassium buffer was 0.52 h. Batra *et al.* (2002) observed that half-life of enzyme from *Bacillus circulans* at 63 °C was 15 h whereas at 65 °C the half-life decreased rapidly to 2 h and the activity was almost entirely lost at 70 °C. However, *Bacillus circulans* has been reported to produce more stable enzyme than that from *Bacillus* sp which the half-life was 83 min at 65 °C (Griffiths and Muir, 1978). The enzyme from *Thermotoga maritima* performed more stable at high temperature for example the half-life times at 70, 80 and 90 °C were 50 h, 16 h and 16 min at pH 6.5 (Kim *et al.*, 2004). Busto *et al.* (1999) mentioned that the presence of protein in the solution may stabilise enzyme activity. Large particle of protein and cell debris can be removed under membrane operation and only small protein particle can pass through the membrane. This might cause less stability of enzyme from membrane-purified source than crude extract.

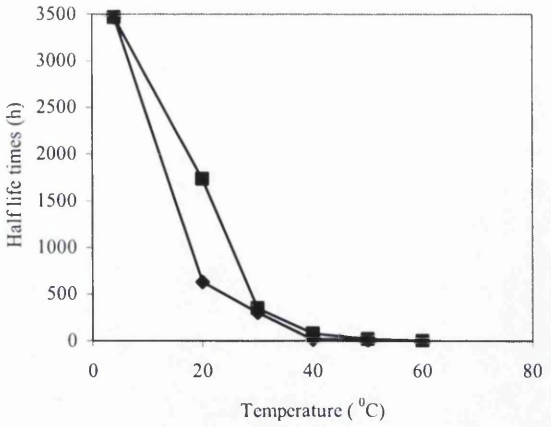


Figure 4.20 *Half-life times of crude enzyme (■) and partially purified enzyme (◆) at different temperatures.*

Although increasing temperatures encourage good β-galactosidase activity, the enzymes in both forms were unstable at high temperature from 40 °C. To minimize loss of enzyme activity, the operational temperature in extraction, purification and utilizing system should be as low as possible, preferable below 10 °C.

4.5.4 Kinetics of β-galactosidase enzyme

The measurements of kinetic from crude and partial purification of β-galactosidase sources were assayed with different concentrations of two substrates, ONPG and lactose (see section 2.13.4 of Materials and Methods). The assay was carried out at temperature 25 °C and pH value of 6.5. Here the activities as a function of substrate concentrations were determined, these data were then linearized using a Lineweaver-Burk plotting (1/[S] versus 1/V) (equation 4.9):

$$\frac{1}{V} = \frac{K_m}{V_{max}} \times \frac{1}{S} + \frac{1}{V_{max}} \qquad \textit{Equation 4.9}$$

The equation plot results in a line with y-axis intercept of $1/V_{max}$ and the slope of K_m / V_{max} values. The results for ONPG substrate are represented in Figure 4.21. The ONPG concentrations tested were from 2 mM to 100 mM. The V_{max} and K_m values of crude

extract were 1.25 μmol/min/ml and 12.63 mM respectively. The V_{max} and K_m values obtained from partially purified form were 1.04 μmol/min/ml and 17.62 mM.

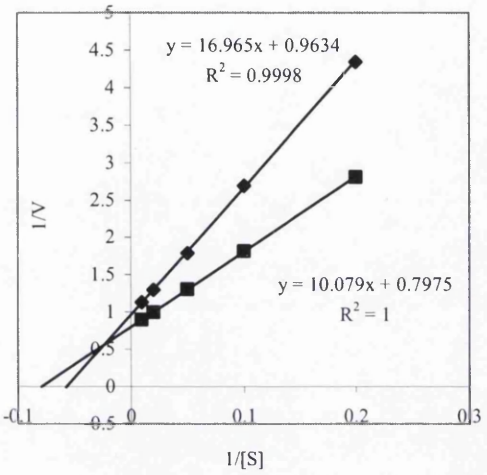


Figure 4.21 *Lineweaver-Burk plots for β-galactosidase from crude extract (■) and partial purification from membrane (◆) when using ONPG as substrate.*

The V_{max} and K_m values for lactose were calculated from the results shown in Figure 4.22. The lactose concentrations used were in range of 5-100 mM. The K_m values obtained for crude and partially purified forms were 23.23 and 27.58 mM with V_{max} 0.91 and 1.34 μmol/min/ml respectively.

As can be seen, the kinetic data obtained from different substrates have different values. Premi *et al.* (1972) found the V_{max} and K_m values of *L. thermophilus* with ONPG were 50 μmol/mg/min and 1.69×10^{-3} μmol while the data with lactose were 42 μmol/mg/min and 0.6×10^{-2} μmol respectively. The kinetic values were dependent on surrounding condition. Garman *et al.* (1996) found the K_m value obtained for β-galactosidase from *L. delbruekii* subsp *bulgaricus* 20056 was 79 mM when determining with lactose concentrations from 1-100 mM in phosphate buffer pH 7.0 containing 2 mM $MgCl_2$. The K_m value of the enzyme from *L. bulgaricus* at pH 6.5, temperature 30 °C was only 4.2 mM (Ohmiya *et al.*, 1977). Kim *et al.* (2004) pointed that the V_{max} and K_m values of β-galactosidase from *Thermotoga maritime* varied with substrate concentration. At lower concentrations up to 10 mM of lactose provided V_{max} 63.3 μmol glucose/min/mg protein and K_m 1.6 mM while at higher

concentration V_{max} and K_m were 139 μmol glucose/min/mg protein and 27.8 mM respectively.

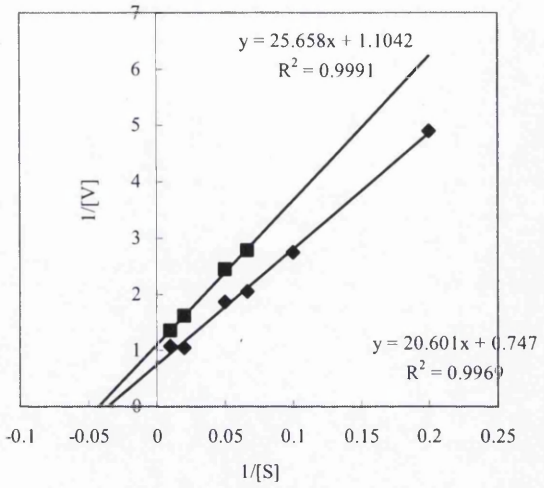


Figure 4.22 *Lineweaver-Burk plots for β-galactosidase from crude extract (■) and partial purification from membrane (◆) when using lactose as substrate.*

4.5.5 Effect of glucose on β-galactosidase activity

Glucose is an end product of the hydrolysis reaction. The effect of glucose at different concentrations (0, 10, 50, 100 mM) on the β-galactosidase activity from the crude extract and partially purified enzymes was determined with ONPG (5, 10, 20, 50 mM) in 100 mM phosphate buffer adjusted pH to be 6.5 with KOH. The ONPG was used as substrate instead of lactose because glucose from lactose hydrolysis interfered with the addition of glucose to the reaction mixture. The reaction rates of the enzymes were determined as mention in enzyme assay using spectrophotometer at 420 nm.

The reaction rates were measured with varying glucose concentrations (mM) in ONPG solution. The reciprocal values of V from crude and partially purified enzymes were plotted against different values of glucose concentrations (Figure 4.23 and 4.24 respectively). Straight lines were fitted and each one represented each ONPG concentration. The amount of ONP produced gradually decreased with increasing glucose concentration. About 13-20% of the activity was inhibited by 100 mM glucose in crude enzyme and about 18% in partially purified enzyme when using ONPG as substrate in

range 5 to 50 mM. From these plots, glucose appeared to be non-competitive inhibitor in the reaction which agreed with the observations in other microbial β-galactosidase (Cowan *et al.,* 1984; Choi *et al.,* 1995; Batra *et al.,* 2002). Ohmiya *et al.* (1977) found that glucose served as a non-competitive inhibitor of β-galactosidase from *L. bulgaricus* in both free enzyme and immobilized forms with the equal K_i value at 230 mM using lactose as substrate while with the enzyme from *Talaromyces thermophilus*, glucose was found to be strong competitive inhibitor of ONPG hydrolysis with K_i 66 mM (Nakkharat and Haltrich, 2006). However, glucose acts as an activator with β-galactosidase from *Kluyeromyces lactis* (Kim *et al.,* 2004) and in *Thermoanaerobacter* TP6B1 over the concentration range 1-10% (Lind *et al.,* 1989).

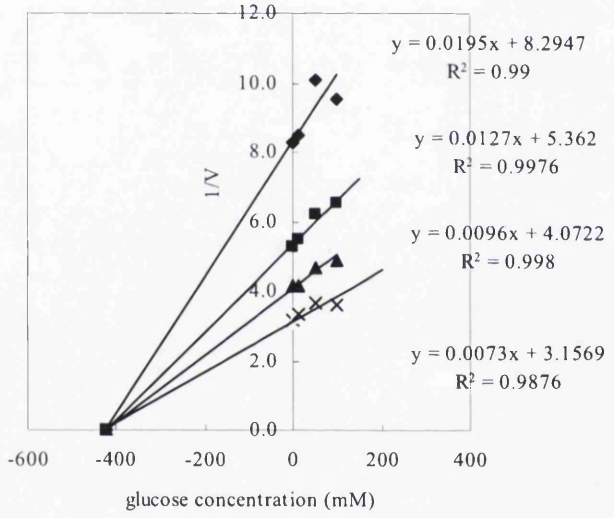


Figure 4.23 *Effect of glucose on the rate of ONP production from crude β-galactosidase. The ONPG was used as the substrate with concentrations at (◆) 5 mM, (■) 10 mM, (▲) 20 mM and (✕) 50 mM. The experiments were carried out at pH 6.5, temperature 25 °C. The data were fitted with linear line and intersected to the left of the 1/V asix. The observed pattern of primary plot was combined with the linearity of the secondary re-plots. The appropriate data fitted were selected based on the most satisfactory correlation coefficient (R^2).*

The K_i value was obtained from straight lines intersected at a point glucose concentration. The K_i from crude enzyme was 424 mM with close to the respective value K_i (439mM) from purified enzyme.

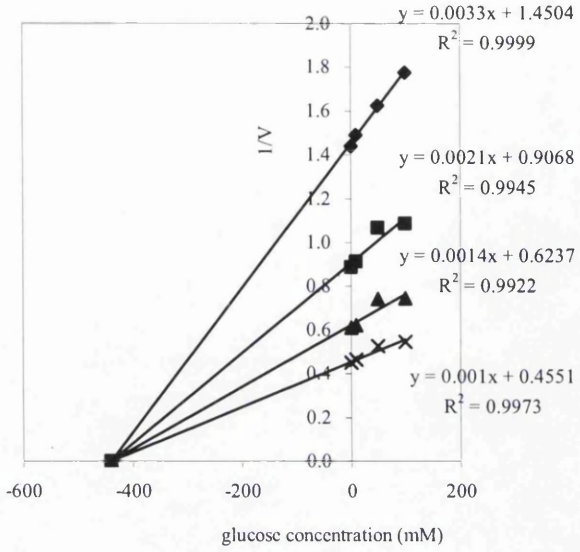


Figure 4.24 *Effect of glucose on the rate of ONP production from partially purified β-galactosidase. The ONPG was used as the substrate with concentrations at (◆) 5 mM, (■) 10 mM, (▲) 20 mM and (×) 50 mM. The experiments were carried out at pH 6.5, temperature 25 °C. The data were fitted as mention in Figure 4.23.*

4.5.6 Effect of galactose on β-galactosidase activity

The effect of galactose on enzyme activity in various concentrations from 0 to 150 mM was studied. The β-galactosidase activity was assayed by monitoring the hydrolysis of lactose at concentrations 20 mM, 50 mM and 100 mM. Different concentrations of lactose and galactose were dissolved in 0.1 M phosphate buffer adjusted pH at 6.5 with KOH. The equal volume of enzyme solution was added into various concentrations of lactose. The samples were taken at different times to measure glucose released from lactose using glucose oxidase solution. Numerical differentiation of glucose concentration versus reaction time was used to determine the initial reaction rate.

The reciprocal values of V from crude and partially purified enzymes were plotted against different values of galactose concentrations (Figure 4.25 and 4.26 respectively). Each straight line represented the influence of galactose on different values of lactose concentration. The addition of galactose up to 10 mM in both enzymes was not obviously inhibited lactose hydrolysis. However, above this concentration the activity of the enzymes gradually decreased.

The K_i value which obtained from straight lines intersected at a point galactose concentration in crude enzyme was about 329 mM and partially purified enzyme was about 440 mM. From the plotted pattern, galactose seemed to act as non-competitive inhibitor for the enzyme.

Many authors stated that β-galactosidase both from mesophile and thermophile sources were competitive inhibition by galactose when measuring the activity with hydrolysis of ONPG (Griffiths and Muir, 1978; Cowan *et al.,* 1984; Berger *et al.,* 1997; Santos *et al.,* 1998; Batra *et al.,* 2002). Ohmiya et al., (1977) found galactose inhibited competitively β-galactosidase from *Kluyveromyces lactis* in lactose hydrolysis reaction and has also been seen with the enzyme from *Aspergillus niger* (Lee and Wacek, 1970; Papayannakos *et al.,* 1993). However, Shukla and Caplin (1993) reported galactose was non-competitive inhibitor of β-galactosidase from *Aspergillus oryzae.*

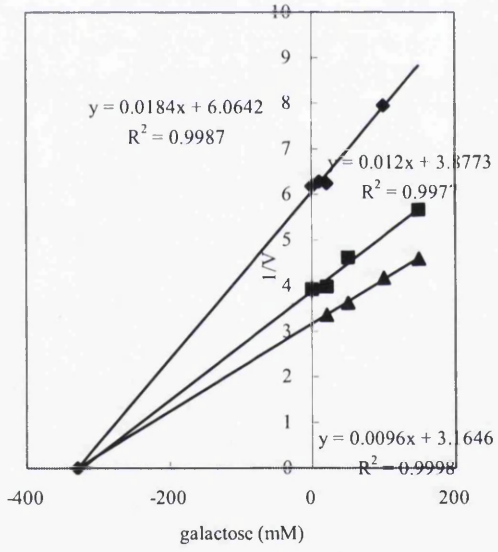


Figure 4.25 *Effect of galactose on the rate of glucose released from crude β-galactosidase. The lactose was used as the substrate with concentrations at (◆) 20 mM, (■) 50 mM and (▲) 100 mM. The experiments were carried out at pH 6.5, temperature 25 °C. The data were fitted as mention in Figure 4.23.*

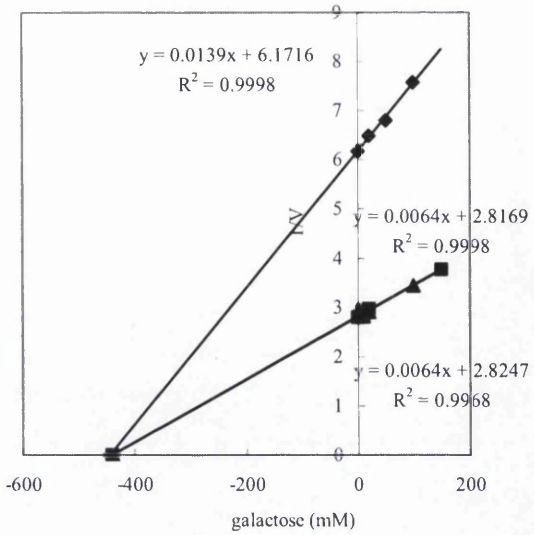


Figure 4.26 *Effect of galactose on the rate of glucose released from partially purified β-galactosidase. The lactose was used as the substrate with concentrations at (◆) 20 mM, (■) 50 mM and (▲) 100 mM. The experiments were carried out at pH 6.5, temperature 25 °C. The data were fitted as mention in Figure 4.23.*

4.5.7 Effect of Na^+ and K^+ on enzyme activity

The effects of K^+ and Na^+ on enzymatic activity varied greatly with different sources of enzyme. The monovalent cations may influence the enzyme activity because these ions can enter the structure of the protein associated on the basis of ionic radius especially Na^+, which is the smallest molecule, being more tightly bound than K^+ (Garman *et al.,* 1996) then these ions cause inducing conformational changes in the enzyme structure (Jurado *et al.*, 2004).

For hydrolysis of ONPG, there was no change with addition of K^+ or Na^+ on the activity of β-galactosidase from *Bacillus* TA-11 (Choi *et al.,* 1995), *Streptococcus thermophilus* (Somkuti and Steinberg, 1979). However, Garman *et al.* (1996) reported that the addition of K^+ activated the β-galactosidase from *S.thermophilus* TS2, *Lb. casei* 20094, *P. pentosaceus* PE39, *B. bifidum* 1901, and *L. lactis* subsp. *lactis* 7962 while the addition of Na^+ increased the activity only the enzyme from *Lb. casei* 20094, *P. pentosaceus* PE39, *B. bifidum* 1901. Cohn (1957) stated that sodium ions enhanced β-galactosidase activity from *E. coli* when using ONPG as a substrate whereas Itoh *et al.* (1980) found both Na^+ and K^+

had an effect on inhibition with β-galactosidase from *L. bulgaricus*. It can be seen that the effect of both ions can differ depending on the sources of enzyme.

In addition to source of enzyme, Kim *et al.* (1997) mentioned that different substrates used also affected the measurement of β-galactosidase activity. For example all metals activated the enzyme from *E. coli* during hydrolysis of ONPG whereas the same ions inhibited lactose hydrolysis (Cohn and Monod, 1951). Thus it would be better to study the activity based on lactose solutions rather than ONPG especially on investigation with any activator or inhibitor.

The effects of Na^+ and K^+ on enzyme activity were determined with 100 mM lactose as a substrate in 0.1 M phosphate buffer pH 6.5 adjusted with KOH. Various concentrations of Na^+ and K^+ in the range from 0 to 50 mM were added to lactose solution. After incubation with enzyme solution for 15 min, β-galactosidase activity was measured by determining glucose released from lactose using glucose oxidase solution. The glucose productions with difference concentrations of Na^+ and K^+ were compared with no addition of these cations.

The relative activity of crude β-galactosidase with the addition of Na^+ and K^+ up to 50 mM is shown in Figure 4.27 and with partially purified β-galactosidase is in Figure 4.28. Increasing concentration of Na^+ affected a decline in activity of both enzymes, indicating Na^+ provided an inhibiting effect on enzyme activity while in the presence of K^+, the activity of enzyme continued to increase with concentrations of K^+. The results indicated the ion acted as an activator with β-galactosidase. Moreover, Kreft and Jelen (2000) monitored the lactose hydrolysis by cryoscopy method and found that the activity of enzyme from *L. delbrueckii* subsp. *bulgaricus* 11842 sonicated in potassium buffer when lactose represented as substrate was higher than when sonicated in the presence of Na^+ ions and sodium seemed to be a strong inhibitor of the β-galactosidase enzyme at this point. Bhowmik *et al.* (1987) said the K^+ ion also stimulated the enzyme from *L. acidophilus*. The effect of these ions also performed the same direction with lactose hydrolysis by the enzyme from *K. fragilis* and *K. lactis* (Mahoney and Adamchuk, 1980; Bernal and Jelen, 1985).

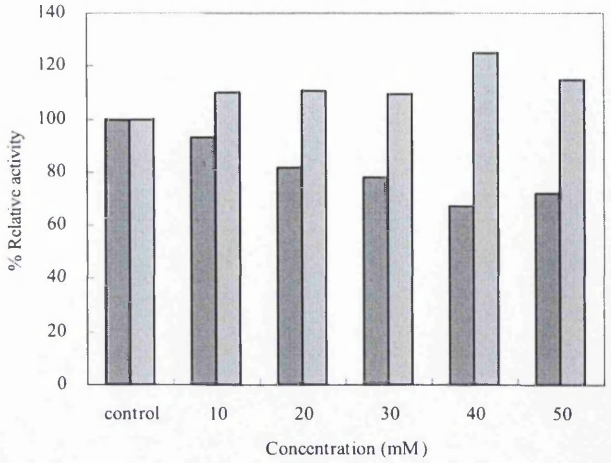


Figure 4.27 *The percent relative activity of crude β-galactosidase with the addition of Na^+ (■) and K^+ (■) in range 0-50 mM in phosphate buffer containing100 mM lactose adjusted pH to be 6.5. The experiments were tested at temperature 25 °C. Relative activity is defined in Figure 4.14.*

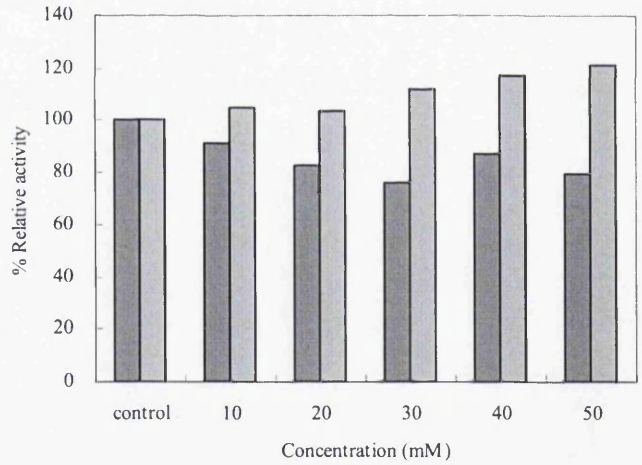


Figure 4.28 *The percent relative activity of partially purified β-galactosidase with the addition of Na^+ (■) and K^+ (■) in range 0-50 mM in phosphate buffer containing 100 mM lactose adjusted pH to be 6.5. The experiments were tested at temperature 25 °C. Relative activity is defined in Figure 4.14.*

4.5.8 Effect of divalent cations Ca^{2+} and Mg^{2+} on enzyme activity

The Ca^{2+} and Mg^{2+}cations are an important component in dairy product. The magnesium content in product varies between processes and during processing while the level of calcium seems to be constant (Garman *et al.,* 1996). It is necessary to observe the effect of Ca^{2+} and Mg^{2+} on the activity of β-galactosidase before using the enzyme in the process.

The experiments were carried out by incubating the enzyme solutions in 0.1 M phosphate buffer pH 6.5 adjusted with KOH containing 100 mM lactose as a substrate with concentrations of Ca^{2+} and Mg^{2+} in range from 0 to 50 mM. After incubation with enzyme solution for 15 min, β-galactosidase activity was measured by determining glucose released from lactose using glucose oxidase solution. The glucose productions with difference concentrations of Ca^{2+} and Mg^{2+} were compared with no addition of these cations.

The influences of Ca^{2+} and Mg^{2+} ions on enzyme activity in terms of the relative activities for both crude and partially purified forms are displayed in Figure 4.29 and 4.30 respectively. The addition of Ca^{2+}ions from 0 to 50 mM decreased the enzyme activity continuously from 100% to 8.7% in crude enzyme and 6.5% in purified enzyme. Although Ca^{2+} ions strongly inhibit the functioning of the enzyme, at the normal pH of milk, almost all of the calcium is bound in colloidal form to the casein micelles (Garman *et al.,* 1996) and less than 10% exists in ionized form. In case of magnesium ions, adding the ion concentrations up to 10 mM did not affect crude β-galactosidase while the activity of partially purified form slightly increased to 114% with 10 mM Mg^{2+} concentration. Above this concentration, activities of both enzymes slightly declined. Thus the Mg^{2+} ions seemed to be a weak inhibitor on the enzyme whereas Garman *et al.* (1996) tested 2 mM Mg^{2+} ion with the β-galactosidase from 6 strains, *L. delbruekii* ssp *bulgaricus* 20056, *L. casei* 20094, *L. lactis*, *B. bifidum* 1901, *S. thermophilus* TS2 and *P. pentosaceus* PE39. The results showed that Mg^{2+} enhanced the rate of lactose hydrolysis from all cases. The Mg^{2+} ion also slightly activated the enzyme from *K. lactis* (Kim *et al.*, 1997) and *K. fragilis* (Mahoney and Whitaker, 1977). The effect of ion on enzyme activity varies between species. Batra *et al.* (2002) studied the effect of Ca^{2+} and Mg^{2+} ions on the enzyme from *B. coagulans* RCS3 at concentration in range 0.5-2.0 mM and found these ions did not inhibit the enzyme activity.

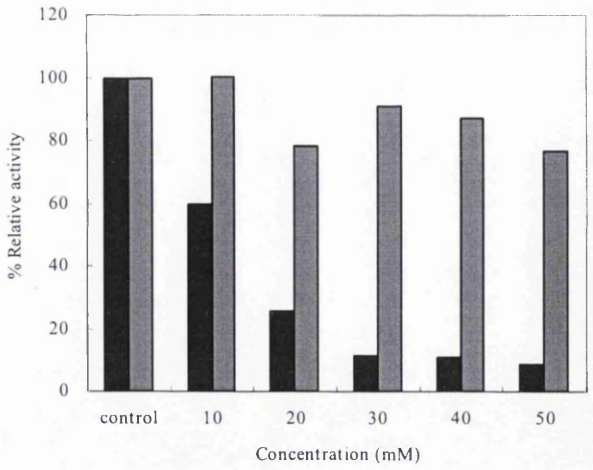


Figure 4.29 *The percent relative activity of crude β-galactosidase with the addition of Ca^{2+} (■) and Mg^{2+} (■) in range 0-50 mM in phosphate buffer containing100 mM lactose adjusted pH to be 6.5. The experiments were tested at temperature 25 °C. Relative activity is defined in Figure 4.14.*

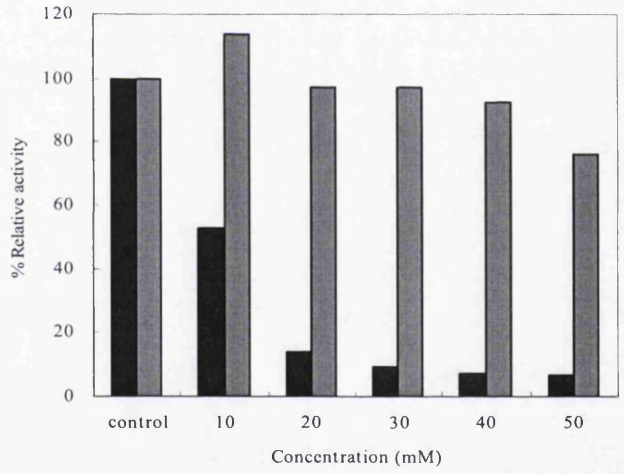


Figure 4.30 *The percent relative activity of partially purified β-galactosidase with the addition of Ca^{2+} (■) and Mg^{2+} (■) in range 0-50 mM in phosphate buffer containing100 mM lactose adjusted pH to be 6.5. The experiments were tested at temperature 25 °C. Relative activity is defined in Figure 4.14.*

4.6 Conclusions

The aim of this chapter was to partially purify β-galactosidase from *L. delbrueckii*. The first stage after producing large quantities of cells was to release the enzyme from the cells using cell disruption. This could be achieved by high pressure homogenization. Two methods were investigated. The first was using ATPS extraction and the second was a combination of membrane filtration. After purification the enzyme activity was characterized. This showed that a stable enzyme preparation could be made and that it suffered significantly from end product inhibition. The following conclusions can be made:

1. The β-galactosidase is an intracellular enzyme which is not released to the outside of cells during fermentation. In addition, *L. delbrueckii* is a Gram positive bacterium and have a very thick cell wall consisting of peptidoglycan. The thick of cell wall can be up to 80 nm (Kaiser, 2006) which gives the cells with very high mechanical strength in comparison with Gram negative bacteria (Samuèlson *et al.*, 2002). The mechanical methods using high pressure for cell disruption was preferred for this study since no addition chemical are used with the enzyme and easy to operate in a large or small scale. The influence of operating pressure from disrupter on the cells was studied. The effective cell disruption was related to the operating pressure. Increasing pressure from 0 to 40 kpsi reduced cell optical density and increased protein released while the enzyme activity released after disruption from pressure 25 kpsi to 40 kpsi remained almost constant. Although high pressure provided great protein released, the pressure can influence the size of cell debris and impact product separation for further task. Then the appropriate pressure used was at 25 kpsi.

2. Different molecular weights of PEG (1500, 3350 and 8000) with potassium dihydrogen phosphate at pH 6.5 were employed for two phase formation in extraction of β-galactosidase. Increasing PEG molecular weights from 1500, 3350 and 8000 at the same concentration resulted in an increase of the β-galactosidase distribution in bottom phase. The partition coefficient normally decreases as the PEG chain length increases (Lima *et al.*, 2002). The phenomenon of the desired enzyme partition in aqueous two phase system favoured the bottom phase when the phase system used was away from critical point of binodal curve. Thereby, raising PEG concentration influenced more β-galactosidase,

soluble proteins and all contaminants partitioned to the salt rich phase as a consequence of their low solubility in PEG-rich phase resulting poor purification.

3. The effect of increasing KH_2PO_4 concentrations caused a decrease enzyme partition in the top phase. It was obviously shown in ATPS of PEG 3350 and PEG 8000 with phosphate concentration from 8.5% w/w the enzyme partitioned bottom phase. For PEG 1500, the concentrations of KH_2PO_4 did not much influence β-galactosidase partition.

4. The addition of NaCl in PEG 3350 and 8000 systems assisted to enhance the β-galactosidase recovery in the bottom phase. In the presence of 1-3% w/w NaCl with PEG 1500 did not alter much partitioning of the enzyme from the top phase as compared with in absent NaCl substance.

5. The influence of pH value was observed to alter the β-galactosidase partition coefficient. At near neutral pH some part of the enzyme moved to the top phase. However, most of the enzyme preferred to be in the bottom phase when changing the pH values above this point.

6. Although this study performed the optimization of ATPS condition for extraction β-galactosidase and cell debris in opposite phases, preferentially the enzyme in the top phase to facilitate further processing (purification), with various molecular weights of PEG, the results of close values between enzyme and protein partition coefficients did not provide effective purification factor because the contaminant proteins were partitioned into the same phase as the β-galactosidase.

Considering the high concentrations of PEG and KH_2PO_4 used, this purification method was seen as inappropriate for enzyme use in the production of low lactose milk.

7. The purification of β-galactosidase was achieved by using crossflow microfiltration and ultrafiltration. The microfiltration membrane with pore size 0.2 μm was used to remove soluble protein from disrupted cells suspension. The protein and enzyme were washed out more from debris to permeate when discontinuos diafiltration was involved in the experiment. About 72% recovery was found after 5 diafiltration volumes of exchange with 2.31 fold purification.

8. During concentration process with 50 kDa molecular weight cut-off crossflow ultrafiltration, most of β-galactosidase and protein were retained by membrane and about 55 % enzyme recovery was found. The purification was 2.91 fold with respect to the crude enzyme. The final volume left in retentate was defined as partially purified enzyme and used in study of enzyme properties.

9. The physical and chemical properties of β-galactosidase from crude and partially purified forms were investigated in this chapter. The residual activity of the enzyme at shaking condition was decreased only 3% in comparison with incubating the enzyme at static condition after 24 h.

10. The β-galactosidase from both forms showed good active on ONPG at neutral pH range from 6.0 to 7.0. The results appeared to be the same when using lactose as a substrate. The most active pH value of both substrates was at 6.5.

11. The activity of β-galactosidase from both crude and partially purified forms in phosphate buffer pH 6.5 increased according to rising temperature when using ONPG as a substrate. The half-life of both forms decreased after exposure at high temperature.

12. The calculated V_{max} and K_m values from two substrates, ONPG and lactose were investigated at temperature 25 °C and pH 6.5. The range of ONPG concentration used was 2-100 mM and lactose from 5-100 mM. The V_{max} values from crude enzyme were 1.25 and 0.91 μmol/mg protein/min and K_m values were 12.63 mM and 23.23 mM respectively. The V_{max} and K_m values from partially purified enzyme were different. The V_{max} values were 1.04 and 1.34 μmol/mg protein/min and K_m values were 17.62 mM and 27.58 mM when ONPG and lactose were applied as substrate respectively.

13. The plots between 1/V and products concentration of lactose hydrolysis, glucose and galactose, pointed that both glucose and galactose acted as non-competitive inhibitor for β-galactosidase. Addition of Na^+ in lactose hydrolysis reduced the enzyme activity while the presence of K^+ promoted β-galactosidase activity. The divalent cations also affected the activity. The Ca^{2+} cation strongly inhibited the activity while the use of Mg^{2+} at concentration above 10 mM appeared to decrease the enzyme activity.

Chapter 5
Lactose hydrolysis

5.1 Introduction

Lactose is a reducing disaccharide sugar. It contains two different monosaccharide units, glucose and galactose, which are joined by a 1,4-beta-glycoside bond between C1 of galactose and C4 of glucose (Figure 5.1). The chemical name for lactose is 4-0-β-D-galactopyranosyl-D-glucopyranose. Lactose is widely found in dairy products such as milk, ice cream, cheese and whey. The sweetness of lactose is less than disaccharide such as sucrose or the monosaccharides such as fructose and glucose and its water solubility is low.

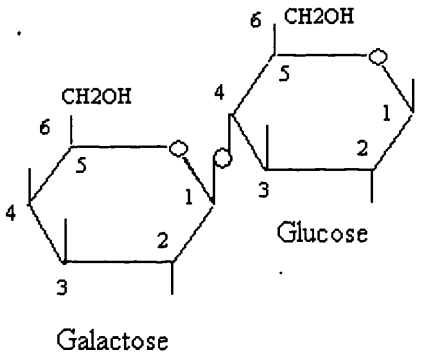


Figure 5.1 *The structure of lactose, galactose binds glucose with 1,4-beta-glycoside bond.*

Lactose can be hydrolyzed either by acid or enzymatic treatment with β-galactosidase. Hatzinikolaou *et al.* (2005) noted that acid hydrolysis process required high acid concentration for completing process and undesirable products, except glucose and galactose, were also produced. The enzymatic hydrolysis of lactose by β-galactosidase produces the constituent monosaccharides, glucose and galactose. Glucose molecule is assumed to be the first to leave the active site of the enzyme, while a covalent galactosyl-enzyme complex can be further hydrolyzed (Kim *et al.*, 2004).

The aim of this study was to investigate the hydrolysis of lactose solutions in a bioreactor, a stirred cell membrane reactor and a membrane bioreactor (MBR) before applying lactose

containing skimmed milk. The performance use of membrane bioreactor, the stability of the enzyme during operation and the effects of substrate concentration, enzyme concentration and flow rate of permeate were evaluated. This study provided an understanding of the reaction with pure lactose solution and assisted to test the operational membrane bioreactor. The experimental data from both bioreactor and MBR were also used for mathematical modeling of the hydrolytic process.

5.2 Lactose hydrolysis in batch bioreactor

The hydrolysis reaction was firstly studied in batch bioreactor since the system was simple and easy to control. The effects of different initial lactose concentrations and enzyme concentrations on lactose hydrolysis were tested in this reactor. The lactose solution was prepared in 10 mM phosphate buffer at pH 6.5. The temperature was controlled at 25 °C. The reaction started when 45 ml of lactose solution was added into bioreactor containing 5 ml of crude enzyme. Total volume of reaction solution was 50 ml.

5.2.1 Effect of lactose concentration

The formation of glucose in batch reactor was produced from three different initial lactose concentrations, 55.5 mM, 83.3 mM and 111.0 mM (Figure 5.2) and the total amount of lactose loading in bioreactor were 2.5, 3.7 and 5.0 mmol respectively. The enzyme concentration in the reactor for this experiment was fixed at 0.28 mg protein/ml reactor.

Increasing lactose concentration resulted in an increase in glucose production and took longer time to reach a steady state. After operation for 360 min, the final yields of glucose production were 1.8, 3.3 and 3.9 mmol and the initial rates of reaction were 0.84, 0.80, and 0.81 μmol/ml/min when lactose concentrations were 55.5 mM, 83.3 mM and 111.0 mM respectively. The initial reaction rates from three tests were not significantly difference. This was probably because the enzyme concentration used was constant and the substrate concentrations were in saturated condition (over K_m value = 23.23 mM, see section 4.5.4), so the initial rate became insensitive with the change of substrate concentration.

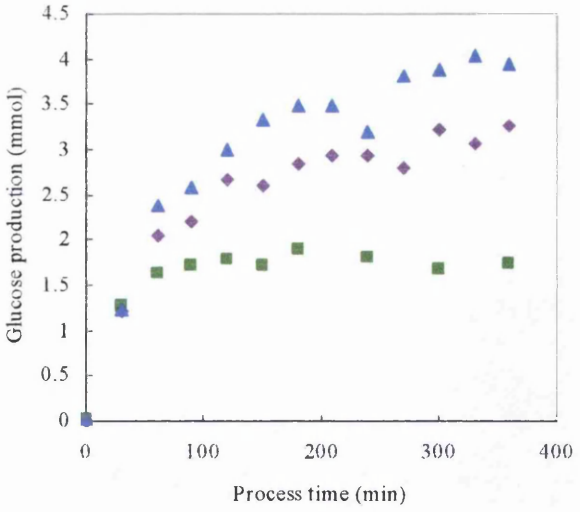


Figure 5.2 *The effect of lactose concentration on glucose production in batch reactor. Total amount of lactose loading in bioreactor were 2.5 mmol (■), 3.7 mmol (◆) and 5.0 mmol (▲). The crude enzyme used for each reactor was 0.28 mg protein/ml. The temperature was controlled at 25 °C. Total volume of reaction solution was 50 ml.*

5.2.2 Effect of enzyme concentration

The effect of enzyme concentration was investigated with a constant lactose concentration at 55.5 mM. The total amount of lactose in each bioreactor set was 2.5 mmol. The condition used was the same as mentioned in section 5.2. Different enzyme concentrations were prepared by diluting crude enzyme with phosphate buffer and displayed in terms of protein concentration. The enzyme concentrations used in the system were in range of 0.06-0.28 mg protein/ml reactor.

The initial rate of reaction varied with enzyme concentrations (Figure 5.3). The rate of reaction was faster when high concentration of the enzyme was employed as shown that the initial rates were 0.28, 0.43, 0.55, 0.64 and 0.84 μmol/ml/min when the concentrations of crude enzyme were 0.06, 0.11, 0.17, 0.22 and 0.28 mg protein/ml reactor respectively. At low enzyme concentration seemed to take longer time to reach the steady state, as can be seen that enzyme concentration at 0.06 mg/ml was almost steady state after 240 min while the concentration at 0.22 mg/ml took shorter time at about 180min. However, the final yields of glucose from varied enzyme concentrations were almost the same. The amount of glucose obtained after operation for 360 min were 1.9, 2.0, 2.0, 1.9 and 1.8

mmol respectively.

The relationship between enzyme concentration and the initial rate of glucose production is plotted (Figure 5.4). The graph shows that the trend of initial rate was linear with the concentration of enzyme. Increasing enzyme concentration resulted in an increase in the rate of productivity.

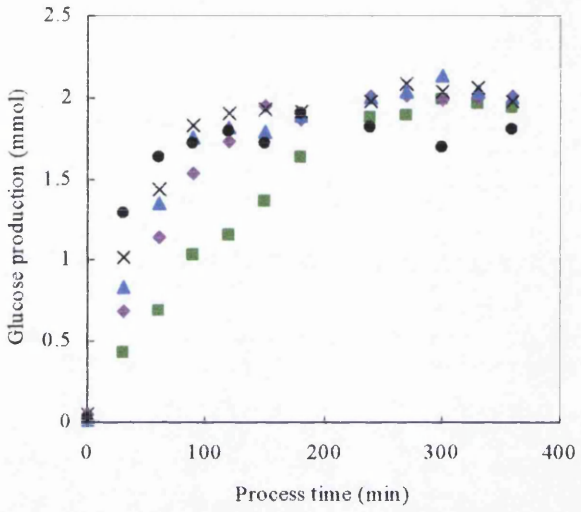


Figure 5.3 *The effect of enzyme concentration on glucose production in batch reactor. Total amount of lactose loading in bioreactor was fixed at 2.5 mmol (concentration 55.5 mM). The crude enzyme in reactor were 0.06 mg/ml (■), 0.11 mg/ml (◆), 0.17 mg/ml (▲), 0.22 mg/ml (×) and 0.28 mg/ml (●). Operating conditions are mentioned in Figure 5.2.*

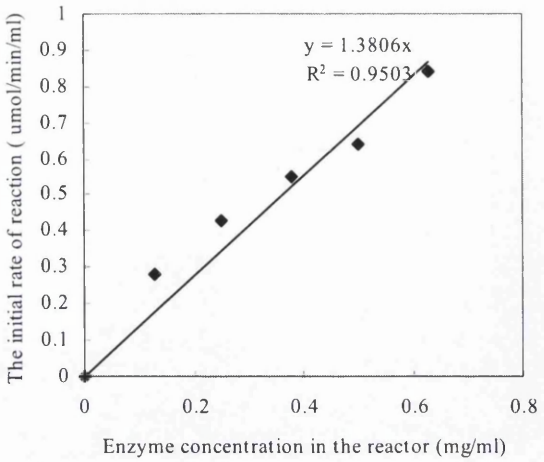


Figure 5.4 *The relationship between enzyme concentrations and the initial rates of glucose production. Operating conditions are mentioned in Figure 5.2.*

5.3 System using stirred cell membrane reactor

The performance of membrane reactor in lab scale for lactose hydrolysis was conducted in stirred cell membrane reactor. The maximum volume of the cell was 50 ml. The operating temperature was controlled at 25 °C. The rejection of the enzyme from stirred cell ultrafiltration membrane reactor and the hydrolysis of lactose by the enzyme in the system were investigated.

5.3.1 The rejection of the enzyme in stirred cell ultrafiltration system

The potentials in retaining and reusing of the enzyme in the reactor are important especially on continuous membrane reactor. This makes enzyme use less expensive than conventional batch bioreactor. The performance of membrane reactor with 30 kDa ultrafiltration membrane molecular weight cut-off in 50 ml maximum process volume reactor was used to determine residual enzyme activity.

The determination of crude enzyme activities in both retentate and permeate after ultrafiltration process was measured at 25 °C for 5 h. The experiment was conducted in the recycling of solution in the reactor loop (see section 2.12.1). The results were expressed in terms of residual activity (%) in retentate and permeate versus process time (Figure 5.5). The calculating residual activities as followed:

$$\text{retentate residual activity (\%)} = \frac{\text{retentate enzyme activity at t=t x 100\%}}{\text{retentate enzyme activity at t=0}} \qquad \textit{Equation5.1}$$

$$\text{permeate residual activity (\%)} = \frac{\text{permeate enzyme activity at t=t x 100\%}}{\text{retentate enzyme activity at t=t}} \qquad \textit{Equation5.2}$$

The residual enzyme activity from crude enzyme in retentate, after operating in ultrafiltration system for 5 h, decreased about 3% and very small amount quantities of β-galactosidase activity, which was less than 0.5% residual activity, were observed in the permeate. The reduction of retentate residual activity may be from fouling at the membrane surface. The efficiency of membrane was obviously shown that β-galactosidase was rejected by this ultrafiltration membrane.

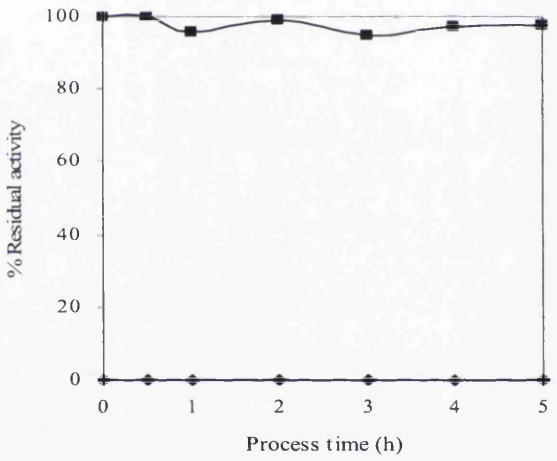


Figure 5.5 *Membrane rejection of β-galactosidase enzyme from crude extract in ultrafiltration membrane reactor with 50 ml maximum process volume reactor. Enzyme activity with 45 mM ONPG was 1.47 μmol/ min/ ml. The temperature was controlled at 25 °C. Total volume of reaction solution was 20 ml. (■) % retentate residual activity and (◆) % permeate residual activity.*

The hydraulic permeability of the membrane before and after filtration was measured (Figure 5.6) in order to investigate the membrane fouling. The deduction of permeate flux of distilled water before and after process at the same pressure might probably because the adsorption or the blockage of the enzyme on the membrane surface or in the pore of

membrane. The reduction of permeability which obtained from calculating the ratio of the slopes before and after filtration was about 39.04%.

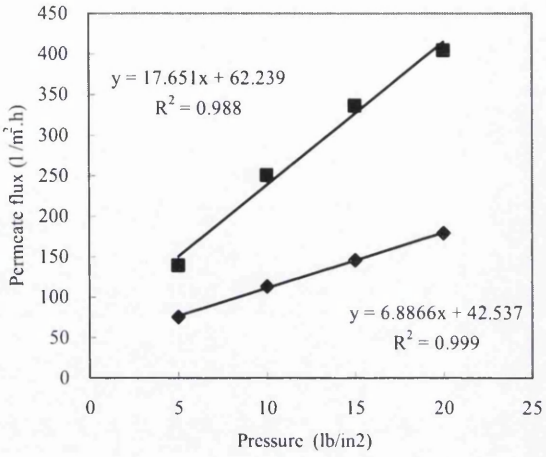


Figure 5.6 *The permeate flux of distilled water before and after enzyme filtration against pressure at 25 °C. (■) before filtration and (◆) after filtration.*

5.3.2 Continuous lactose addition in ultrafiltration stirred cell reactor

These experiments were conducted by continuous lactose addition in reactor. The initial 10 ml of lactose at concentration 124.9 mM was loading to the stirred filter cell. The crude enzyme was then added and the reaction hydrolysis was last for 2 h before filtration. After a volume of permeate was filtered equal to the volume of substrate added. The retentate and permeate were sampled to measure glucose production, enzyme activity and protein concentration. Fresh substrate, same concentration with first time, was added into the stirred filter cell containing the retentate. The long term batch hydrolysis experiments were carried out for 5 cycles.

The results of glucose production with different proportions of enzyme and lactose solution are shown in Figure 5.7 (enzyme: lactose solution was 10 ml: 10 ml) and Figure 5.8 (enzyme: lactose solution was 30 ml: 10 ml).

The initial rate of glucose production in reactor before filtration was 0.56 μmol/ml/min in the first two hours (Figure 5.7). After that the rate of glucose production slowed down and obviously dropped after 360 min due to the loss of enzyme after sampling. The total yield

of glucose found in permeate increased according to process time and was higher than in retentate. The percent average recovery glucose in permeate was 63.6% while in the retentate was about 31.0% in first 360 min.

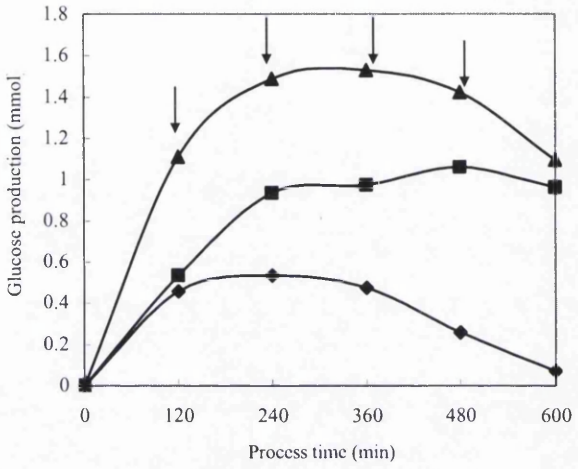


Figure 5.7 *Total glucose production in a 50 ml stirred cell ultrafiltration reactor in a batch substrate addition mode. The ratio of enzyme against lactose solution was 1:1 ml. Total volume in reactor was 20 ml. Initial lactose concentration was 124.9 mM. Then another fresh 124.9 mM of lactose were added in every 2hours (↓). (▲) total glucose in reactor, (◆) glucose in the retentate, (■) glucose in the permeate solution. Enzyme concentration in the reactor was 1.4 mg protein/ml reactor. The molecular weight cut-off of membrane was 30 kDa with area of 13.4 cm^2, nitrogen pressure in the cell was at 30 psi. The temperature was controlled at 25 ^oC.*

In case of the ratio of enzyme against lactose solution 3:1 ml, the initial rate of glucose production in reactor before filtration was 0.81 μmol/ml/min in the first two hours (Figure 5.8), then the rate of bioconversion slowed down. More than 62% of glucose retained in retentate and less than 31% of glucose was found in permeate solution. Although higher enzyme loading in reactor accelerated the rate of bioconversion, the glucose recovery in permeate comparing with the result from Figure 5.7 was lower. It seemed that enzyme acted as a barrier with ultrafiltration membrane for permeation of glucose or glucose was probably binding with the enzyme.

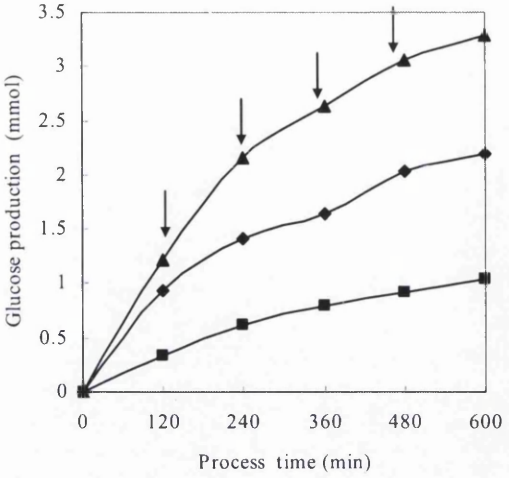


Figure 5.8 *Total glucose production in a 50 ml stirred cell ultrafiltration reactor in a batch substrate addition mode. The ratio of enzyme against lactose solution was 3:1 ml. Total volume in reactor was 40 ml. Initial lactose concentration was 124.9 mM and afterwards another fresh 124.9 mM of lactose were added in every 2hours (↓). (▲) total glucose in reactor, (◆) glucose in the retentate, (■) glucose in the permeate solution. Enzyme concentration in the reactor was 2.1 mg protein/ml reactor. The operating conditions are mentioned in Figure 5.7.*

To evaluate the possibility of enzyme leakage from the membrane, the activity of the enzyme was measured with 45 mM ONPG in phosphate buffer pH 6.5 at 25 ^{o}C. After termination of each filtration, the retentate and permeate solution from stirred cell ultrafiltration reactor were collected to check the residual activity in retentate and the percentage of enzyme transmission.

The percentages of residual enzyme activity in retentate and enzyme transmissions from enzyme loading one and three times with lactose solution are presented in Figure 5.9. The activity of enzyme from batch enzyme loading ratio volume 1:1 ml decreased more sharply than batch enzyme loading ratio volume 3:1 ml. Each filtration cycle the activity was lose average 22% and 12% from enzyme loading ratio 1:1 and 3:1 respectively while the enzyme transmission in both cases were not found. These results indicated that no enzyme leakage from the membrane was detected and the reasons in drop of enzyme activity in retentate were probably due to loss the amount of enzyme during sampling or enzyme adsorption on membrane.

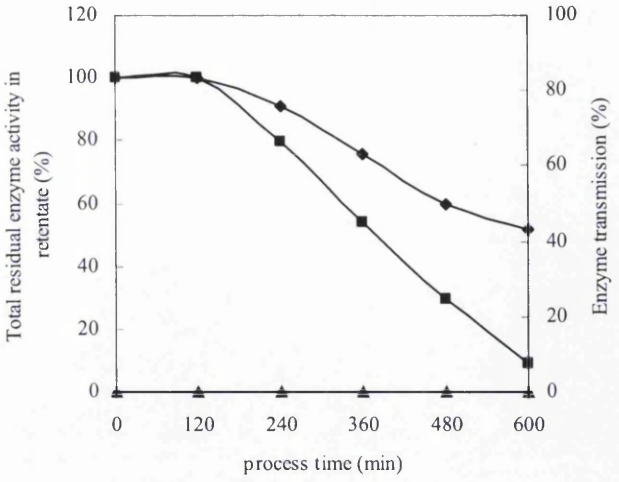


Figure 5.9 *The enzyme activity with 45 mM ONPG during the process time of lactose hydrolysis in a 50 ml stirred cell ultrafiltration reactor in a batch substrate addition mode. Initial lactose concentration was 124.9 mM and afterwards another 124.9 mM of lactose were added every 2hours. (■) and (◆) were enzyme activities in retentate when the reactor contained ratio of enzyme against lactose solution were 1:1 ml and 3:1 ml respectively. (▲) and (×) were enzyme in the permeate when the reactor contained ratio of enzyme against lactose solution were 1:1 ml and 3:1 ml respectively. The operating conditions are mentioned in Figure 5.7.*

Adsorption of protein on the membrane in both cases, the ratio of enzyme loading with lactose solution 1:1 ml and 3:1 ml, were investigated by measuring the protein content from Folin assay in retentate and permeate. The results performed in terms of the percentage of residual protein in retentate and protein transmission (Figure 5.10). The residual protein content in both retentates were fluctuate slightly and very small quantities of protein were detected in permeate. The permeate residual protein from batch ratio 1:1 ml was about 1.0-2.1% while from batch 3:1 ml was about 0.1-0.4%. These results showed that most proteins were retained on the membrane and the enzyme acted as barrier for protein permeate.

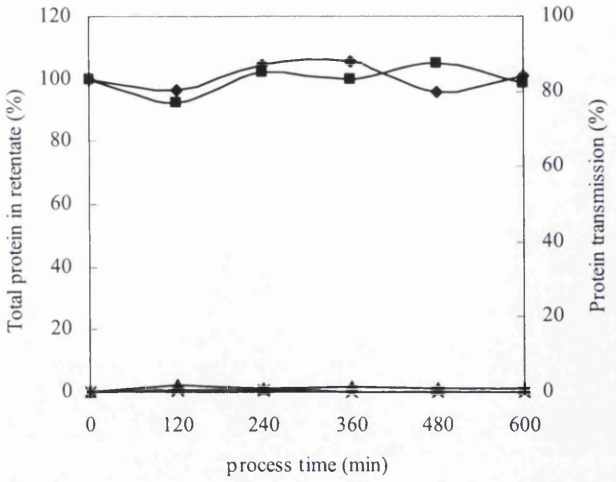


Figure 5.10 *The residual protein and protein transmission during the process time of lactose hydrolysis in a 50 ml stirred cell ultrafiltration reactor in a batch substrate addition mode. Initial lactose concentration was 124.9 mM and afterwards another 124.9 mM of lactose were added in every 2 hours. (■) and (◆) were % of residual protein in retentate when the reactor contained ratio of enzyme against substrate 1:1 and 3:1 respectively. (▲) and (×) were protein transmission (permeate) when the reactor contained ratio of enzyme against substrate 1:1 and 3:1 respectively. The operating conditions are mentioned in Figure 5.7.*

5.4 Characterization of 5 l membrane bioreactor

The study of membrane characterization was carried out to determine membrane resistance, the influences of pressure and temperature during operation

5.4.1 Effect of pressure on permeate flux

The permeability of water and phosphate buffer solution through crossflow membrane was measured in order to analyze the behavior of reactor system. Initially, 2.5 l of testing solution were filled into retentate tank. The experiments were conducted at temperature 25 ^{o}C. During operating system, the permeate from membrane was collected and measured the flow rate using a graduated cylinder and a stopwatch. The average flow rate was used for flux calculation.

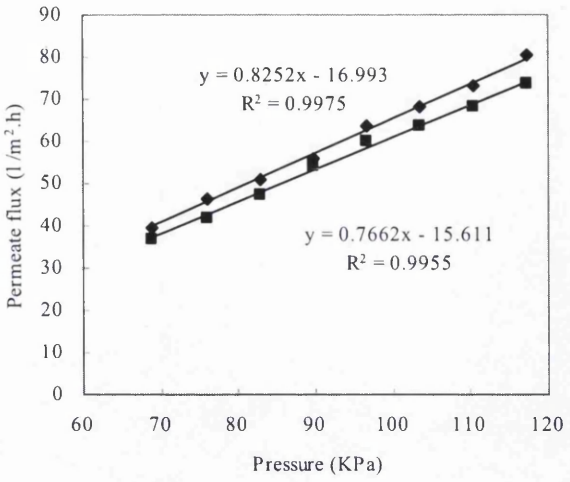


Figure 5.11 *The effect of pressure on permeate flux during ultrafiltration of water (◆) and 10 mM KH_2PO_4 solution (■) in the system at constant temperature 25 °C.*

The effect of pressure on the permeate flux for both water and 10 mM KH_2PO_4 buffer solution pH 6.5 is shown in Figure5.11. Flux values from both linearly increased with increasing pressure. For pure water the permeate flux increased from 39.68 to 80.32 $l/m^2.h$ with an increase in pressure from 69 to 117.2 KPa while operating with KH_2PO_4 solution the flux slightly lower from 36.77 to 73.55 $l/m^2.h$ with pressure from 69 to 117.2 KPa respectively.

The membrane resistance (R_m) was determined from the relationship between pure water flux and pressure. According to Darcy's law, the permeate flux of water is:

$$J = \frac{\Delta P}{\mu R_m} \qquad \textbf{Equation 5.3}$$

where J is the permeate flux ($l/m^2.h$), $\triangle P$ is the transmembrane pressure (Pa), μ is viscosity of water (Pa. s), R_m is membrane resistance (m^{-1}).

Transmembrane pressure (TMP) is calculated from following equation:

$$TMP = \frac{(P_{inlet} + P_{outlet})}{2} - P_{permeate} \qquad \textbf{Equation 5.4}$$

where P_{inlet} is the inlet pressure, P_{outlet} is the outlet pressure which cannot be measured and was assumed to be the same value as the inlet pressure, $P_{permeate}$ is the permeate pressure.

The calculated R_m value from the inverse of slope of water flux (hydraulic membrane permeability, $L_p = J/\triangle P = 1/\mu R_m$) in Figure 5.11 was estimated to be 4.88×10^6 m^{-1} when the viscosity of water was 8.937×10^{-4} Pa. s at the experimental temperature.

5.4.2 Effect of temperature on permeate flux

The effect of temperature on the permeate flux for both water and 10 mM KH_2PO_4 buffer solution pH 6.5 at constant pressure 10 psi is displayed in Figure 5.12. The relationship between permeate flux and temperature was linear for both liquid. The water flux increased from 34.84 to 48.06 $l/m^2.h$ and the flux of phosphate buffer was from 32.26 to 44.19 $l/m^2.h$ when the temperature was increased from 20.5 to 35.0 °C. Dina Afonso *et al* (2004) said the permeation flux usually increases 3-4% when the temperature rise every degree.

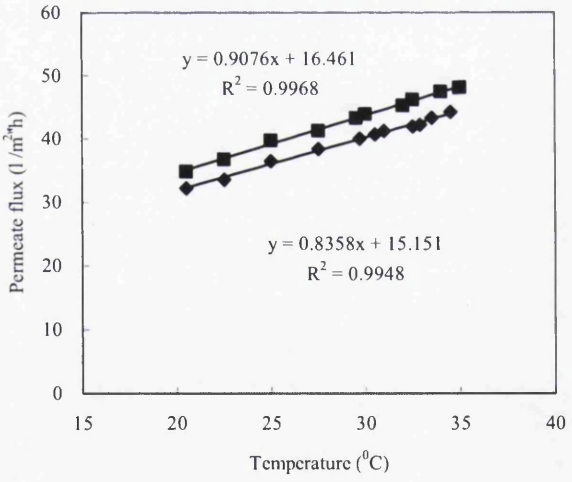


Figure 5.12 *The effect of temperature on permeate flux during ultrafiltration of water (◆) and 10 mM KH_2PO_4 solution pH 6.5 (■) in the system at constant pressure 10 psi.*

Increasing permeate flux with temperature was probably due to change in the physical properties such as viscosity, diffusivity and density of the liquid in the system. Increasing

temperature directly affected a decrease in viscosity of liquid whereas the intrinsic membrane resistance remained constant. The relation between viscosity and flux was shown according to equation 5.3 and this lead to an increase in membrane hydraulic permeability.

5.5 Stability of enzyme in operational membrane bioreactor

The study in ability of the enzyme to retain catalytic activity under specific operating condition is very important for the efficient and economical use. Enzymes are normally not stable during storage and their activities are gradually decrease or lost with time. Retaining in stability depends on surrounding condition. Operating the enzyme in MBR might be subjected to leakage and easy losses as a result of prolonged process time and lot of effects from the system. So it is necessary to consider the operational stability of the enzyme in the reaction system in order to handle or at least protect the enzyme from fast deactivation.

The stability of β-galactosidase in operational MBR was carried out with and without addition of nitrogen gas in the system so as to analyze the effect of gas-liquid interface on enzyme activity. The measurement of the enzyme operational stability was set up in the reactor without the interfering of substrate and product inhibition. The experiment was operated in batch mode with recirculating permeate in the system. The crude enzyme with 10 mM KH_2PO_4 buffer pH 6.5 was loaded in the system. The operation temperature was fixed at 25 °C and the system was processed for 9 h with the inlet pressure of membrane at 10 psi. Every an hour, the enzyme activity and protein concentration were determined by reacting aliquots of permeate and retentate using 45 mM ONPG and Folin method respectively. The activity during the time was compared with the initial activity and expressed the result in terms of its residual activity (%) as can be calculated following equation 5.1 and 5.2.

The percentages of residual activity and process time for β-galactosidase in MBR with nitrogen gas and air are presented in Figure 5.13. The enzyme activity in retentate from both conditions was gradually decreased through time. After 9 h the enzyme in nitrogen system appeared to stabilize better than in the air. The residual enzyme activity lost approximately 15% of its initial activity in the presence of gas whereas about 34% was deactivated in air. The slight enzyme activity, less than 0.5%, was found in both permeate.

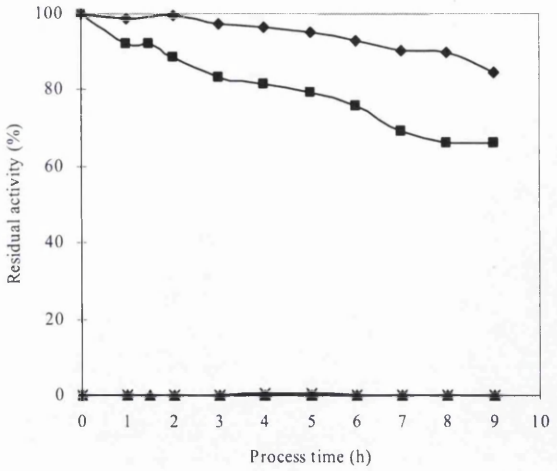


Figure 5.13 *The residual activity of β-galactosidase in ultrafiltration membrane reactor with nitrogen and air conditions versus time. The maximum process volume reactor was 5 l. Total volume of reaction solution consisted of 250 ml crude enzyme and 2250 ml phosphate buffer pH 6.5. Enzyme concentration was 0.28 mg protein/ml reactor. The temperature was controlled at 25 °C and the inlet pressure was at 10 psi. (◆) and (×) were residual activity (%) in retentate and permeate in the presence of nitrogen gas. (■) and (▲) were residual activity (%) in retentate and permeate in the presence of air.*

The half-life value of β-galactosidase in MBR was calculated from the slope in Figure 5.14 according to the equation 4.8. The half-life time in the presence of nitrogen condition was 40.8 h which was 2.7 times higher than in air. The results showed operating in nitrogen condition appeared to improve stability of enzyme rather than atmospheric condition.

However, in comparison to the results of half-life time from incubation the enzyme at 20-30 °C with time in batch reactor (Figure 4.18) or even residual activity from operating the enzyme in 50 ml stirred cell ultrafiltration system (Figure 5.5), the enzyme seemed to be more stable with these conditions than operating in the MBR. The enzyme in membrane bioreactor system might easily lose its activity caused by a variety of effects exist in the reactor such as gas-liquid interface, shear stresses due to the flow across the membrane device, pumping the stream from the tank to membrane device by the recycling pump, and especially from enzyme adsorption on the membrane surface. Petzelbauer *et al.* (2002) said that adsorption on the membrane was found to happen during the start-up of the reactor. Typically, 0.1-0.2 mg protein/cm^2 of the membrane were required to occupy the

membrane surface as it assisted to prevent the binding of the enzyme and loss of activity.

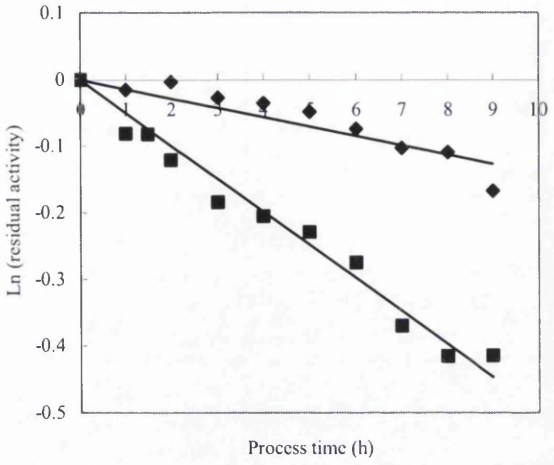


Figure 5.14 *The effect of nitrogen on operational stability of β-galactosidase in MBR. Operation conditions are described in Figure 5.13. (◆) is with nitrogen, (■) is in air.*

The protein concentration in system was determined in order to check if there was leakage or breakage of β-galactosidase from the membrane (Figure 5.15). Detecting the protein in permeate was not found. Thus there was no loss of β-galactosidase observed from system during operating time. The residual protein in retentate for both conditions did not have a significant change with their initial concentrations while the activity of the enzyme reduced gradually with time. Thus it can be interpreted that the main cause in loss of enzyme activity with this system was probably not from the adsorption on the membrane. The enzyme might be deactivated by force associated with the operation membrane reactor system i.e. exposure to pumps, foaming other air/liquid and solid/liquid interfaces. The fluxs of permeate in both conditions (Figure 5.15) were relatively stable with process time with little loss of activity.

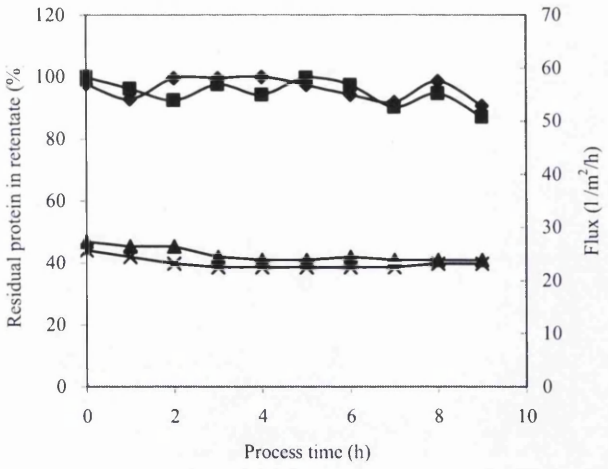


Figure 5.15 *Plots of residual protein in retentate (%) during operating MBR system (◆) with nitrogen, (■) with air and flux from the system (▲) with nitrogen, (✕) with air versus process time. Operation conditions are described in Figure5.13.*

5.6 Lactose hydrolysis in 5 l MBR

The performance of lactose hydrolysis using a pilot scale membrane reactor was investigated after study the possibility in small scale stirred cell membrane reactor and characteristics of membrane reactor including the retaining and stability of enzyme in the reactor. In this membrane reactor, the glucose production in batch mode operated in permeate recycling with continuous adding lactose every 180 min was investigated. In addition, the continuous mode with different initial lactose concentrations, enzyme concentrations and flow rates were studied as well.

5.6.1 Investigation of lactose hydrolysis in fed batch mode MBR

The hydrolysis of lactose in MBR was firstly tested in batch mode because it was easy to operate and control. Batch hydrolysis experiment in MBR was carried out by first adding 2250 ml of 55.5 mM lactose in the reactor tank. After adjusting the temperature in the system at 25 °C, 250 ml enzyme solution was added. The membrane bioreactor was operated in permeate recycling. The permeate flux was controlled by the permeate pump at 12.9 ml/min. The pressure of inlet membrane was set at 10 psi. After operation the system for 180 min, fresh lactose solution at the same concentration and volume with first loading was added every 180 min. The system was operated for 540 min. The samples were

collected periodically for glucose measurement.

Figure 5.16 displays the total glucose production in the system versus process time. A sharp increase in the reaction rate of lactose hydrolysis was observed at the initial 180 min operation. The calculated initial rates of appearance of each 180 min reaction were 0.93, 0.87 and 0.63 μmol/ml/min. The decrease in initial rate was probably from enzyme deactivation in the system or the reaction was approaching equilibrium. The fractional hydrolyzed conversions of lactose every 180 min in each cycle were 0.82, 0.89 and 0.85 respectively.

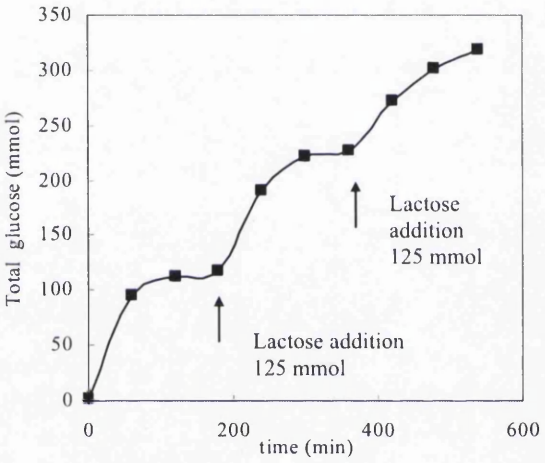


Figure 5.16 *The production of glucose on lactose hydrolysis in fed batch membrane reactor by crude enzyme. The initial lactose substrate concentration was* 55.5 mM. *The same concentration of lactose solution was added every 180 min as indicated. The enzyme concentration in the reactor was 0.35 mg protein/ml reactor. The temperature was controlled at 25 °C.*

5.6.2 Investigation of lactose hydrolysis in continuous mode MBR

The effect of the continuous operation mode on enzymatic transformation in MBR was carried out using different lactose concentrations, enzyme concentrations and flow rates of permeate. To start operating, 2250 ml of lactose solution was loaded into the reactor tank with substrate solution at the required concentration. After adjusting the temperature to 25 °C, 250 ml of the enzyme was added. Mixing substrate and the enzyme was achieved by recirculation of the reactor volume. The total working volume was fixed at 2500 ml.

Further details of operating the MBR in continuous mode are given in section 2.12.3 (see Materials and Methods).

5.6.2.1 Effect of lactose concentration

Three different initial lactose concentrations, 55.5 mM, 83.3 mM and 111.0 mM were investigated. During the operation, the permeate flow rate was maintained at 12.9 ml/min (a mean residence time of 194 min). The experiments were conducted for 540 min.

The glucose concentrations in both retentate and permeate through the process time were very similar (data not shown) and implied that the concentration polarisation had negligible effect on flux and retention of glucose.

The glucose production in permeate as a function of reaction time with different substrate concentrations is shown in Figure 5.17. It can be clearly seen that the glucose concentration in permeate increased with increasing initial lactose concentration. At low lactose concentration (55.5 mM), the reaction was reached steady state after 90 min while higher concentration took longer time. The average glucose concentrations in permeate after operation were 35.7, 48.8 and 56.4 μmol/ml at different lactose concentrations of 55.5 mM, 83.3 mM and 111.0 mM respectively.

5.6.2.2 Effect of enzyme concentration

The effect of enzyme concentration on the reaction rate was determined by carrying out runs at different protein concentrations of crude enzyme. The experiments were conducted at fix lactose concentration (55.5 mM). The permeate flow rate was controlled at 12.9 ml/min.

The formation of glucose using different concentrations of enzyme is shown in Figure 5.18. The enzyme concentration strongly affected the release of product. The glucose production was positively related to enzyme concentration. The glucose concentrations in permeate after operation were about 17.3, 26.9 and 35.7 μmol/ml when the concentrations of crude enzyme were 0.06, 0.14 and 0.28 mg protein/ml reactor respectively.

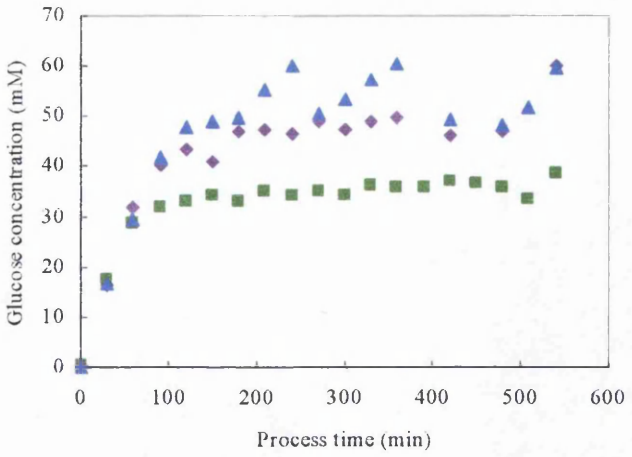


Figure 5.17 *The effect of lactose concentration on glucose production in continuous MBR. The initial lactose concentrations were 55.5 mM (■), 83.3 mM (◆) and 111.0 mM (▲). The concentration of crude enzyme was 0.28 mg protein /ml reactor. The total volume reaction in MBR was 2500 ml which consisted of 2250 ml lactose solution and 250 ml crude enzyme. The operating temperature was controlled at 25 °C. The permeate flow rate was controlled at 12.9 ml/min.*

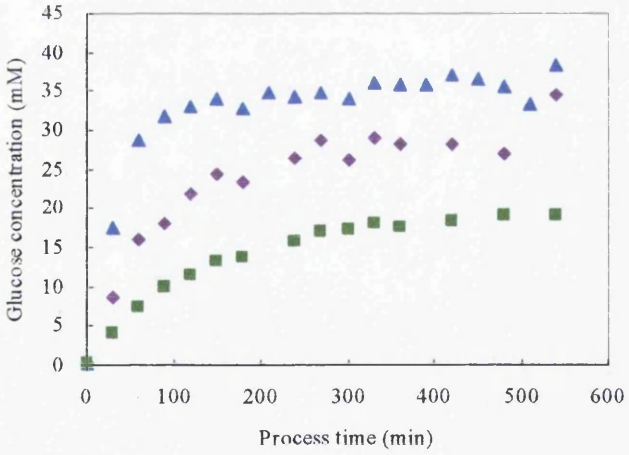


Figure 5.18 *The effect of enzyme concentration on glucose production in continuous MBR. The protein concentrations of crude enzyme were 0.06 mg/ml (■), 0.14 mg/ml (◆), and 0.28 mg/ml (▲). The lactose concentration was fixed at 55.5 mM. Operating conditions are mentioned in Figure 5.17.*

5.6.2.3 Effect of permeate flow rate

The effect of permeate flow rate on glucose production as a function of reaction time was investigated. The results in change of the flow rate affected the mean residence time of the substrate during hydrolysis (Figure 5.19). The lactose concentration was prepared at constant 55.5 mM. The glucose concentrations after operation at flow rate 8 ml/min, 12.9 ml/min, and 25 ml/min were on average 36.6, 35.7 and 32.1 μmol/ml respectively. The results can also be shown in terms of productivity of glucose (Figure 5.20) which was calculated as followed;

Productivity (μmol/min)=Glucose concentration (μmol/ml) x Flow rate (ml/min)

Equation 5.5

The productivity of glucose was therefore directly dependent on the flow rate. Increasing the flow rate from 8, 12.9 to 25 ml/min resulted in productivity of about 293, 461 and 803 μmol/min respectively.

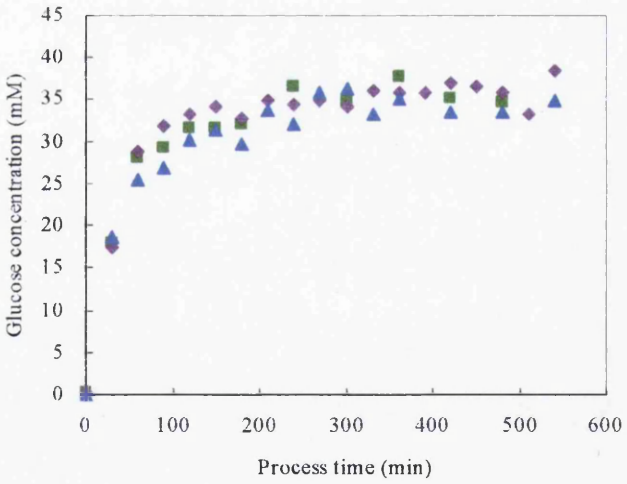


Figure 5.19 *The effect of flow rate on glucose production in continuous MBR. The flow rates were 8 ml/min (■), 12.9 ml/min (◆), and 25 ml/min (▲). Operating conditions are mentioned in Figure 5.16.*

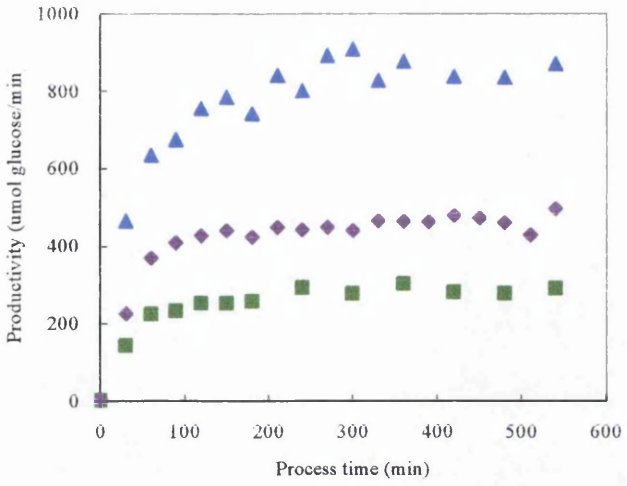


Figure 5.20 *The effect of flow rate on productivity of glucose in continuous membrane bioreactor. The flow rates were 8 ml/min (■), 12.9 ml/min (◆), and 25 ml/min (▲).*

5.7 Mathematical modelling for lactose hydrolysis

A mathematical modelling study was employed to describe and investigate various aspects of reaction occurring in the system during lactose hydrolysis. The use of model also assists the prediction of further reactions and would probably lead to the design of better strategies for the optimization of the process.

The mechanism of lactose hydrolysis has been recently investigated in the formation of galacto-oligosaccharide (GalOS), a potentially important material associated with prebiotic formulations. As known that, β-galactosidase hydrolyzes lactose to monosaccharides, glucose and galactose. During hydrolysis, after glucose molecule leaves the active site of the enzyme, a covalent galactosyl-enzyme complex can be further hydrolyzed (Kim *et al.*, 2004). Zhou *et al.* (2001) mentioned the enzyme-galactosyl complex is transferred to an acceptor containing a hydroxyl group. In a diluted lactose solution condition, water can be more competitive as an acceptor than sugars such as lactose and glucose. Therefore, free galactose is formed and released from the active site. In contrast, at high lactose concentration, lactose has more chance to serve as an acceptor, binding with the galactosyl-enzyme complex to synthesis GalOS by transgalactosylation reaction. In addition to lactose, glucose can also act as a better acceptor for transgalactosylation

reaction to form galactosyl-glucose disaccharide (Kim *et al.*, 2004). The structure of GalOS can be in form of disaccharides, trisaccharides and higher saccharides. However, oligosaccharides found during lactose hydrolysis are almost entirely disaccharides which are directly formed from monosaccharide and degradation of trisaccharide (Mahoney, 1998). The synthesis of GalOS depends on the source of the enzyme, lactose concentration and the reaction conditions used in the process (Petzelbauer *et al.*, 2002). High substrate concentration results in high concentration of GalOS.

The oligosaccharide formation also relates to lower residence times when the lactose is only partially hydrolysed (Prenosil *et al.*, 1987; Mozaffar *et al.*, 1986). Lopez-Leiva and Guzman (1995) reported that oligosaccharides were formed as intermediate products with a maximum concentration at residence time of 5-15 seconds during hydrolysis of milk whey permeate. At longer reaction times the oligosaccharide tended to disappear and degrade to monosaccharides. The formation of GalOS as transient reaction products is also found during lactose hydrolysis before conversion to main products, glucose and galactose, by the enzyme from *Talaromyces thermophilus* CBS 236.58 (Nakkharat and Haltrich, 2006).

According to GalOS being an intermediate product during short time, the kinetic model on lactose hydrolysis concerned on the formation of GalOS may be negligible when operating long period. Zhou *et al.* (2003) said that most models describing β-galactosidase reaction had neglected GalOS.

Many kinetic models have been proposed for lactose hydrolysis using β-galactosidase by several authors. Different sources of the enzyme and analyzed conditions use different fitted kinetic models due to type of inhibition are shown in Table 5.1.

Enzyme source	T(°C)	$C_{lactose}$ (mM)	C_{enzyme} (mg/l)	Buffer	Kinetic model	Reference
E.coli	25-37	0.5-3	3.08	0.1M phosphate buffer pH 7.0	Competitive inhibition by galactose	Wadiak and Carbonell, 1975.
K.fragilis	5-40	69.4-194.3	2.3-11.7	BM buffer pH 6.5	Competitive inhibition by galactose	Santos *et al.*, 1998
K.fragilis	43	146-292	21-88 (protein)	0.025M phosphate buffer pH 6.86	Competitive inhibition by galactose	Carrara and Rubiolo, 1996.
K.fragilis	37	139	2-2.5 mg/ml dried cells	Tris-maleate-NaOH buffer pH 7.0	Competitive inhibition by galactose and non-competitive by glucose	Chen *et al.*, 1985.
A.oryzae	37	25-500	-	pH 5.0	Noncompetitive inhibition by galactose	Chen *et al.*, 1985.
A.oryzae	30	50	-	milk	Competitive inhibition by galactose	Portaccio *et al.*, 1998
A.niger	40-60	14.4-144	47.2-472 UI	pH 3.6	Competitive inhibition by galactose	Papayannakos *et al.*, 1993.
A.niger	8-60	73-584	10-10000	lactic acid buffer pH 4.0	Competitive inhibition by galactose	Yang and Okos, 1989.

Table 5.1 *Summary on kinetic models proposed for hydrolysis of lactose by β-galactosidase (cited in Santos et al., 1998).*

The enzyme kinetic studies (Table 5.1) were on *E. coli*, yeast and fungi and fit models based on Michaelis-Menten kinetics with product inhibition, competitive and non-competitive product inhibition, have been proposed. However, the properties of the enzyme or even kinetic mechanism can be different depending on sources of the enzyme and an environmental condition of the enzyme production. The kinetic model of β-galactosidase from lactic acid bacteria especially from *L. delbrueckii* ssp *bulgaricus* has not been studied in detail. All possible classical models for the enzyme from this bacterium were therefore investigated.

The aim of this study was to use the model to predict the reaction of lactose hydrolysis by crude β-galactosidase in both batch reactor and continuous membrane bioreactor. The experimental data measuring hydrolysis described above (section 5.2.1-5.2.2 for batch reactor and 5.6.2.1-5.6.2.3 for continuous MBR) were investigated with models.

The kinetic parameters of model were estimated from experimental data by using nonlinear regression analysis with SCIENTIST software (MicroMath Scientist Software, Inc.).The data from experiments were fitted to the model so as to evaluate unknown constants. The differences between the experiment data and calculated data were compared and recalculated in the optimization routine and fed again to the integration step until achieving a minimal value by using Least Squares method from the software. The correlation coefficient (R^2) was then used to assess the degree of fit of the model with each experimental data. The programs for model used in the software are written in Appendix 2.

5.7.1 Mathematical modelling of lactose hydrolysis in batch system

After complete hydrolysis of lactose, monosaccharides, glucose and galactose are formed. Following equation can be used;

$$\frac{-d[Lac]}{dt} = \frac{d[Glu]}{dt} = \frac{d[Gal]}{dt} \qquad \textit{Equation 5.6}$$

where $-d[Lac]/dt$ is the lactose utilization rate (mmol/l.min), $d[Glu]/dt$ and $d[Gal]/dt$ are the glucose and galactose production rates (mmol/l.min) respectively.

The product formation can be expressed in terms of the kinetic rate of reaction. This indicates that the rate of glucose formation is proportional to the reaction rate of enzyme.

$$\frac{d[Glu]}{dt} = r \qquad \textit{Equation 5.7}$$

The lactose hydrolysis in batch reactor was studied using different initial lactose concentrations and enzyme concentrations. Many kinetic models were tested and compared the results with the experimental data from different lactose concentrations.

5.7.1.1 Mathematical modelling on initial rate of reaction and lactose concentration

The experimental results between lactose concentrations and initial rates of reaction were compared with the proposed model curve based on the Michaelis–Menten equation. The simplest model without inhibition was used due to a negligible effect of product inhibition on reaction during initial phase. The model is on the basic of following mechanism:

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

where E is enzyme, S is substrate, P is product, k_1 is forward reaction rate constant for the formation of ES, k_{-1} is reverse reaction rate constant for the breakdown of ES to E+S and k_2 is reaction rate constant for the breakdown of ES to E+P.

Kinetic equation can be expressed as:

$$r = \frac{V_{max} S}{K_m + S} \qquad \textit{(Model I)}$$

Michaelis–Menten without inhibition (Segel, 1993)

when $Km = \frac{k_{-1} + k_2}{k_1}$ and $V\max = k_2[E]_t$

where r is the initial reaction rate (mmol/l.min), V_{max} is the maximum reaction rate (mmol/l.min), S is the concentration of substrate (mmol/l), K_m is the Michaelis–Menten constant for substrate (mmol/l), $[E]_t$ is total enzyme concentration.

The relationship between initial rates of reaction and different concentrations of both lactose and ONPG were measured in the phosphate buffer pH 6.5, temperature 25 °C (Figure5.21). The initial rates of product formation were positively related to the initial substrate concentrations. Increasing in the rate of reaction was observed when substrate concentration was increased and clearly shown in the range of low concentration with sheer slope. However, at high substrate concentration especially significantly greater than K_m, the reaction rate generally becomes insensitive to the change of substrate concentration. The lines through the data points were calculated according to the Model I. The experimental data from both substrates fitted well with the model.

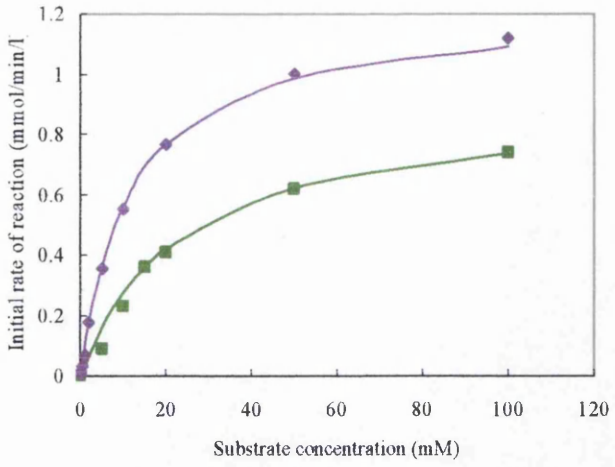


Figure 5.21 *Initial rate of product formation at different substrate concentrations. (■) and (◆) are from experimental data when lactose and ONPG were applied as substrate respectively and lines drawn were derived from the model I.*

The maximum initial rate of glucose production when lactose used as substrate was 0.91 μmol/min/ml. The K_m value calculated from experimental data according to equation of Lineweaver-Burk plotting (from Figure 4.22) and used in this model was 23.23 mM (R^2 = 0.995). In case of ONPG as substrate (from Figure 4.21), the maximum initial rate of reaction was 1.22 μmol/min/ml and K_m value was 11.86 mM (R^2 = 0.999).

5.7.1.2 Kinetic model selection

The experimental data on glucose production from three different initial lactose concentrations (55.5 mM, 83.3 mM and 111.0 mM) in the phosphate buffer pH 6.5, temperature 25 °C were compared with the simulated results from four possible models based on Michaelis–Menten equation and another one was from Haldane equation.

Michaelis–Menten kinetic equation without inhibition

There are many proposed models based on Michaelis–Menten kinetic describing the reaction rate of lactose hydrolysis by β-galactosidase. The simple one without concerning on product inhibition was considered. The kinetic equation can be expressed as Model I (see section 5.7.1.1)

Figure 5.22 shows the value of glucose production obtained from experiments and predicted from Model I. The points represented the experimental data whereas simulated results from models were drawn as lines. The K_m value used in this model was fixed at 23.23 mM while the V_{max} value and substrate concentration used for calculating model were from experimental data. The results showed large differences between model value and data with R^2 in range 0.763-0.874.

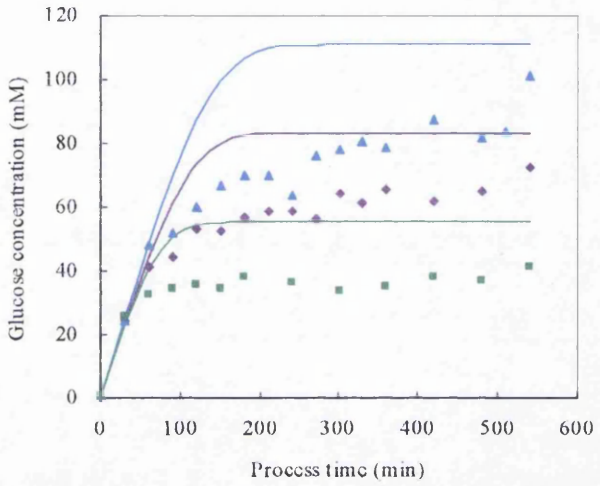


Figure 5.22 *Comparison between experimental data and model I (no inhibition) on the effect of lactose concentration on glucose production from batch reactor. The experimental data of glucose production were performed as points when lactose concentrations were 55.5 mM (■), 83.3 mM (◆) and 111.0 mM (▲). The results from model were drawn as lines 55.5 mM (—), 83.3 mM (—) and 111.0 mM (—).*

Michaelis–Menten kinetic equation with end product inhibitor

Many research have reported that the activity of β-galactosidase is able to be inhibited by galactose (Griffiths and Muir, 1978; Cowan *et al.*, 1984; Berger *et al.*, 1997; Santos *et al.*, 1998; Batra *et al.*, 2002). The reaction rate is decreased when the product formation increases.

Galactose acts as a competitive end product inhibitor, galactose directly competes with the substrate in forming enzyme complex. The reaction steps can be described as follows:

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

$$E + P \underset{}{\overset{K_i}{\rightleftharpoons}} EP$$

Kinetic equation can be expressed as:

$$r = \frac{V_{max} S}{K_m\left(1+\frac{P}{K_i}\right)+S} \qquad \textbf{(Model II)}$$

Competitive inhibition (Segel, 1993)

If galactose acts as a non-competitive end product inhibitor (as a result from section 4.5.6), the reaction occurs by the inhibitor binds to either the enzyme or the enzyme-substrate complex without affecting the binding of the substrate as displayed follows:

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

$$E + P \underset{}{\overset{K_i}{\rightleftharpoons}} EP$$

$$ES + P \underset{}{\overset{K_i}{\rightleftharpoons}} ESP$$

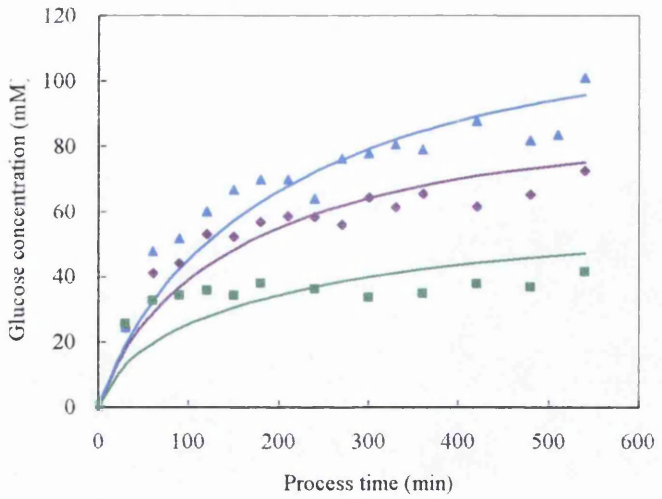
Kinetic equation can be expressed as:

$$r = \frac{V_{max} S}{K_m\left(1+\frac{P}{K_i}\right)+S\left(1+\frac{P}{K_i}\right)} \qquad \textbf{(Model III)}$$

Non-competitive inhibition (Segel, 1993)

where r is the reaction rate (mmol/l.min), V_{max} is the maximum reaction rate (mmol/l.min), S is the concentration of substrate (mmol/l), P is the concentration of product (mmol/l), K_m is the Michaelis–Menten constant for substrate (mmol/l), K_i is galactose inhibition constant (mmol/l).

(a)



(b)

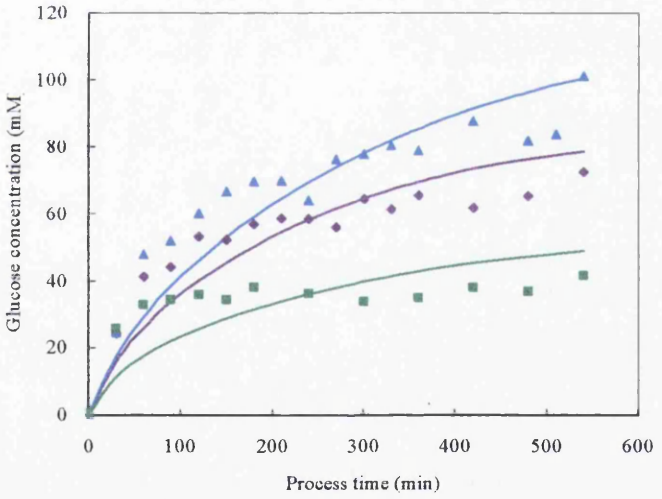


Figure 5.23 *Comparison between experimental data and model II (a), model III (b). The experimental data of glucose production were performed as points when lactose concentrations were 55.5 mM (■), 83.3 mM (◆) and 111.0 mM (▲). The results from model were drawn as lines.*

The comparison of experimental data and calculating data from Model II and Model III are displayed in Figure 5.23 (a) and (b) respectively. The K_m value used in this model was fixed at 23.23 mM. The V_{max} value and substrate concentration used for calculating model

were from experimental data. The parameter K_i value from section 4.5.6 (K_i = 329 mM) was simulated with both models and it did not fit with the experimental data. In addition to this value, the parameter K_i value was also evaluated by fitting the experimental data with models. The K_i values for best fitting from Model II were in range 2.5-7.5 and from Model III were in range 5.2-23.7 with R^2 in range 0.928-0.988 (Figure 5.23 (a) and (b)). Both models did not fit well with experimental data.

Michaelis–Menten kinetic equation with both product inhibitors

In addition to galactose as an inhibitor, the other product of lactose hydrolysis, glucose, is able to act as an inhibitor. The kinetic equation for mixed inhibition can be expressed as;

$$r = \frac{V_{max} S}{K_m\left(1+\frac{P}{K_i}+\frac{P}{K_i'}\right)+S\left(1+\frac{P}{K_i}+\frac{P}{K_i'}\right)} \qquad \textit{(Model IV)}$$

Non-competitive inhibition from both end products (Ladero et al., 2001)

where r is the reaction rate (mmol/l.min), V_{max} is the maximum reaction rate (mmol/l.min), S is the concentration of substrate (mmol/l), P is the concentration of product (mmol/l), K_m is the Michaelis–Menten constant for substrate (mmol/l), K_i is galactose inhibition constant (mmol/l), K_i' is glucose inhibition constant (mmol/l).

The glucose production obtained from experiments and predicted from Model IV is compared in Figure 5.24 with the K_m 23.23 mM and K_i galactose at 329 mM. According to the result from section 4.5.5, glucose reacted with ONPG as a non-competitive inhibitor. However, the K_i' of glucose reacted with lactose was unknown. The guess K_i' value was evaluated by fitting the experimental data with models. The K_i' values for best fitting between the Model IV and three set of experimental data were in range 5.2-25.6 with R^2 in range 0.927-0.980.

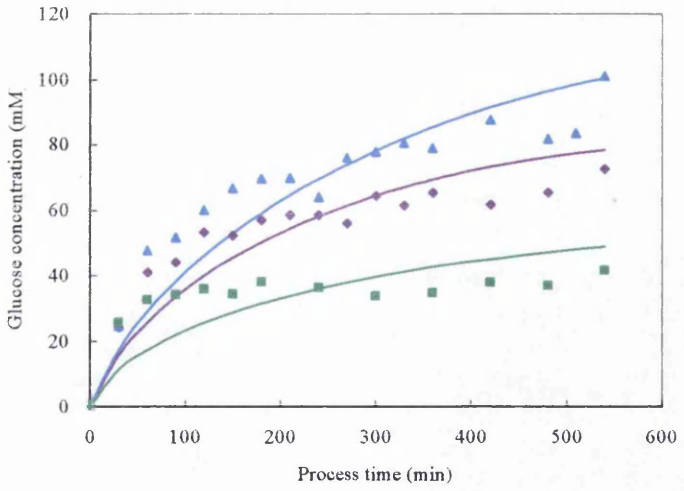


Figure 5.24 *Comparison between experimental data and model IV on the effect of lactose concentration on glucose production from batch reactor. The experimental data of glucose production were performed as points when lactose concentrations were 55.5 mM (■), 83.3 mM (◆) and 111.0 mM (▲). The results from model were drawn as lines.*

Reversible reaction-Haldane kinetic equation

Although Michaelis–Menten model is generally used for evaluation in initial rate of reaction and in fact, all enzyme reactions are to some degree reversible. Another model based on Haldane kinetic equation was used in this study. The model is on the basic of following mechanism:

$$E + S \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} ES \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} E + P$$

The reaction might be measured in either direction. The constants for forward and reverse reactions according to above equation can be calculated as followed;

Forward direction

$$Km = \frac{k_{-1} + k_2}{k_1} \quad \text{and} \quad V\max_f = k_2[E]_t$$

Reverse direction

$$Kp = \frac{k_2 + k_{-1}}{k_{-2}} \quad \text{and} \quad V\max_r = k_{-1}[E]_t$$

Therefore, the overall equilibrium constant for the reaction (K_{eq}) can be expressed in terms of maximum rate of reactions and constant rates from both directions as followed;

$$K_{eq} = \frac{V_{\max f}.K_p}{V_{\max r}.K_m}$$

The rate of reaction then relates to equilibrium constants. The expression can be written as follows:

$$r = \frac{V_{\max f}\left(S - \frac{P}{K_{eq}}\right)}{K_m\left(1 + \frac{P}{K_p}\right) + S} \qquad \textbf{(Model V)}$$

Reversible reaction (Haldane) (Segel, 1993)

where r is the reaction rate (mmol/l.min), $V_{max\ f}$ is the maximum reaction rate in the forward direction (mmol/l.min), $V_{max\ r}$ is the maximum reaction rate in the reverse direction (mmol/l.min), S is the concentration of substrate (mmol/l), P is the concentration of product (mmol/l), K_m is the Michaelis–Menten constant for substrate (mmol/l), K_p is Michaelis–Menten constant for product (mmol/l), K_{eq} is Haldane relationship.

The performance of glucose production obtained from experiments and predicted from Haldane equation (Model V) are compared in Figure 5.25. The K_m value used was at 23.23 mM. The V_{max} value varied depending on the amount of enzyme while the unknown kinetic parameters (K_p and K_{eq}) according to Haldane kinetic equation were estimated by fitting the model equation with the experimental results. The obtained K_p and K_{eq} values were 24.62 mM and 3.4 respectively.

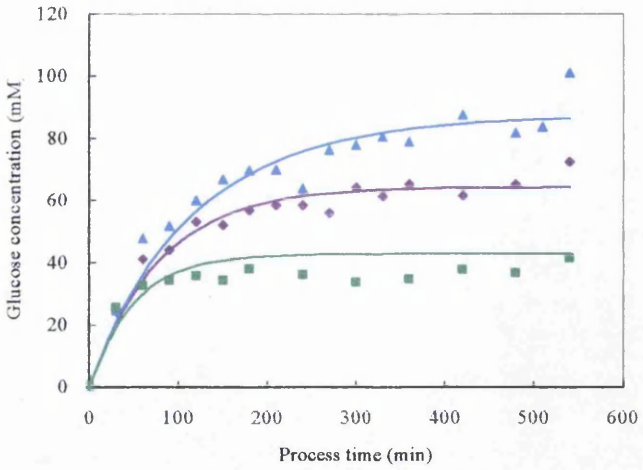


Figure 5.25 *Comparison between experimental data and model V on the effect of lactose concentration on glucose production from batch reactor. The experimental data of glucose production were performed as points when lactose concentrations were 55.5 mM (■), 83.3 mM (◆) and 111.0 mM (▲). The results from model were drawn as lines.*

As shown in Figure 5.25, the data from experiment of lactose concentrations at 83.3 mM and 111.0 mM were good agreement with simulated model. The R^2 values of both experiments with the model were 0.996 and 0.992 respectively. While the results from experiment at concentration 55.5 mM were slightly underestimated from predicted model ($R^2 = 0.980$).

Table 5.2 *Summary of parameters used and correlation coefficient (R^2) from five models.*

Model	Parameter used						R^2 of Lactose concentration (mM)			
	V_{max}	K_m	K_i	K'_i	K_p	K_{eq}	55.5	83.3	111.0	average
I	0.98-1.19	23.23	-	-	-	-	0.763	0.874	0.842	0.826
II	0.98-1.19	23.23	329	-	-	-	0.946	0.989	0.988	0.974
III	0.98-1.19	23.23	329	-	-	-	0.928	0.977	0.980	0.962
IV	0.98-1.19	23.23	329	5.2-25.6	-	-	0.927	0.977	0.980	0.961
V	0.98-1.19	23.23	-	-	24.62	3.4	0.980	0.996	0.991	0.989

The degree of fit of the model with each experimental data was expressed in terms of correlation coefficient (R^2). The parameter used and comparison of R^2 from experimental data with different five models are shown in Table 5.2. The average fitting degrees from

five models were 0.83, 0.97, 0.96, 0.96 and 0.99 respectively. The results seemed to correlate well with Model V rather than the models based on Michaelis–Menten equation (Model I-IV). Then the reversible reaction-haldane kinetic model (Model V) was directly used as a model for predicting product formation from lactose hydrolysis in this study.

5.7.1.3 Model simulation in batch mode with different enzyme concentrations

The experimental data of glucose production from different concentrations of enzyme with a constant initial lactose concentration at 55.5 mM were compared with the data from Haldane equation (Model V) as performed in Figure 5.26. The points represented the experimental data whereas simulated results from models were drawn as lines.

The initial rate of reaction varied with enzyme concentration. The rate of reaction was faster when high concentration of the enzyme was employed. Good agreement between the model V prediction and the experimental data were obtained. The R^2 values of nonlinear regression between experimental results and the model for these four experiments enzyme concentration were 0.996, 0.994, 0.995 and 0.997 when the concentrations of crude enzyme were 0.06 mg/ml, 0.11 mg/ml, 0.17 mg/ml, and 0.22 mg/ml respectively.

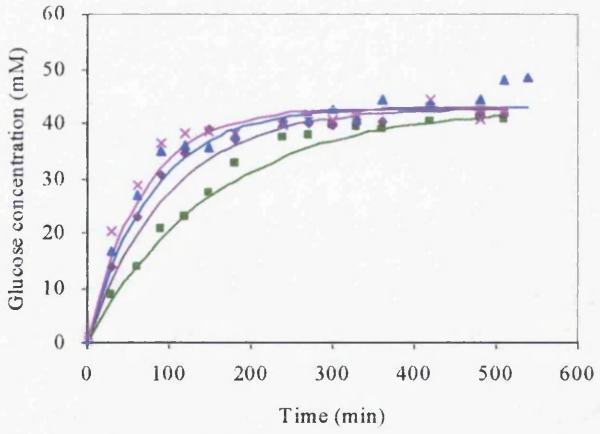


Figure 5.26 *Comparison of glucose production between experimental data from batch bioreactor and the model. The initial lactose concentration was fixed at 55.5 mM. The experimental data of glucose production were performed as points with various enzyme concentrations 0.06 mg/ml (■), 0.11 mg/ml (◆), 0.17 mg/ml (▲) and 0.22 mg/ml (×). The results from model were drawn as lines.*

5.7.2 Continuous membrane reactor

The continuous membrane reactor was assumed to behave likely as a continuous–stirred-tank reactor (CSTR). The mathematical model for this system was developed from the fundamental principle of mass balance. The flow rate of feeding was adjusted to equal the flow rate of outflow. The product balance can be written as follows:

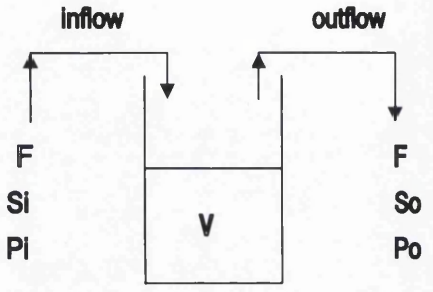


Figure 5.27 *The schematic of continuous system. F is flow rate (ml/min), S is substrate concentration (mM), P is product concentration (mM), V is reactor volume (ml), i is inflow and o is outflow.*

Rate of product accumulation = product input + product formation – product output

Mathematically, equation above can be written as follows:

$$V\frac{dP}{dt} = FP_i + rV - FP_o \qquad \textbf{Equation 5.8}$$

According to Figure 5.27, the substrate is added to the reactor at a constant flow rate and removed equally from the system. The product is formed during the reaction and removed from the reactor a constant rate ($P_i = 0$). In this study, [Glu] is assumed equal to $[Lac]_o$ – [Lac]. Then- $dS/dt = dP/dt = r$. The equation therefore can be written as:

$$V\frac{dP}{dt} = -FP_o + rV \qquad \textbf{Equation 5.9}$$

$$\frac{dP}{dt} = -D_pP + r \qquad \textbf{Equation 5.10}$$

where dP/dt is the rate of product formation, D_p is dilution rate (min^{-1}) which

equal to F / V.

The effect of the continuous mode on the enzymatic transformation in MBR was displayed at different lactose concentrations, enzyme concentrations and flow rates of permeate. The results from experiments were applied to the model developed in equation 5.10. The proposed reversible reaction model from batch reactor was combined with mass balance equation to predict the lactose hydrolysis occurred in continuous membrane reactor. Operating in the reactor was assumed to behave likely as a CSTR. The experimental data were compared with results from model. The kinetic parameters, K_m, K_p and K_{eq} used in the model expressed the same values in batch reactor. The fits of the model to the experimental data in continuous membrane reactor are displayed in Figure 5.28, 5.29 and 5.30.

5.7.2.1 Effect of lactose concentration

The production of glucose as a function of reaction time using different substrate concentrations from the experiments and predicted values by model is performed in Figure 5.28. The experimental data from lactose concentrations at 55.5 mM and 83.3 mM correlated well with the model while the data from lactose 111.0 mM was slightly fluctuate. The R^2 values obtained between data and model were 0.998, 0.993 and 0.994 respectively.

5.7.2.2 Effect of enzyme concentration

Figure 5.29 presents the formation of glucose from experimental data and simulated model as a function of reaction time with different concentrations of enzyme. The permeate flow rate was controlled at 12.9 ml/min. The lactose concentration was fixed at 55.5 mM. The model predictions correlated well with data from experiments. The R^2 values obtained from this simulation were 0.998, 0.992 and 0.997 when the enzyme concentrations were 0.06, 0.14 and 0.28 mg/ml respectively.

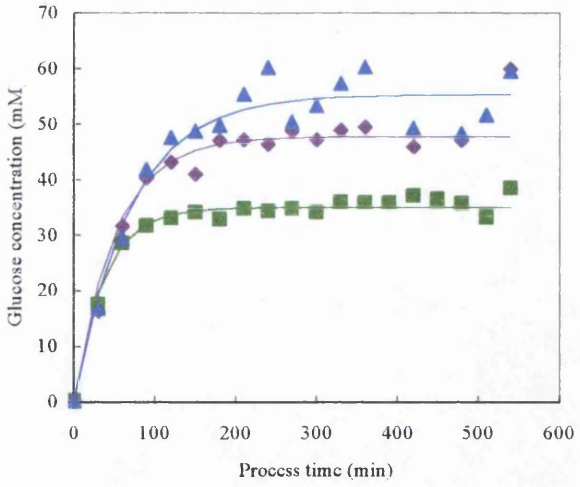


Figure 5.28 *Comparison of glucose production as a function of reaction time between experimental data and the model from continuous membrane reactor. The experimental data of glucose production were performed as points when lactose concentrations were 55.5 mM (■), 83.3 mM (◆) and 111.0 mM (▲). The results from model were drawn as lines.*

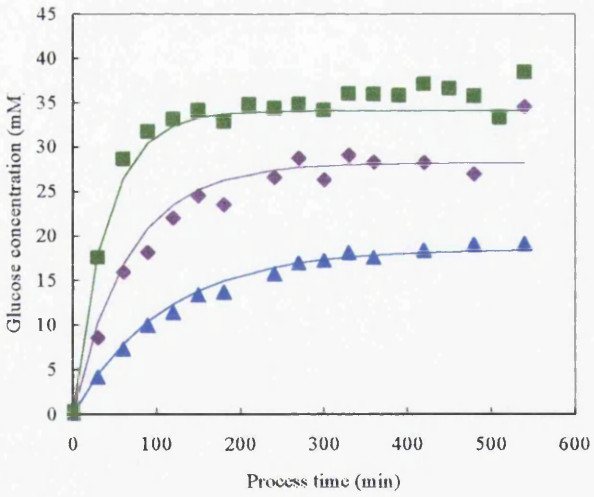


Figure 5.29 *Comparison of glucose production as a function of reaction time between experimental data and the model from continuous membrane reactor. The experimental data of glucose production were performed as points with different enzyme concentrations 0.06 mg/ml (▲), 0.14 mg/ml (◆), and 0.28 mg/ml (■). The results from model were drawn as lines.*

5.7.2.3 Effect of flow rate

The data of glucose production from experiments were compared with the values from model. The results are shown in Figure 5.30. The lactose concentration was prepared at constant 55.5 mM. The glucose productions after operation at flow rate 8 ml/min, 12.9 ml/min and 25 ml/min were about 36.6, 35.7 and 32.1 mM respectively. The obtained R^2 values were in range 0.993-0.998.

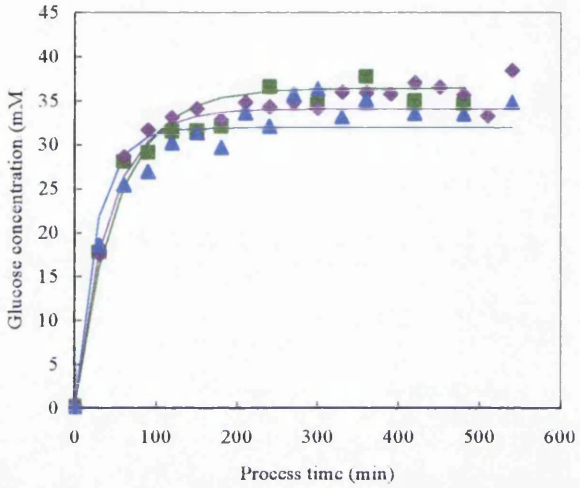


Figure 5.30 *Comparison of glucose production as a function of reaction time between experimental data and the model from continuous membrane reactor. The experimental data of glucose production were performed as points with different flow rates 8 ml/min (■), 12.9 ml/min (◆) and 25 ml/min (▲). The results from model were drawn as lines.*

The correlation coefficient (R^2) values between reversible reaction model based on the principle of mass balance and experimental data from continuous MBR with different operating conditions are concluded in Table 5.3. The R^2 values indicated that data fitted well with the proposed model.

Different operating condition in MBR	Correlation coefficient (R^2)		
	Condition 1	Condition 2	Condition 3
Lactose concentration	0.998	0.993	0.994
Enzyme concentration	0.998	0.992	0.997
Permeate flow rate	0.993	0.998	0.994

Table 5.3 *Conclusion of correlation coefficient (R^2) from different operating conditions in MBR.*

Condition 1, 2 and 3 for lactose concentration are the initial concentration of lactose at 55.5, 83.3 and 111.0 mM respectively (from section 5.7.2.1).

Condition 1, 2 and 3 for enzyme concentration are enzyme at 0.06, 0.14 and 0.28 mg/ml respectively (from section 5.7.2.2).

Condition 1, 2 and 3 for permeate flow rate are flow rate at 8, 12.9 and 25 ml/min respectively (from section 5.7.2.3).

5.8 Conclusions

1. The stability of β-galactosidase in operational MBR in nitrogen condition provided more stable and prevented the loss of activity better than atmospheric condition. This can be interpreted that the effect of gas-liquid interface might influence the activity of the enzyme. In addition, the enzyme stability from the system decreased quickly in comparison with incubation from batch reactor (Figure 4.18). Many effects such as flow of liquid across the membrane device, pumping and adsorption of the enzyme on membrane surface might associate with deactivation of the enzyme.

2. The effect of substrate concentration and enzyme concentration on the continuous MBR were in agreement with the results observed in batch bioreactor, as the glucose production increased with substrate concentration. The rate of reaction increased directly with enzyme concentration and the reaction reached steady state earlier as enzyme concentration increased.

Although high amount of enzyme assisted an increase in glucose production, the increase of enzyme loading decreased the solute diffusivity as can be seen from section 5.3.2. At high enzyme loading, the glucose concentration observed in permeate was much lower

than in retentate while in case of lower enzyme loading the glucose easily passed through the membrane, so the concentration in both permeate and retentate were not great difference.

The change of permeate flow rate in continuous MBR resulted in the variation of residence time of the substrate in the system. Increasing the permeate flow rate from 8 to 25 ml/min increased the productivity of glucose.

3. In comparison between the experimental data from batch bioreactor (Figure 5.2) and continuous MBR (Figure 5.17) in Table 5.4, the results show lactose hydrolysis in batch provided higher glucose concentration than in MBR. The conversion rate of various lactose concentrations, from 55.5 to 111.0 mM, for batch reactor was in range 0.66-0.78 while in continuous MBR was from 0.51-0.64. However, the volumetric productivity in continuous MBR provided better results than in batch bioreactor under the same experimental conditions after operation 360 min.

Lactose concentration (mM)	Batch bioreactor			Continuous MBR		
	55.5	83.3	111.0	55.5	83.3	111.0
Glucose concentration (mM)	36.8	65.4	79.0	35.7	48.8	56.4
Conversion ratio (average)	0.66	0.78	0.71	0.64	0.59	0.51
Volumetric productivity (μmol/min/ml)	0.10	0.18	0.22	0.18	0.25	0.29

Table 5.4 *Comparison experimental data on glucose production in batch bioreactor and continuous MBR.*

In case of batch bioreactor, the volumetric can be calculated as;

$$\text{Volumetric productivity} = \frac{\text{Amount of glucose production } (\mu\text{mol})}{\text{Reaction time (min)} \times \text{Reactor volume (ml)}}$$

Equation 5.11

In case of continuous MBR, the volumetric can be calculated as;

$$\text{Volumetric productivity} = \frac{\text{Glucose production } (\mu\text{mol/ml}) \times \text{Flow rate (ml/min)}}{\text{Reactor volume (ml)}}$$

Equation 5.12

4. After review models, a new model gave the best fit for the enzymatic transformation of lactose solution in buffer pH 6.5, temperature 25 °C in both batch reactor and continuous membrane bioreactor. The experimental data correlated well with the kinetic model concerned on reversible reaction from Haldane model (Model V). The real values of unknown parameter, K_{eq} and K_p, calculated from the experiment were not investigated since they were out of scope in this study. Therefore, these values were only guess to make the data fit with the model.

In case of continuous membrane reactor, applying the basic principles of mass balance was combined with kinetic equation. The kinetic parameters were determined by fitting data with model. The model was tested using experimental data from a system operating with different lactose concentrations, enzyme concentrations and flow rates of permeate from continuous membrane reactor. Good agreement between the model predictions and the experimental data were obtained with R^2 values ranging from 0.992 to 0.998 (Table 5.3).

Chapter 6

Hydrolysis of lactose containing skimmed milk

6.1 Introduction

The composition of milk varies considerable within a species and its pH is in range from 6.5-6.9. Milk normally consists of water, milk fats, milk proteins, carbohydrates, minerals and vitamins as shown in Table 6.1.

Composition	Molecular weight	Diameter (nm)
Water	18	0.3
chloride ion	35	0.4
calcium ion	40	0.4
Lactose	342	0.8
α-lactalbumin	14500	3
β-lactalbumin	36000	4
blood serum albumin	69000	5
casein micelles	107-109	25-130
Fat		2000-10000

Table 6.1 *Molecular weights and diameters of milk components (Cheryan and Alvarez, 1995).*

Milk fats are mostly triglycerides. Triglycerides are composed of three fatty acids covalently bound to a glycerol molecule by ester bonds. Fats present in form of small globules of triglyceride suspended in water. Each globule is surrounded by bilayer of phospholipids, which prevents the globules from clumping together by repelling other fat globules and attracting water. This lipid bilayer assists to stabilize the fat globules remain as an emulsion in environment of milk. Normally, fat found in milk is from 3.5-6.0% depending on the source of milk.

Milk proteins consist of two major groups which are casein micelles and whey proteins, soluble non-casein protein. The concentration of protein in milk varies from 3-4%. Casein is a major protein of milk and disperses as a great number of solid particles in suspension. These particles are called micelles, and the dispersion of the micelles in the milk is typical colloidal suspension. Casein micelles can be separated from milk by centrifugation at a

very high speed. Once casein has been removed, all other protein left in milk are referred to be whey proteins. The major whey proteins are α-lactalbumin and β-lactalbumin. α-lactalbumin is used in the synthesis of lactose while the function of β-lactalbumin is unknown. Other whey proteins are immunoglobulins, serum albumin and also including hormones, growth factors, and nutrient transporters (Hurley, 2006).

In addition to lactose as a major carbohydrate in milk, other carbohydrates found in milk are amino sugars, sugar phosphates, oligosaccharides, sugar nucleotides, glucose and galactose. The concentration of lactose in milk is normally about 5% while glucose and galactose are found much low concentration around 12-14 mg per 100g.

The major minerals found in milk are calcium and phosphorus (Table 6.2) and they are found in association with the casein especially at the normal pH of milk more than 90% of calcium is bound in colloidal form to the casein micelles and less than 10% is in ionized form.

Minerals	mg/ 100 ml	Vitamins	μg/ 100 ml
potassium	138	vitamin A	30.00
calcium	125	vitamin D	0.06
chloride	103	vitamin E	88.00
phosphorus	96	vitamin K	17.00
sodium	58	vitamin C	1.70
sulfur	30	vitamin B1	37.00
magnesium	12	vitamin B2	180.00
trace minerals	<0.1	vitamin B6	46.00
		vitamin B12	0.42

Table 6.2 *Minerals and vitamins concentration found in milk (Wattiaux, 2006).*

The aim of this work was to remove large particles in milk such as milk fats and some proteins by a membrane separation technique and produce a stream of low molecular weight material in permeate solution. The permeate UF skimmed milk was further used in study of lactose hydrolysis by the enzyme in both bioreactor and membrane bioreactor. The obtained model of glucose production from lactose solution hydrolysis in Chapter 5 was directly investigated for hydrolysis of lactose containing skimmed milk. The model simulation was compared with the experimental data in both bioreactor and MBR.

6.2 Preparation of substrate from skimmed milk using MF and UF

Skimmed milk (Tesco Stores Ltd., UK) was prepared by dissolving 10% w/v dried skimmed milk powder in distilled water. The dried skimmed milk consisted of protein 35%, carbohydrate of which sugar 50%, fat 1.1%, sodium 0.6% and trace vitamins. The first step was to filter the skimmed milk by microfiltration to give a clear fat in lactose solution. 16.5 l of skimmed milk was filled into the initial feed container and processed in the 0.2 μm membrane filtration (Figure 2.10). The system was operated at constant feed pressure at 10 psi and temperature at 25 °C. The skimmed milk was drawn down through the tank and into the stainless steel piping by feed pump. The pump pressurized the membrane system. The skimmed milk was pumped up to MF membrane. The permeate passed out through the membrane and 13 l was collected while 3.5 l was retentate and passed back to feed tank.

The permeate solution was then followed with 10 kDa membrane ultrafiltration process. 13 l of solution from MF system was feed into the tank and drawn into the stainless steel piping then passed through the UF membrane (Figure 2.11). The operating pressure was 10 psi and the temperature during running was at 25 °C. 2.46 l of retentate was collected from the tank and 10.54 l solution passed through the membrane and collected as permeate. The permeate and retentate solutions from both MF and UF were sampled to determine lactose and protein composition.

After filtration skimmed milk with MF membrane, most of milk fats and some of milk proteins, according to particle size, were retained in the system as shown in Table 6.3. About 81.9% of protein was in retentate and only 5.7% was found in permeate. The loss of protein was probably absorbed on the surface of membrane. While most of lactose easily passed through the system (Table 6.4). It was found that about 70% of lactose was in permeate and around 23.7% was in retentate. The rest may be absorbed on the membrane with protein and milk fat. The color of permeate solution was clear yellow-green solution while the retentate was white and sticky.

The second filtration with UF membrane was in order to reduce the amount of milk protein and obtained mainly carbohydrate in the permeate solution. About 27.3% protein left in the retentate, 59.5% of protein was found in permeate and the rest (about 13%) lost in the system. While most of lactose approximately 81% passed through the membrane system.

The rejection of lactose during MF and UF processes was probably due to lactose binding with protein and some peptide or the protein in solution might act as barrier which influenced the permeation of lactose.

Table 6.3 *The mass balance of protein after MF and UF separations*

Sample	Protein concentration (g/l)	Total volume (l)	Total protein balance (g)	Protein unaccounted for (%)
Initial feed	38.02±1.69	16.5	627.33±27.95	
Microfiltration				12.43±1.20
retentate MF	146.78±4.59	3.5	513.73±16.06	
permeate MF	2.74±0.24	13	35.62±3.08	
Ultrafiltration				13.16±2.93
retentate UF	3.96±0.57	2.46	9.74±1.40	
permeate UF	2.01±0.19	10.54	21.19±2.00	

Table 6.4 *The mass balance of lactose after MF and UF separations*

Sample	Lactose concentration (g/l)	Total volume (l)	Lactose balance (g)	Lactose unaccounted for (%)
Initial feed	50.86±1.54	16.5	839.19±25.45	
Microfiltration				5.89±1.69
retentate MF	56.80±4.08	3.5	198.80±14.27	
permeate MF	45.46±1.52	13	590.98±19.70	
Ultrafiltration				0.88±0.18
retentate UF	43.51±0.45	2.46	107.03±1.10	
permeate UF	45.42±1.68	10.54	478.73±17.68	

6.3 Investigation of permeate UF skimmed milk hydrolysis in batch bioreactor

The hydrolysis of lactose in skimmed milk in batch reactor was studied using different lactose concentrations, enzyme concentrations and temperatures. Two forms of the enzyme, crude enzyme and partially purified enzyme were used in the bioreactor. The reaction started when 20 ml of lactose containing skimmed milk solution was added into bioreactor containing 20 ml of the enzyme. The total volume of reaction solution was 40 ml. Samples

were taken at intervals and the reactions were stopped by 0.1 M HCl before determining glucose concentration.

6.3.1 Effect of lactose concentration

The hydrolysis of various lactose concentrations with a constant enzyme concentration was carried out in the enzyme bioreactor in order to find out the most effective yield of glucose. Different lactose concentrations were prepared simply by diluting the original permeate UF skimmed milk with 10 mM potassium phosphate buffer pH 6.5. The temperature in the system was controlled at 25 °C.

6.3.1.1 Crude enzyme

The effect of lactose concentration containing skimmed milk was studied from 6.7, 13.3, 26.7, 66.7, and 133.4 mM. The total amounts of lactose loading in bioreactor were 0.13, 0.27, 0.53, 1.33 and 2.67 mmol respectively. The enzyme concentration in the reactor was fixed at 1.40 mg protein/ml. The process was last for 360 min.

The amount of glucose produced from crude enzyme as a function of different lactose concentrations is performed in Figure 6.1. It can be clearly seen that the glucose production increased with an increase in lactose concentration. At low lactose concentrations from 6.7 to 26.7 mM, the steady state was reached in about 45 min while higher concentration took longer about 180min. After enzymatic hydrolysis was conducted for 360 min, the final yields of glucose products were 0.12, 0.26, 0.50, 1.24, 2.60 mmol and the initial rates of glucose production were 0.36, 0.54, 0.60, 0.91 and 0.95 μmol/min/ml when lactose loading in bioreactor were 0.13, 0.27, 0.53, 1.33 and 2.67 mmol respectively.

The obtained K_m and V_{max} values from the relationship between initial rates reaction of crude β-galactosidase and lactose concentrations (Figure 6.2) were 12.84 mM and 1.05 μmol/min/ml respectively.

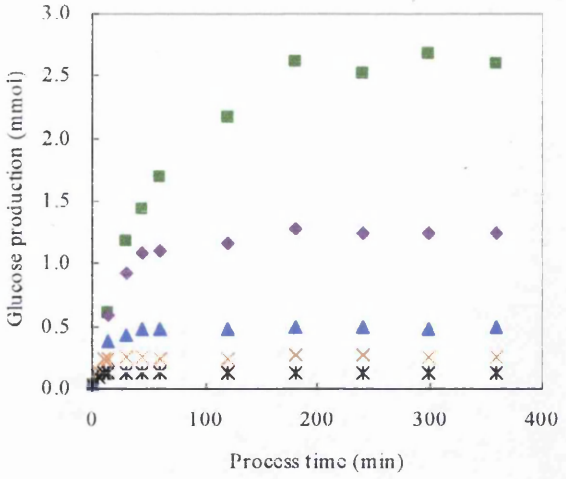


Figure 6.1 *Effect of lactose concentration on glucose production by crude enzyme solution. The temperature was controlled at 25 °C. The crude enzyme used for each reactor was 1.40 mg protein/ml. Total volume of reaction solution was 40 ml. The amount of lactose skim milk loading in bioreactor were 0.13 mmol (∗), 0.27 mmol (×), 0.53 mmol (▲), 1.33 mmol (◆) and 2.67 mmol (■).*

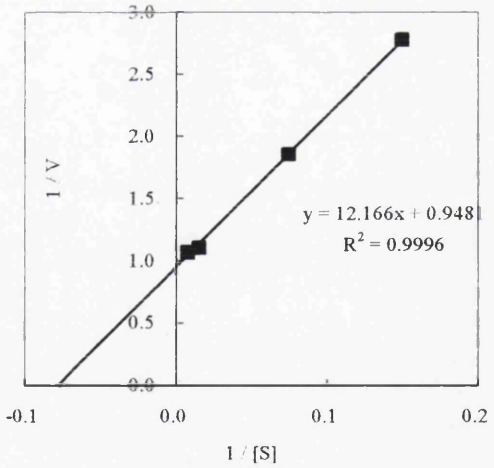


Figure 6.2 *Lineweaver-Burk plot of initial rate of crude β-galactosidase versus lactose concentration (K_m =12.84 mM and V_{max} =1.05 μmol/ min /ml). Operating conditions are mentioned in Figure 6.1.*

6.3.1.2 Partially purified enzyme

The influence of lactose concentration on glucose production from partially purified enzyme is displayed in Figure 6.3. The trend of glucose production was in agreement with the results obtained from crude enzyme. After operation for 360 min, the final yields of glucose product were 0.10, 0.21, 0.45, 1.12 and 2.39 mmol and the initial rates of glucose production were 0.53, 0.76, 0.80, 0.95 and 0.94 μmol/ml/min when the amounts of lactose loading in bioreactor were 0.13, 0.27, 0.53, 1.33 and 2.67 mmol respectively.

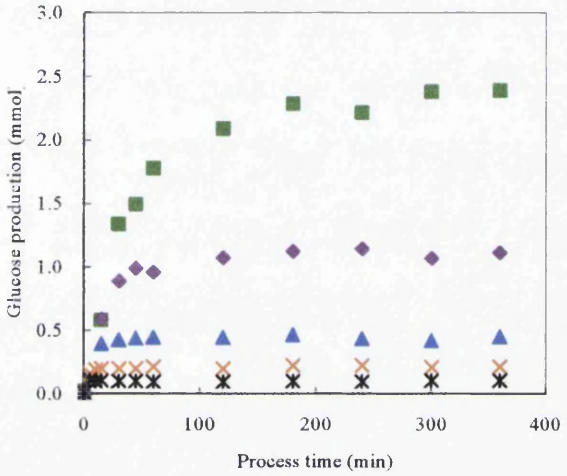


Figure 6.3 *Effect of lactose concentration on glucose production by partially purified enzyme solution. The temperature was controlled at 25 °C. The partially purified enzyme used for each reactor was 0.24 mg protein/ml. Total volume of reaction solution was 40 ml. The amount of lactose skim milk loading in bioreactor were 0.13 mmol (✱), 0.27 mmol (×), 0.53 mmol (▲), 1.33 mmol (◆) and 2.67 mmol (■).*

The apparent K_m and V_{max} values for partially purified enzyme obtained from Lineweave-Burk plot between the initial rates of reaction versus lactose concentrations were 6.85 mM and 1.09 μmol/min/ml respectively (Figure 6.4).

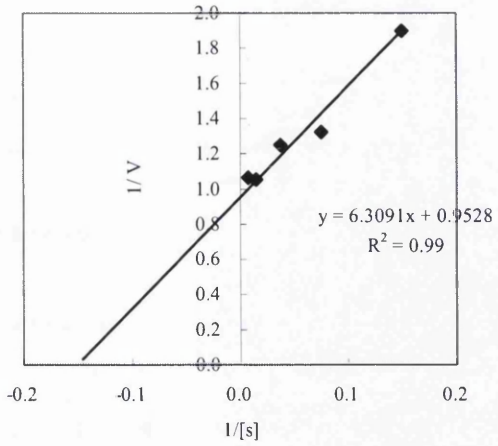


Figure 6.4 *Lineweaver-Burk plot of initial rate of partially purified β-galactosidase versus lactose concentration (K_m =6.85 mM and V_{max} =1.09 μmol/min/ml). Operating conditions are mentioned in Figure 6.3.*

The amount of lactose loading in mmol (concentration, mM)	The amount of glucose (mmol) in bioreactor at 180 min		Conversion ratio	
	crude enzyme	partially purified enzyme	crude enzyme	Partially purified enzyme
2.67 (133.4 mM)	2.62	2.29	0.98	0.86
1.33 (66.7 mM)	1.28	1.12	0.96	0.84
0.53 (26.7 mM)	0.50	0.46	0.94	0.87
0.27 (13.3 mM)	0.26	0.22	0.96	0.81
0.13 (6.7 mM)	0.12	0.10	0.92	0.77

Table 6.5 *Summary of lactose conversion (is defined as ratio between the amount of glucose produced and the amount of initial lactose loading) in skimmed milk on constant crude and partially purified enzyme activities at 180 min, all experiments were carried out at 25 °C.*

The conversions of lactose containing skimmed milk by two forms of the enzyme, crude and partially purified enzyme, after operation for 180 min are summarised in Table 6.5. Lactose conversion is defined as the ratio between formed glucose concentration and initial lactose concentration in reactor. After the reaction was almost steady-state, the conversion rates of various lactose concentrations from 6.7 to 133.4 mM with crude

enzyme were not much changed and in range 0.92-0.98 while with partially purified enzyme were slightly lower in range 0.77-0.87. Although the substrate concentrations used differed even high concentration, the rates of conversion determined in enzyme solution were almost the same for each form of the enzyme.

6.3.2 Effect of enzyme concentration

The enzymes from both crude and partially purified forms were prepared in each three different concentrations by diluting in phosphate buffer. The hydrolysis reaction was conducted with a constant concentration of lactose from the original permeate UF skimmed milk. The concentration of lactose containing skimmed milk was 133.4 mM.

6.3.2.1 Crude enzyme

The bioconversion of glucose production by different crude enzyme concentrations is shown in Figure 6.5. The productions of glucose were 2.02, 2.44 and 2.60 mmol at 360 min when the enzyme concentrations were 0.28, 0.70 and 1.40 mg protein/ml reactor respectively. It was shown that low enzyme concentration provided less glucose production than with product from higher enzyme concentration at the same operating time. The initial rates of glucose production were 0.27, 0.61 and 0.95 μmol/min/ml when the enzyme concentrations were 0.28, 0.70 and 1.40 mg protein/ml reactor respectively.

6.3.2.2 Partially purified enzyme

The glucose production by partially purified enzyme is performed in Figure 6.6. Different enzyme concentrations from 0.05, 0.12 and 0.24 mg/ml generated glucose at 1.95, 2.38 and 2.39 mmol and the initial rates of glucose production were 0.23, 0.50 and 0.94 μmol/min/ml. The initial rate of high enzyme concentration was higher and reached steady state faster than low enzyme concentration as can be seen that enzyme concentration at 0.24 mg/ml was almost steady state after 180 min while the rate of enzyme at 0.05 mg/ml was still increasing. Therefore, increasing the enzyme concentration significantly reduced the reaction time of lactose hydrolysis.

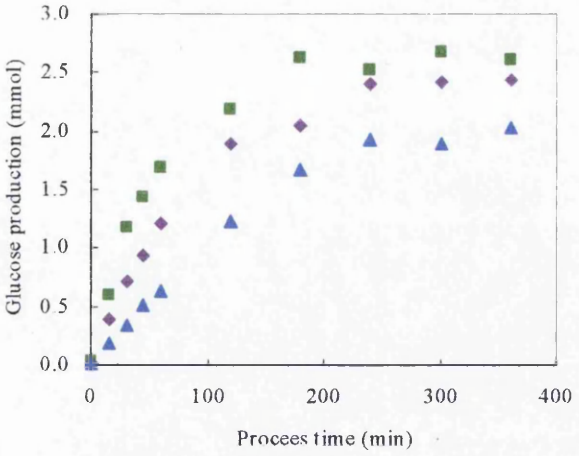


Figure 6.5 *Effect of enzyme concentration from crude extract on the glucose production. Lactose concentration was 133.4 mM. The temperature was controlled at 25 °C. Total volume of reaction solution was 40 ml. Enzyme concentrations were 0.28 mg/ml (▲), 0.70 mg/ml (◆) and 1.40 mg/ml (■).*

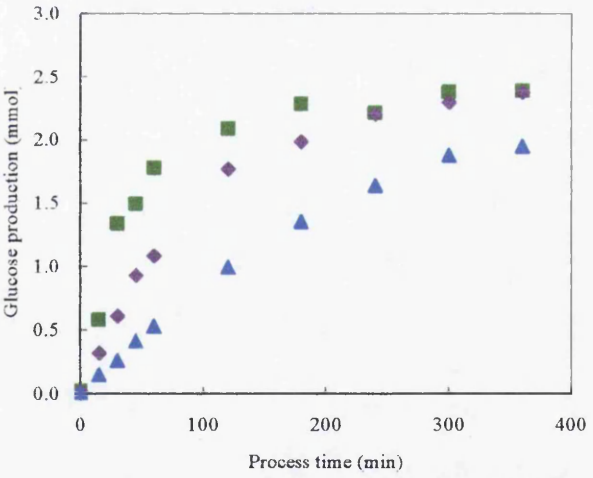


Figure 6.6 *Effect of enzyme concentration from partially purified form on the glucose production. Lactose concentration was 133.4 mM. The temperature was controlled at 25 °C. Total volume of reaction solution was 40 ml. Enzyme concentrations were 0.05 mg/ml (▲), 0.12 mg/ml (◆) and 0.24 mg/ml (■).*

The relationship between enzyme concentrations and the initial rates of glucose production in both crude enzyme and partially purified enzyme are plotted in Figure 6.7. The concentration of enzyme was related straightforward to the initial rate. Increasing enzyme

concentration increased the rate of productivity.

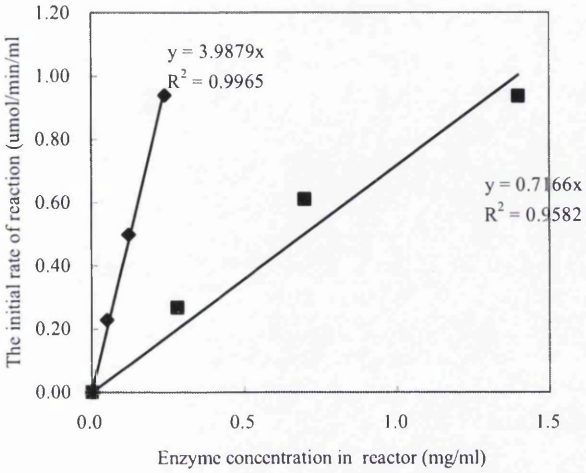


Figure 6.7 *The relationship between enzyme concentrations and the initial rates of glucose production from crude extract enzyme (■) and partially purified enzyme (◆).*

6.3.3 Effect of temperature

The activity of β-galactosidase on hydrolysis of lactose containing skimmed milk was assayed at different incubation temperatures ranging from 25-40 °C. The enzyme activity was expressed in terms of glucose production for 360 min. The total working volume in bioreactor was 40 ml. The concentration of lactose containing skimmed milk was 133.4 mM.

6.3.3.1 Crude enzyme

The glucose production from crude enzyme at different incubation temperatures is shown in Figure 6.8. The rates of glucose production during the beginning period were 0.95, 1.09, 1.24 and 1.59 μmol/min/ml at operating temperature 25 °C, 30 °C, 35 °C and 40 °C respectively. Although high temperature slightly increased the rate of reaction, after the reaction almost reached steady state the temperature did not strongly affected glucose production. When the reaction was last for 360 min, the glucose was released 2.60, 2.62, 2.64 and 2.60 mmol at temperature 25 °C, 30 °C, 35 °C and 40 °C respectively.

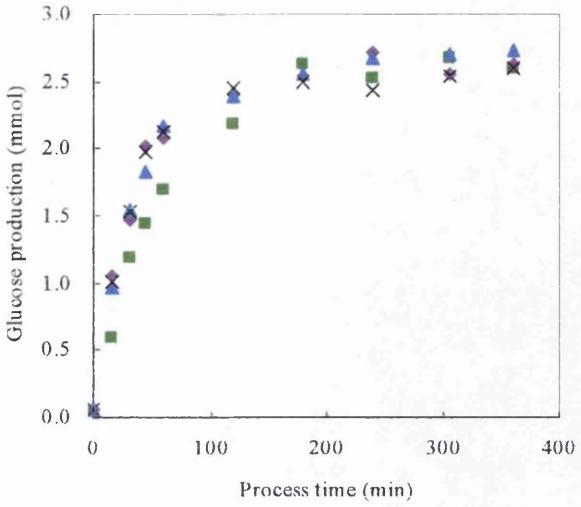


Figure 6.8 *Effect of temperature on glucose production by crude enzyme solution. The crude enzyme concentration was fixed at 1.40 mg protein/ ml. Lactose concentration from skim milk was 133.4 mM. Total volume of reaction solution was 40 ml. The temperatures were tested at 25 °C (■), 30 °C (◆), 35 °C (▲) and 40 °C (×).*

The reaction rate on temperature for crude β-galactosidase was observed following Arrhenius plots in the temperature range 25-40 °C. The activities data were plotted in terms of Log versus 1/T x10 $^{-3}$(K) to give a straight line with a slope of –Ea/2.3R. The ideal gas constant (R) was 8.314 J. $K^{-1}mol^{-1}$. The activation energy of crude enzyme is calculated from Figure 6.9 as 25.79 kJ/mol with the correlation coefficient of R^2 =0.96. Calculated values for temperature coefficient (Q_{10}), which is the rate increases by raising the temperature 10 °C, derived from reaction rate data was 1.53.

Vasiljevic and Jelen (2003) displayed the energy of activations for lactose hydrolysis in lactose and skimmed milk preparations at temperature 30-60 °C using β-galactosidase from *L. delbrueckii* 11842 were 28.68 and 23.71 kJ/mol respectively, from *L. delbrueckii* ssp *lactis* 3078 were 23.94 and 26.57 kJ/mol while the activation energy of the reaction of lactose hydrolysis in 5% w/v lactose for temperatures ranging from 20-55 °C using the enzyme from *Kluyveromyces fragilis* reached to 39.9 kJ/mol (Matioli *et al.*, 2001).

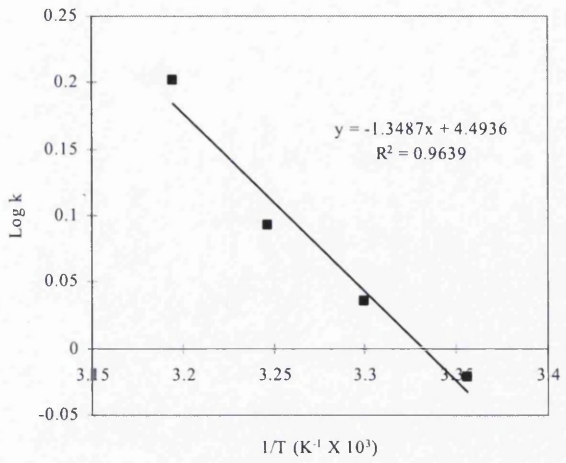


Figure 6.9 *Arrhenius plot for the effect of reaction temperature versus the rate of lactose hydrolysis from crude enzyme.*

6.3.3.2 Partially purified enzyme

The effect of temperature on lactose hydrolysis by partially purified enzyme was conducted and expressed in terms of glucose production (Figure 6.10). Four different temperatures 25 °C, 30 °C, 35 °C and 40 °C gave the initial rates of glucose production as 0.94, 1.22, 1.44 and 1.58 μmol/min/ml and the amounts of glucose generated after 360 min were 2.39, 2.61, 2.58 and 2.60 mmol respectively.

The Arrhenius plot of log activities versus the inverse of absolute temperatures was applied to quantify the temperature dependence of the rate constant from purified enzyme (Figure 6.11). The calculated activation energy from the slope of plots at 25-40 °C was 26.74 kJ/mol with the correlation coefficient of R^2 = 0.96. The activation energy of purified enzyme was slightly different from crude enzyme. The calculated Q_{10} from reaction rate data was 1.53. The Q_{10} values in both crude and purified enzymes were low because most enzymatic reactions tend to provide a value about 2 (Holden and Storey, 1998). This might because the β-galactosidase activity was not strongly affected by temperature change.

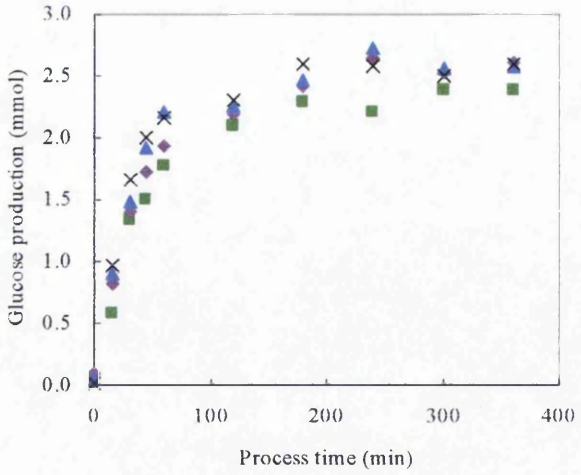


Figure 6.10 *Effect of temperature on glucose production by partially purified enzyme. Enzyme concentration was fixed at 0.24 mg/ml. Lactose concentration from skim milk was 133.4 mM. Total volume of reaction solution was 40 ml. The temperatures were tested at 25 °C (■), 30 °C (◆), 35 °C (▲) and 40 °C (×).*

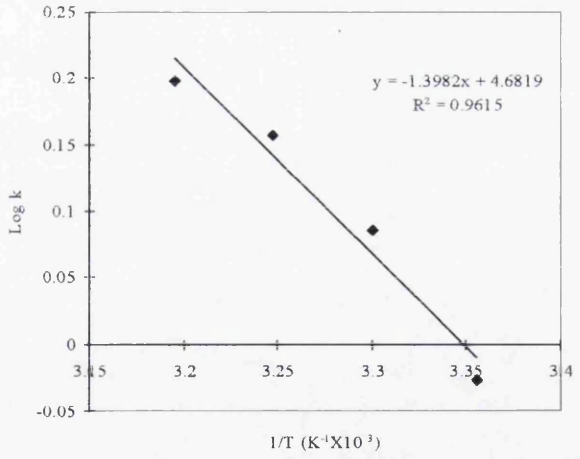


Figure 6.11 *Arrhenius plot for the effect of reaction temperature versus the rate of lactose hydrolysis from purified enzyme.*

6.3.4 Hydrolysis of permeate UF skimmed milk in fed batch bioreactor

The continuous glucose production from lactose hydrolysis by crude enzyme and partially purified enzyme in batch bioreactor was determined (Figure 6.12). The initial lactose concentration in the reactor was 133.4 mM (2.67 mmol). According to previous results, the rate of glucose production increased before reaching steady state at 180 min. In this experiment, further addition of 133.4 mM lactose solution was added into reactor every 180 min. The total amount of lactose loading in bioreactor was 8.01 mmol. The experiments were last for 600 min.

The glucose production from crude and purified enzyme continuously increased during operating time. The initial rates of reaction every 180 min after adding fresh substrate into crude enzyme reactor were 0.31, 0.18 and 0.08 μmol/min/ml and the yields of glucose were 1.61, 3.16 and 4.89 mmol with a conversion of 60%, 59% and 61% respectively. The initial rates from purified enzyme were 0.66, 0.31 and 0.18 μmol/min/ml and the yields of glucose were 2.33, 4.29 and 6.62 mmol with a conversion of 87%, 80% and 82% respectively. The rates of production were decreased probably due to product inhibition and loss of enzyme from sampling.

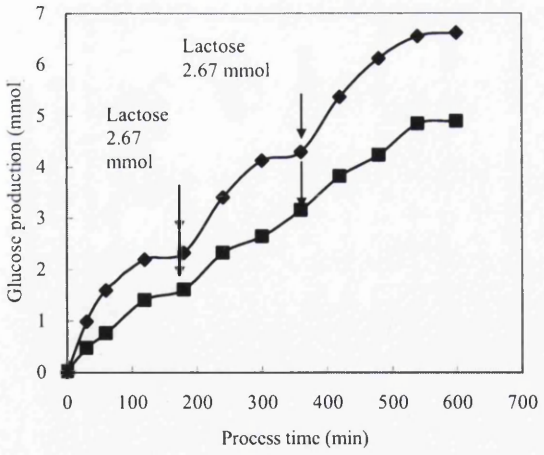


Figure 6.12 *The production of glucose on lactose hydrolysis in fed batch reactor by crude enzyme (■) and partially purified enzyme (◆). The initial substrate concentration was 133.4 mM. Further fresh 133.4 mM lactose solution from skim milk was added every 3 hours (↓). The crude enzyme concentration was 0.28 mg protein/ml reactor and from purified enzyme was 0.24 mg protein/ml reactor. The temperature was controlled at 25 °C.*

6.3.5 Effect of amount of enzyme loading

The effect of crude enzyme loading was determined in different proportions of enzyme against a constant volume of permeate UF skimmed milk with lactose concentration at 133.4 mM. The optimal proportion between enzyme and substrate is very important for the efficient use of lactose hydrolysis process.

The lactose hydrolysis was performed in stirred enzyme bioreactor at 25 °C for four different proportions of enzyme. The ratios between enzyme and substrate in these experiments were 1:1, 2:1, 3:1 and 4:1. The protein concentration of crude enzyme was 2.80 mg/ml. After adding the crude enzyme into the reactor containing substrate, the samples were taken at interval to measure the glucose production. The results are shown in Figure 6.13. Different ratios provided different initial rates of glucose production. The rates were 0.81, 0.97, 1.09 and 1.19 μmol/min/ml from ratios of 1:1, 2:1, 3:1 and 4:1 respectively. Low ratio tended to take longer time to reach steady–state as can be clearly seen that ratio 1:1 spent more than 180 min while ratio at 4:1 took about 60 min to reach the state. The reactions were last for 360 min and final yields of glucose production were 2.65, 2.61, 2.65 and 2.60 mmol with ratio of 1:1, 2:1, 3:1 and 4:1 respectively.

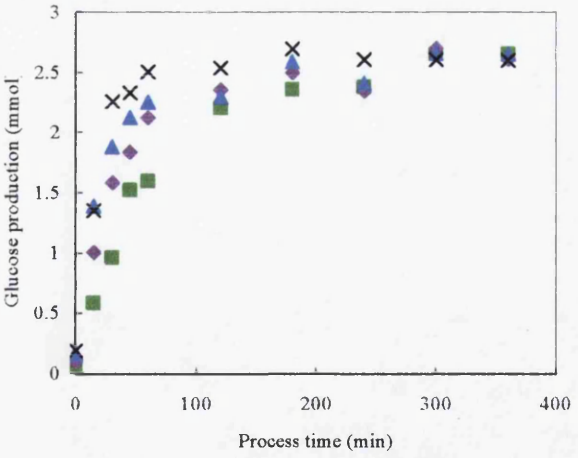


Figure 6.13 *Effect of enzyme loading from crude extract on the glucose production. Lactose concentration was 133.4 mM. The temperature was controlled at 25 °C. The protein concentration of crude enzyme was 2.80 mg/ml. The proportions between crude enzyme and substrate were 1:1 (■), 2:1(◆), 3:1(▲) and 4:1(×). The reactions monitored for 360 min.*

6.4 Investigation of permeate UF skimmed milk hydrolysis in 5 l continuous MBR

The continuous lactose hydrolysis in skimmed milk by β-galactosidase from *L. delbruekii* was carried out in 5-l MBR at different amounts of crude enzyme loading and permeate flow rates. The total working volume in reactor was fixed at 2500 ml. The procedure is described in section 2.12.3. Samples from permeate were taken periodically during continuous process and the reactions were stopped by 0.1 M HCl before determining glucose concentration. All experiments were conducted for 360 min.

6.4.1 Effect of enzyme loading

Two different amounts of crude enzyme, with the total final concentration at 0.28 and 0.56 mg/ml reactor, were added into MBR. The concentration of lactose was fixed at 126.1 mM (283.61 mmol). During the operation, the permeate flow rate was maintained at 12.9 ml/min (resident time was about 194 min).

The glucose production in permeate as a function of different amounts of enzyme loading is shown in Figure 6.14. The results of glucose concentration in both retentate and permeate through the process time for both amounts of the enzyme were very slightly different (data not shown). Therefore, the concentration polarisation of the enzyme and substrate concentration on the membrane did not influence the retention of solution significantly.

It was obviously seen that the glucose production in permeate increased with an increase in the amount of enzyme loading. The glucose concentrations in permeate after operation were 54.73 and 63.97 μmol/ml while the productivities were about 706 and 825 μmol glucose/min when the enzyme adding was 0.28 and 0.56 mg/ml reactor respectively.

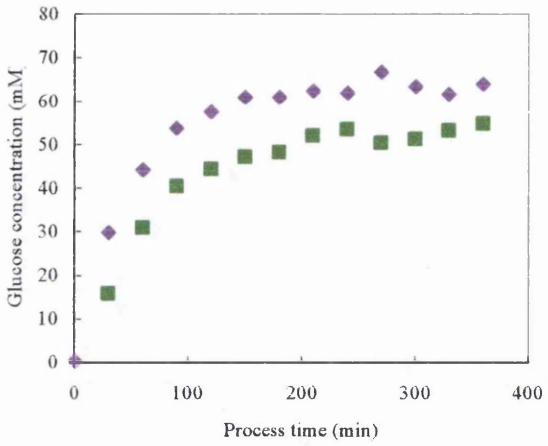


Figure 6.14 *The effect of enzyme loading on glucose production in continuous MBR. The concentrations of crude enzyme were 0.28 mg/ml (■), 0.56 mg/ml (◆). Lactose concentration was fixed at 126.1 mM. The temperature was controlled at 25 °C. The total volume reaction in MBR was 2500 ml.*

6.4.2 Effect of permeate flow rate

Three different permeate flow rates, 8 ml/min, 12.9 ml/min and 25 ml/min (residence times were 313, 194 and 100 min respectively) were investigated on hydrolysis reaction. The experiments were conducted with lactose in permeate UF skimmed milk at concentration approximately 126.1 mM and the concentration of the enzyme was 0.28 mg/ml reactor.

The relationship between glucose production in terms of concentration and process time, and productivity with process time are presented in Figure 6.15 and 6.16 respectively. At flow rate 8 ml/min, 12.9 ml/min, and 25 ml/min the glucose concentrations after operation were approximately 67.7, 54.7 and 44.7 mM respectively while the productivities were about 541, 706 and 1117 μmol glucose/min. It can be seen that at low permeate flow rate, (longer residence time) resulted in an increase in glucose production but the productivity was decreased.

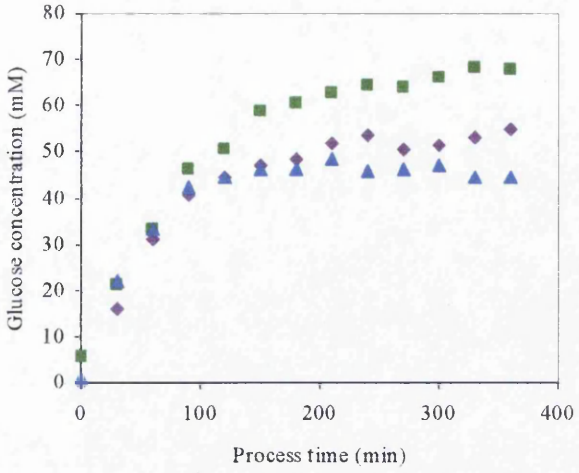


Figure 6.15 *The effect of flow rate on glucose concentration in continuous MBR. The concentrations of crude enzyme were 0.28 mg/ml reactor. Lactose concentration was fixed at 126.1 mM. The flow rates were 8 ml/min (■), 12.9 ml/min (◆), and 25 ml/min (▲). Operating conditions are mentioned in Figure 6.14.*

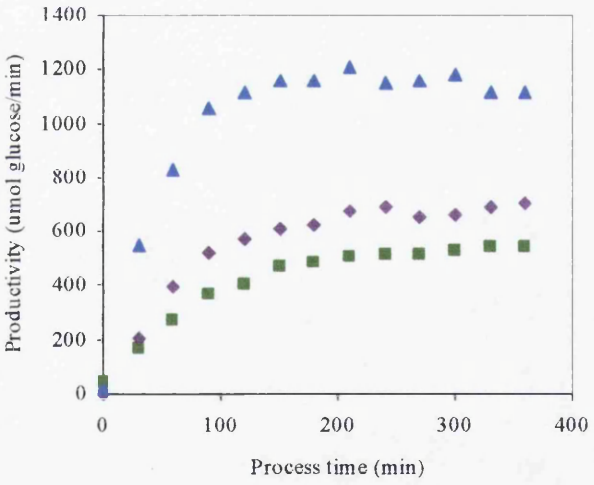


Figure 6.16 *The effect of flow rate on productivity of glucose in continuous membrane bioreactor. The flow rates were 8 ml/min (■), 12.9 ml/min (◆), and 25 ml/min (▲).*

In addition the effect of permeate flow rate on membrane-bound enzyme in the MBR system was studied (data not shown). The activity of the enzyme in phosphate buffer was measured with ONPG solution during interval and was found that the enzyme activity did

not change according to the permeate flow rate. This indicated that there was no adsorption of the enzyme onto the membrane occurred during the process.

Some of experimental data on hydrolysis reaction in both batch bioreactor and continuous MBR were selected and summarized to assess the potential of enzymatic reaction in these reactors (Table 6.6), although the operating conditions were different and difficult to compare.

The amount of crude enzyme used in both reactors and both source of substrates was about 0.27 units where 1 unit (U) is defined as 1 μmol of glucose released from hydrolysis of lactose in skimmed milk per min per ml at 25 °C pH 6.5. Considering the hydrolysis of lactose from different sources, the conversion rate of lactose hydrolysis from skimmed milk source in both bioreactor and MBR seemed to be lower than the rate observed from lactose solution. The solution prepared from skimmed milk contained some proteins and ions in addition to lactose, so these particles probably influenced the enzyme by binding with the active site of enzyme and decreased its activity. The batch bioreactor for both sources of lactose provided higher conversion than from continuous MBR. However in comparison of volumetric productivity, which is calculated as the amount of glucose produced in 1 min reaction time divided by the given reactor volume, the performance in continuous MBR gave better results than in the batch bioreactor.

Novalin *et al.* (2005) introduced a process called membrane-diffusion reactor for direct lactose hydrolysis in skimmed milk without UF step before enzymatic conversion. The result is also summarised in Table 6.6. The 120 U/ml of Maxilact from *Kluyvermyces lactis* was circulated in a hollow fiber membrane reactor system and the skimmed milk containing lactose concentration at 141.5 mM was pumped through the hollow fiber module with flow rate at 165 ml/min, temperature at 23±2 °C. The enzymatic conversion took place in the module and the samples were collected at the end of module. The average conversion rate was 0.78 and the volumetric productivity was approximately 16.65 μmol/min/ml. As compared to the β-galactosidase from *L. delbrueckii*, the process is much more productive. This is mainly due to the amount of enzyme present almost 500 times more and 12 times more flow rate as compared to *L. delbrueckii* with 60 times greater productivity. To match the productivity the *K. lactis* system, the *L. delbrueckii* system would require at least 50 times more enzyme of the used at present.

Table 6.6 *Summary of the results on lactose hydrolysis in reactors*

Reactor	Bioreactor	Bioreactor	MBR	MBR	Membrane- diffusion reactor (Novalin *et al.*, 2005)
cut off (kDa)			50 kDa MWCO UF	50 kDa MWCO UF	10 kDa MWCO UF
surface area (m^2)			0.186	0.186	4.9
volume of solution (ml)	50	40	2500	2500	
Substrate					
Type	lactose solution	lactose containing skimmed milk (Tesco)	lactose solution	lactose containing skimmed milk (Tesco)	skimmed milk (Lactoprot, Austria)
initial lactose concentration (mM)	111.0	133.4	111.0	126.1	141.5
Enzyme					
Source	crudeβ-galactosidase from *L. delbruekii*	crude β-galactosidase from *L. delbruekii*	crude β-galactosidase from *L. delbruekii*	crude β-galactosidase from *L .delbruekii*	Maxilact from *Kluyvermyces lactis*
amount of enzyme (U/ml)	0.27	0.27	0.27	0.27	120
Condition					
mode of operation	Batch	Batch	Continuous	continuous	continuous
temperature (°C)	25	25	25±2	25±2	23±2
flow rate (ml/min)			12.9	12.9	165
operation time (h)	6	6	6	6	6 to 8
Conversion ratio (average)	0.71	0.76	0.51	0.43	0.78
Volumetric productivity (μmol/min/ml)	0.22	0.18	0.29	0.28	16.65

6.5 Mathematical modelling for lactose hydrolysis containing skimmed milk

It was clearly shown in section 5.7 that the reversible reaction-Haldane kinetic model predictions correlated well with the experimental data from lactose hydrolysis in both batch bioreactor and continuous MBR. Then, this model was used directly to predict the performance of lactose hydrolysis containing skimmed milk.

In case of batch bioreactor, the rate of lactose utilization was equal to the rate of product formation. Therefore, the reaction rate is calculated from equation 5.6 with model V (Chapter 5).

$$\frac{-d[Lac]}{dt} = \frac{d[Glu]}{dt} = \frac{d[Gal]}{dt} = r \qquad \textit{Equation 5.6}$$

$$r = \frac{V_{\max f}\left(S - \frac{P}{K_{eq}}\right)}{K_m\left(1 + \frac{P}{K_p}\right) + S}$$

Reversible reaction (Haldane) (Segel, 1993)

where -d[Lac]/dt is the lactose utilization rate (mmol/l.min), d[Glu]/dt and d[Gal]/dt are the glucose and galactose production rates (mmol/l.min), r is the reaction rate (mmol/l.min), $V_{max\,f}$ is the maximum reaction rate in the forward direction (mmol/l.min), $V_{max\,r}$ is the maximum reaction rate in the reverse direction (mmol/l.min), S is the concentration of substrate (mmol/l), P is the concentration of product (mmol/l), K_m is the Michaelis–Menten constant for substrate (mmol/l), K_p is Michaelis–Menten constant for product (mmol/l), and K_{eq} is Haldane relationship.

In case of continuous MBR, the consideration of the model for lactose containing skimmed milk hydrolysis was the same principle as lactose hydrolysis in Chapter 5 (see section 5.7.2). The rate of product formation can be calculated as mentioned in equation 5.10.

$$\frac{dP}{dt} = -D_P P + r \qquad \textit{Equation 5.10}$$

where dP/dt is the rate of product formation, D_p is dilution rate (min^{-1}) which equal to F / V.

6.5.1 Model simulation in batch bioreactor with different lactose concentrations

The model was simulated with the experimental data from batch bioreactor using crude enzyme (Figure 6.17) and partially purified enzyme (Figure 6.18) in different lactose concentrations. The enzyme concentration of both forms was constant through the experiments. The points represented the experimental data whereas simulated results from models were drawn as lines.

In case of crude enzyme (Figure 6.17), the K_m value was determined from experimental data according to equation of Lineweaver-Burk plotting (see section 6.3.1.1) and used in this model was 12.84 mM. The V_{max} value and substrate concentration used with the model were from experimental data. The unknown kinetic parameters (K_p and K_{eq}) were estimated by fitting the model equation with the experimental results. The obtained K_p and K_{eq} values were 20 mM and 0.95 respectively. The model gave a fit with correlation coefficient (R^2) 0.977, 0.976, 0.993, 0.992 and 0.995 when lactose concentrations in solution were 6.7, 13.3, 26.7, 66.7, and 133.4 mM respectively.

The kinetic parameters used for partially purified enzyme in the model varied from the values obtained from crude enzyme. The K_m value for this form of enzyme was 6.85 mM (see section 6.3.1.2). After fitting the model equation with the experimental data, the best estimated K_p and K_{eq} values were 15 mM and 0.79 respectively. Figure 6.18 shows the model correlated with experimental data. The observed R^2 values were 0.960, 0.983, 0.993, 0.993 and 0.995 when lactose concentrations used were 6.7, 13.3, 26.7, 66.7, and 133.4 mM respectively.

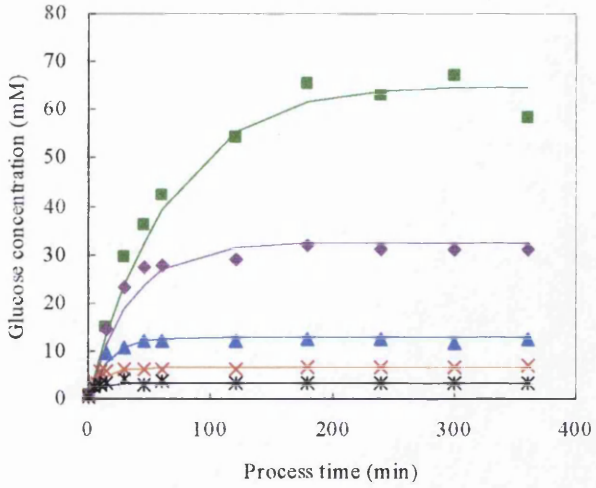


Figure 6.17 *Comparison of glucose production by crude enzyme as a function of reaction time between experimental data and the model from batch bioreactor. The experimental data of glucose production were performed as points when lactose concentrations were 6.7 mM (✳), 13.3 mM (×), 26.7 mM (▲), 66.7 mM (◆) and 133.4 mM (■). The results from model were drawn as lines.*

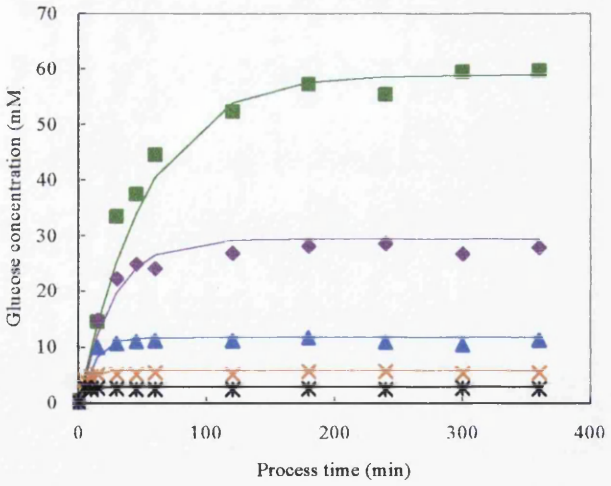


Figure 6.18 *Comparison of glucose production by partially purified enzyme as a function of reaction time between experimental data and the model from batch bioreactor. The experimental data of glucose production were performed as points when lactose concentrations were 6.7 mM (✳), 13.3 mM (×), 26.7 mM (▲), 66.7 mM (◆) and 133.4 mM (■). The results from model were drawn as lines.*

6.5.2 Model simulation in batch bioreactor with different enzyme concentrations

The experimental data of glucose production from different enzyme concentrations with constant initial lactose concentration at 133.4 mM were compared with the data from reversible reaction model. The results are shown in Figure 6.19 for crude enzyme and Figure 6.20 for partially purified enzyme.

Good agreement between the model predictions and the experimental data in both enzyme forms were obtained. The R^2 values of nonlinear regression between experimental results and the model were 0.989, 0.998 and 0.994 when crude enzyme concentrations were 0.28 mg/ml, 0.70 mg/ml, and 1.40 mg/ml respectively. In case of partially purified enzyme, the R^2 values were 0.994, 0.996 and 0.991 when enzyme concentrations were 0.05 mg/ml, 0.12 mg/ml, and 0.24 mg/ml respectively.

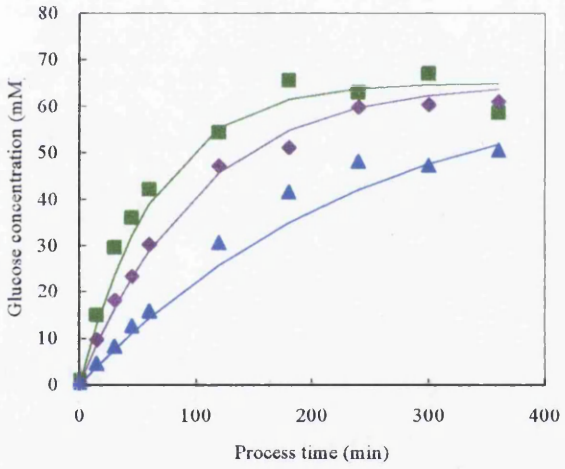


Figure 6.19 *Comparison of glucose production between experimental data from batch bioreactor and the model. The initial lactose concentration was fixed at 133.4 mM. The experimental data of glucose production were performed as points with various crude enzyme concentrations 0.28 mg/ml (▲), 0.70 mg/ml (◆) and 1.40 mg/ml (■). The results from model were drawn as lines.*

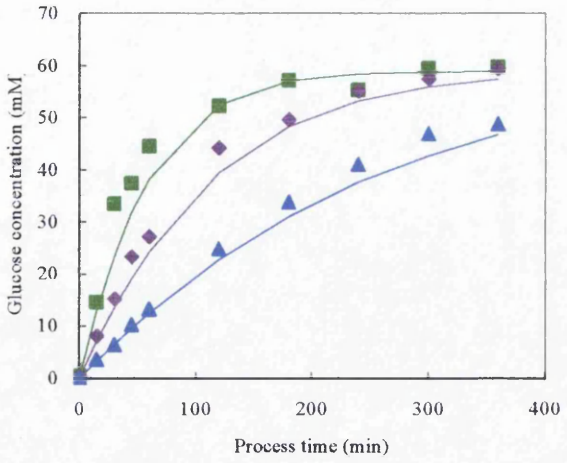


Figure 6.20 *Comparison of glucose production between experimental data from batch bioreactor and the model. The initial lactose concentration was fixed at 133.4 mM. The experimental data of glucose production were performed as points with various partially purified enzyme concentrations 0.05 mg/ml (▲), 0.12 mg/ml (◆) and 0.24 mg/ml (■). The results from model were drawn as lines.*

6.5.3 Model simulation in continuous MBR with different permeate flow rates

The experiment data and predicted values from the model on glucose production in continuous MBR with different permeate flow rates is shown in Figure 6.21. The lactose concentration containing skimmed milk was fixed at about 126.1 mM and the crude enzyme concentration was about 0.28 mg/ml reactor. The permeate flow rate varied from 8 ml/min, 12.9 ml/min and 25 ml/min. The model did not fit well with the experimental data as can be seen with R^2 values 0.964, 0.991 and 0.974 respectively.

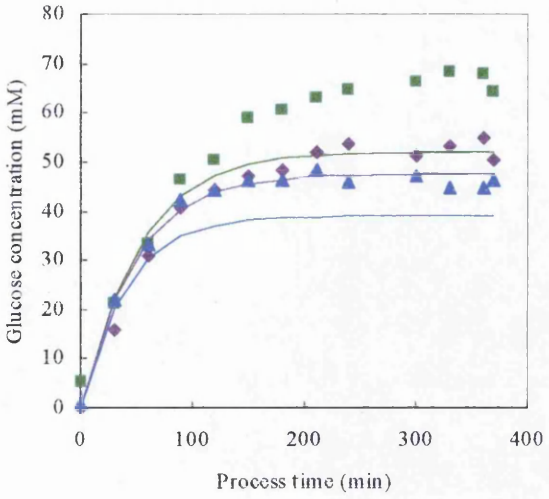


Figure 6.21 *Comparison of glucose production as a function of reaction time between experimental data and the model from continuous membrane reactor. The experimental data of glucose production were performed as points with different flow rate 8 ml/min (■), 12.9 ml/min (◆), and 25 ml/min (▲). The results from model were drawn as lines.*

Table 6.7 shows the percent deviation of glucose production at steady state between the observed experimental data and model data when changing the permeate flow rate.

Permeate flow rate (ml/min)	Observed product (mM)	Predicted product (mM)	Deviation (%)
8	66.6	52.1	21.8
12.9	52.9	47.4	9.5
25	45.7	38.9	14.9

Table 6.7 *Comparison of glucose production at steady state between the observed and predicted data for each permeate flow rate.*

6.6 Conclusions

1. After filtration skimmed milk through 0.2 μm microfiltration membrane, most of milk fats and some of milk proteins, according to particle size, were mostly retained in the membrane system and less than 10% passed through the membrane while some loss of

protein was probably adsorbed on the surface of membrane. More than 70% lactose passed through the membrane. The color of solution in permeate was clear yellow-green solution while the retentate was white and sticky.

The hollow fibre ultrafiltration with 10 kDa MWCO was applied as a second filtration for permeate microfiltration solution so as to reduce the amount of milk protein and obtained mainly carbohydrate in the permeate solution. However, the small proteins and peptides were able to pass through and detected after ultrafiltration. And about 80% lactose was recovered after ultrafiltration process.

2. The effects of different lactose concentrations, enzyme concentrations and operation temperatures in batch bioreactor with two forms of the enzyme, crude and partially purified forms, were investigated. The product formations from both forms of the enzyme increased with substrate concentration and enzyme concentration. The rate of reaction increased directly with enzyme concentration and the reaction reached steady state earlier as enzyme concentration increased. The production after hydrolysis also increased (see section 6.3.2, the conversion rate increased from 0.76 to 0.97). Novalin *et al.* (2005) found adding more Maxilact in membrane-diffusion reactor from 60 to 120 U/ml enhanced the percentage of lactose conversion in skimmed milk from 57% to 78% approximately. Using the β-galactosidase from *L. delbrueckii* with high concentration should easily match these figures.

The trend of the results was in agreement with the results observed in hydrolysis of lactose solution in Chapter 5. However, the conversion rate of lactose to glucose observed in lactose containing skimmed milk was slightly lower (as seen in Table 6.6 the conversion from lactose solution was 0.78 and from lactose in skimmed milk was 0.76). The possible reason might because in addition to lactose, some small protein and ions were left in the substrate solution, so these particles probably influenced the enzyme by binding with the active site of enzyme and decreased its activity.

The β-galactosidase activity was not strongly affected by temperature in range 25-40 °C. High temperature slightly increased the rate of reaction. After the reaction almost reached steady state the temperature did not strongly affected glucose production.

From Table 6.5, the rate of conversion from lactose to glucose at the same substrate

concentration, crude enzyme gave higher than partially purified enzyme with the conversion rate in range 0.92-0.98.

3. The hydrolysis of lactose containing skimmed milk was also studied in continuous MBR system with different enzyme loadings and permeate flow rates. The detected glucose production in the retentate and permeate from MBR were not significantly different. It was concluded that the concentration polarisation of the enzyme and substrate concentration on the membrane did not influence the retention of sugar solution.

At low permeate flow rate provided long resident time and increased the contact time between the enzyme and substrate resulted in an increase in glucose concentration in solution, but when consideration in terms of productivity, at low flow rate operation gave less productivity than higher one.

The performance in continuous MBR showed significantly better results on volumetric productivity than in the batch bioreactor. On the benefits of continuous MBR such as the capability in recovery and reuse of the enzyme from the reaction, reduce the time cost of restart the process and the ease of product separation. The MBR seemed to be more attractive system and feasible for commercial production purpose.

4. The previous fit model from hydrolysis of lactose synthetic solution in Chapter 5 was directly applied with the reaction in lactose containing skimmed milk. The experimental data on glucose production from both batch bioreactor and continuous MBR were compared with the model prediction. The results were correlated well as previous results on batch but not for MBR. And according to substrate solution contained several suspensions such as ions, small proteins and some peptides apart from lactose, these probably affected the conformation of the enzyme and binding at the active site of the enzyme. Then the activity of the enzyme was changed. As can be seen from the results of changing substrate concentration in batch bioreactor, the calculated kinetic parameter (K_m) from Lineweave-Burk plot was 12.84 mM when the substrate was lactose containing skimmed milk while in case of lactose solution the K_m value was higher at 23.23 mM. In addition, the parameter values used in this model were different from values used in lactose hydrolysis (Chapter 5) since the characteristic of substrates were varied.

Chapter 7

Overall conclusions and recommendations

The aims of this project were to investigate the production and separation β-galactosidase from *L. delbrueckii* and then use the enzyme to transform lactose in skimmed milk. This was chosen as it is a dairy organism and could be produced on whey. The feasibilities of enzyme purification using aqueous two phase system and membrane separation were investigated. This showed the membrane purification gave an active stable extract. The calculation of kinetic parameters were achieved by using Lineweave-Burk plot and the studies of environmental conditions on the activity showed optimum pH, half-life time with various temperatures, the inhibition by some cations, glucose and galactose. Then the performances of β-galactosidase on lactose transformation in enzyme bioreactor and in MBR were studied. To describe the kinetics of the system, mathematical models were used to analyse the experimental data and confirmed that Haldane kinetic fitted the data. The data showed that lactose hydrolysis in skimmed milk could be described in batch but not well in MBR. Specific points of discussion are given under the proceeding sections.

7.1 Growth of *Lactobacillus delbrueckii* ssp *bulgaricus* NCTC 11778

The growth performance of *L. delbrueckii* on various concentrations of nutrient compositions was determined with starting point pH 6.5 on the basis of providing high cell density and specific growth rate. The effects of yeast extract (see section 3.2.1) and lactose (see section 3.2.2) obviously played an important role affecting the cell growth. Although soy peptone which enriched with amino acid, did not greatly influence the growth, it was still needed in the medium since this strain has limit on biosynthetic abilities.

The most suitable concentration of KH_2PO_4 (see section 3.2.3) was in range 0.25% to 0.5%. In case of sodium acetate (see section 3.2.4), the cell yield increased positively in proportion to sodium acetate concentration but above 0.5% did not show great different yield. Therefore at 0.5% sodium acetate in medium was satisfied for supporting the growth. Generally, triammonium citrate is able to act as a source of carbon for synthesis of some essential cell constituent. Although increasing concentration of citrate from the experiment seemed to inhibit the growth of culture (see section 3.2.5), low concentration of citrate

needed to be added into medium to maintain cell balance. The presence of tween80 also slightly affected the specific growth rate and cell yield (see section 3.2.6). The effect of magnesium and manganous sulphate were not investigated only adequate levels of these elements based on used in MRS medium were directly applied in the medium.

Therefore the ingredients of final growth medium consisted of yeast extract 10 g/l, soy peptone 10 g/l, lactose 20 g/l, KH_2PO_4 2.5 g/l, sodium acetate 5 g/l, triammonium citrate 2 g/l, tween80 1 ml/l, magnesium sulphate 0.2 g/l and manganous sulphate 0.05 g/l. This medium produced the desirable specific growth rate of the cells at 0.35 $h^{-1}$and was employed for optimum pH growth experiment.

The study on the effect of pH medium against the cell growth was conducted in 5-l batch fermenter at 37 °C (see section 3.3). The optimum pH was range from 5.0 to 5.5. Although at pH 5.0 provided slightly higher cell density, the pH value at 5.5 was chosen for cultivating in 140-l pilot fermenter because growth time to stationary phase was shorter.

Although not studied in this work, the growth on sweet whey should also be possible allowing the microbe to be produced within the dairy on a waste product reducing the production cost.

After harvesting cells from pilot scale cultivation by MF membrane system, the enzyme released from cells by using high pressure homogenizer was determined (see section 4.2). Increasing operating pressure resulted in decreasing cell optical density and increased protein released while the enzyme activity released after disruption from pressure 25 kpsi to 40 kpsi (172 MPa-275 MPa) remained almost constant. Although high pressure provided high protein and enzyme released, the pressure can influence the size of cell debris and impact product separation for further task. Then the appropriate pressure used was selected at 25 kpsi (172 MPa).

7.2 Purification and properties of β-galactosidase

Two different methods for enzyme separation, aqueous two phase system (ATPS) and membrane separation were investigated.

The preferential separation in ATPS was the enzyme partitioned in the top phase while the cell debris partitioned in the other phase in order to facilitate further purification process.

Increasing the molecular weight of PEG from 1500, 3350 and 8000 in comparison to the same concentration with a fix concentration of KH_2PO_4 at pH 6.5 (see section 4.3.2) performed a tendency of the β-galactosidase recovery concentrated in the bottom salt-rich phase with cell debris. The enzyme preferred partitioning in salt-rich phase rather than in the top PEG-rich phase. Lima *et al.* (2002) mentioned that the partition coefficient of enzyme normally decreased as the PEG chain length increases and these experimental results seemed to be agreed with these findings.

The phenomenon of the desired enzyme partition in ATPS favoured the bottom phase when the phase system used was away from critical point of binodal curve. Thereby, raising PEG concentration influenced more β-galactosidase, soluble proteins and all contaminants partitioned to the salt rich phase as a consequence of their low solubility in PEG-rich phase resulting poor purification.

The trend in the result was decreased when the concentrations of KH_2PO_4 were increased (see section 4.3.3). It was obviously seen in PEG 3350 and 8000 the partition coefficient reduced quickly and the enzyme favourably partitioned to the bottom phase when the system composed of KH_2PO_4 from 8.5% to 9% w/w while in case of PEG 1500, the concentrations of KH_2PO_4 did not much influence β-galactosidase partition. The explanation of this phenomenon was probably because increasing KH_2PO_4 concentration made the bottom phase became more negatively charge while the top PEG-rich phase became more positively charge. Thus most proteins which are positively charge will partition to the bottom phase.

The feasibility of enhancing the potential of enzyme recovery was determined using the addition of NaCl (see section 4.3.4). It was found that adding NaCl in the PEG 3350 or 8000 systems assisted the β-galactosidase partitioned in the bottom phase rather than the top phase. The reason was probably because in general salts can change the electrostatic charge of the system and cause the molecules with positive charge to prefer the salt-rich phase. In case of PEG1500, the presence of NaCl from 1-3% w/w did not much alter partitioning of the enzyme from the top phase as compared with in absent NaCl substance.

Different pH values condition can affect the charge in the side chain of protein leading to a net charge modification of overall macromolecule then changing its partitioning properties. The influence of pH value was observed to alter the β-galactosidase partition coefficient

(see section 4.3.5). At near neutral pH some part of the enzyme moved to the top phase. However, most of the enzyme still preferred to be in the bottom phase when changing the pH values above this point. The best partition coefficients of enzyme in PEG 3350 and 8000 were obtained at pH value 6.5 while in PEG 1500 was in range 5.5-6.5.

Although some experimental results from ATPS performed the partitioning of the enzyme preferred in the top PEG-rich phase, the results between enzyme and protein partition coefficients in different conditions did not provide effective purification factor because the contaminant proteins were partitioned into the same phase as the β-galactosidase. At best, the purification factor of 2.08 was observed. A further consideration on the industrial scale is the expense of the two phase system, including cost of disposal of waste materials and the problem on the contamination of skimmed milk by phosphate or PEG. As such ATPS was no longer considered as a separation method for further experiments in this project.

The combination of MF and UF were applied to improve enzyme separation instead of ATPS. The MF membrane with pore size 0.2 μm was used to remove soluble protein from disrupted cells suspension. The enzyme and protein were washed out from the cell debris with discontinuos diafiltration technique (see section 4.4.1). The percentage of enzyme removal obviously increased from 27% to 82% and protein removal gradually raised from 9% to 30% with an increase in volume diafiltration. After 5 diafiltration volumes of exchange the approximate enzyme recovery in total permeate was 72% with 2.31 fold purification.

Then the MF permeate solution was concentrated in UF system with 50 kDa MWCO membrane (see section 4.4.2). Most of β-galactosidase and protein were retained by membrane. The recovery and purification of the β-galactosidase after MF and UF processes were about 55% for recovery and giving an overall purification of 2.91 over the crude enzyme. The final volume left in retentate was defined as partially purified enzyme and used in study of enzyme properties.

This purification was considered at least equivalent to ATPS and it involved only physical separation, no addition of chemicals or alteration of the physical conditions. There is potential for further optimization of the process by selection of different membrane system with better cut off molecular weight characteristics.

The properties of β-galactosidase from crude extract and partially purified enzyme from membrane system were then characterised. The β-galactosidase from both forms showed maximum activity on ONPG and lactose at pH 6.5 (see Figure 4.14 and Figure 4.15).The activity of β-galactosidase increased with the rising temperature from 20 to 65 °C (see section 4.5.3). Although at high temperature enhanced the reaction rate of enzyme, the stability at high temperature decreased rapidly due to thermal degradation. Therefore, it was crucial to consider the appropriate temperature of storage handling and used.

The determination of enzyme kinetics was based on the Michaelis-Menten equation and of Lineweaver-Burk transformation plots. The calculated V_{max} and K_m values from the plot in two substrates, ONPG and lactose were investigated (see section 4.5.4). The range of ONPG concentration used was 2-100 mM and lactose from 5-100 mM. The V_{max} values from crude enzyme were 1.25 and 0.91 μmol/min/ml and K_m values were 12.63 mM and 23.23 mM respectively. In case of partially purified enzyme, the V_{max} values were 1.04 and 1.34 μmol/min/ml and K_m values were 17.62 mM and 27.58 mM when ONPG and lactose were applied as substrate respectively. The plots between 1/V and product concentrations of lactose hydrolysis pointed that both glucose and galactose acted as non-competitive inhibitor for β-galactosidase (see section 4.5.5 and 4.5.6 respectively).

The addition of monovalent cations, Na^+ and K^+ (see section 4.5.7) were thought able to induce conformational changes in the enzyme structure (Jurado *et al.*, 2004). It was found that adding Na^+ in lactose hydrolysis reduced the activity while the presence of K^+ promoted the activity. The divalent cations also affected the activity (see section 4.5.8). The Ca^{2+} cation strongly inhibited the activity while using Mg^{2+} at concentration above 10 mM appeared to decrease the enzyme activity.

7.3 Hydrolysis of lactose solutions

The hydrolysis reaction with a lactose synthetic solution in small scale bioreactor was studied. It was found that the yield of glucose production from batch bioreactor depended on the lactose concentration and enzyme concentration.

After investigation in batch bioreactor, the reaction was further tested in pilot scale MBR. The MBR based on the difference in molecular weight between the enzyme, lactose and the hydrolysis products. The soluble β-galactosidase enzyme was retained in the retentate

side of the membrane, where it was in contact with the substrate, while low molecular weight particles, such as lactose and its hydrolyzed products, were small enough to permeate through the membrane. The stability of β-galactosidase in operational pilot scale MBR was also determined in the condition with and without nitrogen (see section 5.5). In case of nitrogen condition, the enzyme was more stable and prevented the loss of activity better than in atmospheric condition. This was probably concluded that the gas-liquid interface influenced the activity of the enzyme. In addition, the enzyme stability from the system decreased quickly in comparison with incubation from batch reactor (see section 4.5.3) and in small scale stirred cell membrane reactor with 30 kDa MWCO (see section 5.3.1). Many effects such as flow of liquid across the membrane device, pumping and adsorption of the enzyme on membrane surface might associate with deactivation of the enzyme.

Different lactose concentrations and enzyme concentrations during MBR operation influenced the glucose production (see section 5.6.2.1-5.6.2.2). The results from MBR were in agreement with the results observed in batch bioreactor (see section 5.2.1-5.2.2). High concentration of lactose and enzyme increased the productivity. The change of permeate flow rate in MBR resulted in the variation of residence time of the substrate in the system. Increasing the permeate flow rate from 8 ml/min (resident time was 313 min) to 25 ml/min (resident time was 100 min) increased the productivity of glucose (see section 5.6.2.3) by 293 to 803 μmol/min.

Although operation hydrolysis in batch system was simple and easy to control, the volumetric product formation from continuous MBR system provided slightly better results than in bioreactor system under the same experimental conditions (see Table 5.4). The continuous MBR is also more attractive system especially consideration in terms of the benefits on commercial purpose such as the capability in recovery and reuse of the enzyme from the reaction, reduce the time cost of restart the process and the ease of product separation.

The enzymatic transformation of lactose solution in buffer pH 6.5, temperature 25 °C by β-galactosidase was modelled in both batch reactor and continuous membrane bioreactor. The experimental data correlated well with the kinetic model based on reversible reaction from Haldane equation (see section 5.7.1.2). In case of MBR, applying the basic principle

of mass balance was combined with kinetic equation. The model was tested using experimental data from a system operating with different lactose concentrations, enzyme concentrations and flow rates of permeate. Good agreement between the model predictions and the experimental data were obtained with R^2 values ranging from 0.992 to 0.998 (see section 5.7.2.1-5.7.2.3).

Clearly, the enzyme loading would significantly increase the productivity and as such the loading should be increased by at least factor of 50. This would match the data published for enzyme from *K. lastic* (Novalin *et al.,* 2005).

7.4 **Hydrolysis of lactose containing skimmed milk**

In preparation for hydrolysis, skimmed milk was first filtered with 0.2 μm MF system. Most of milk fats and some of milk proteins, according to particle size, were retained in the membrane system while lactose and low molecular weight particles passed through the membrane. To reduce the amount of milk proteins and obtained mainly carbohydrates in the permeate solution, the UF system with 10 kDa MWCO was applied as a second filtration for permeate MF solution. However, small proteins and peptides were able to pass through and detected after UF. About 80% lactose was recovered after UF process in the permeate (see section 6.2.).

The product formations from both preparations of the enzyme in batch bioreactor increased with substrate concentration and enzyme concentration (see section 6.3.1 and 6.3.2 respectively). The rate of reaction increased directly with enzyme concentration and the reaction reached steady state earlier. The trend of the results was in agreement with the results observed in Chapter 5. However, the conversion rate of lactose to glucose observed in lactose containing skimmed milk was slightly lower. The possible reason might because apart from lactose, some small proteins and ions were suspended in the substrate solution, so these particles probably influenced the enzyme by binding with the active site of enzyme and altered the kinetics slightly.

The temperature in range 25-40 °C did not strongly influenced β-galactosidase activity. High temperature slightly increased the rate of reaction but almost certainly effect the membrane filtration by increasing flux at high temperatures. After the reaction reached steady state the final yields product were not significantly different (see section 6.3.3).

The hydrolysis of lactose containing skimmed milk was also studied in continuous MBR system with different enzyme loadings and permeate flow rates (see section 6.4.1 and 6.4.2 respectively). The detected glucose production in the retentate and permeate from MBR were not significantly different. It was concluded that the concentration polarisation of the enzyme and substrate concentration on the membrane did not influence the retention of solution.

Operating MBR at low permeate flow rate, which provided long resident time and increased the contact time between the enzyme and substrate, resulted in greater glucose concentrations than when operating at high permeate flow rate. However, consideration in terms of productivity, at low flow rate operating gave less productivity than higher one.

The model fit obtained from Chapter 5 correlated well with the experimental data from lactose containing skimmed milk in batch bioreactor (see section 6.5.1-6.5.2) but not for continuous MBR (see section 6.5.3). The possible reason for poor fit in this case might because the suspended ions, small proteins and some peptides in solution apart from lactose affected the conformation of the enzyme. Then the activity of the enzyme was changed as can be seen from the results of changing substrate concentration in batch bioreactor. The K_m value for lactose containing skimmed milk was 12.84 mM while the K_m from lactose solution was higher at 23.23 mM. In addition, the unknown parameter values used in this model were different from values used in lactose hydrolysis (Chapter 5) since the characteristic of substrates differed. Another possible reason for poor correlation was the distribution of enzyme within the MBR.

7.5 Commercial prospective for operating a process for low lactose milk

The process for commercial plant was then proposed on the basis of previous results to assess the possibility whether this work fits to an industrial scale. Therefore, the aim of this section is to evaluate the cost of product, low lactose milk from the proposed process.

7.5.1 Proposed process for low lactose milk

The basis of this design is to produce low lactose milk according to Figure 7.1 about 20,000 l/day (12 hour working). The overall processing of milk consists of 4 main steps as followed;

1. Skimmed milk separation

In skimmed milk separation step, 10,000 l of skimmed milk would be fed into UF membrane system. The idea is large particle solution such as milk fat and some protein would be left about 1,000 l in membrane system whereas about 9,000 l of small molecular weight permeate solution, mainly contained carbohydrate, pass through the membrane system.

2. Enzyme preparation

To gain about 50% lactose hydrolysis, 10% of crude enzyme from total working volume in MBR would be add to the system. Therefore, 250 l of crude enzyme would be prepared and used in MBR. *L. delbrueckii* would be grown on 2,500 l of diluted UF permeate skimmed milk containing 2% lactose with addition of 1% yeast extract and 1% soy peptone. The temperature would be at 37 °C and pH at 5.5 using 10 M NaOH. The cultivation would take about 8 h, the cells would be then harvested by MF system using diafiltration technique to remove medium. The concentrated cell slurry would then be disrupted by pressure homoginizer at 25 kpsi and the enzyme was removed from debris by MF system. The crude enzyme could then be added to MBR system.

3. Lactose hydrolysis in MBR

The total working volume of MBR would be set at 2,500 l. The UF permeate skimmed milk would continuously feed to the MBR. The permeate flow rate from MBR system would set at 1,000 l/h.

4. Milk reconstitution.

The obtained permeate would be divided into two parts. Half of them would be disposed since the taste of final product might be too concentrated on the sweetness from monosaccharide if all permeate used. So, only half of the permeate would be returned and diluted with water at the same amount of disposal. Then the solution retained in MF membrane during milk separation process and diluted permeate product from MBR would be treated with heat at high temperature for a short time after reconstitution in the mixing tank. The final product would contain lactose about 1.5-1.75 % or about 70% reduction in lactose.

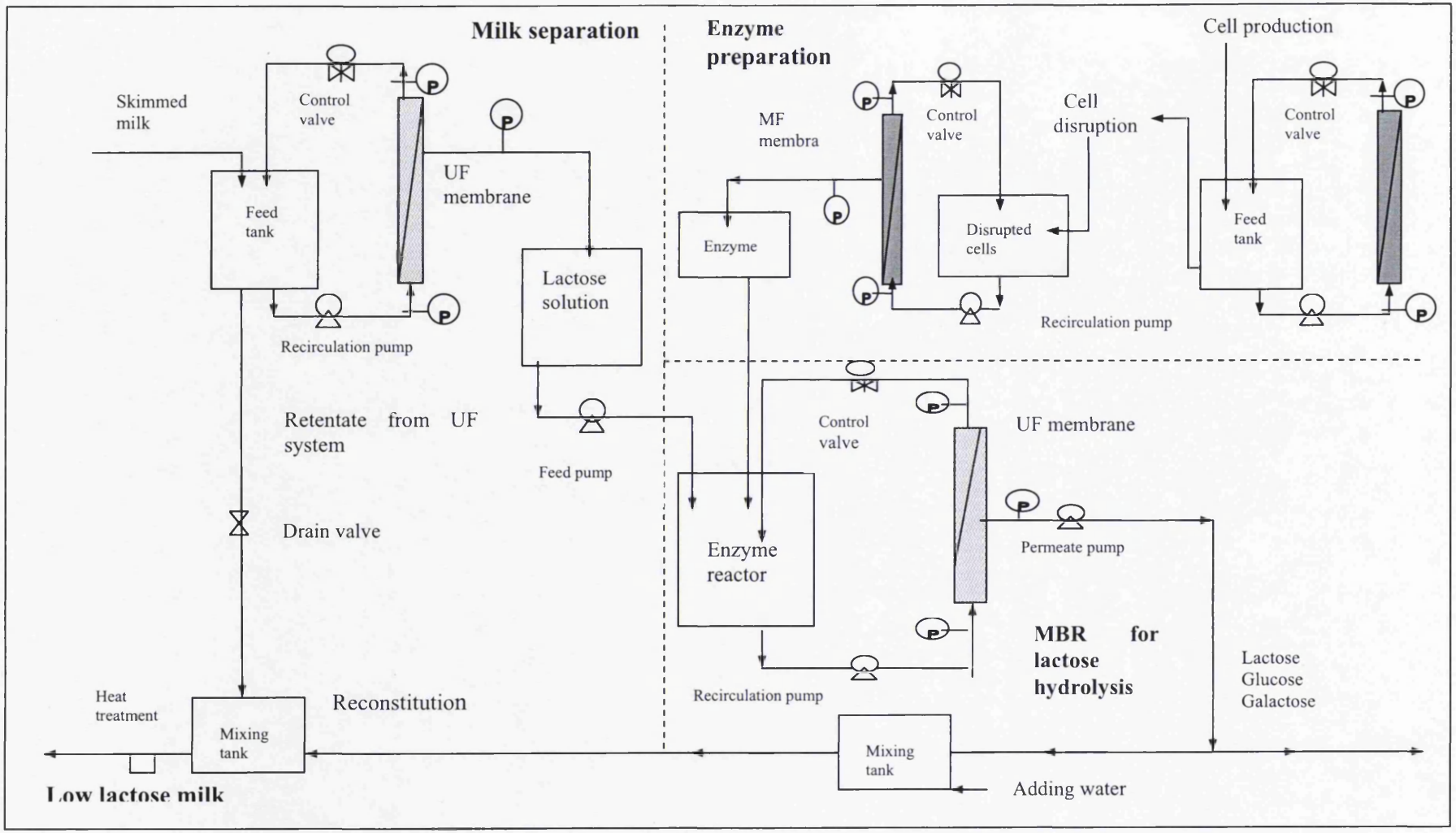


Figure 7.1 *Scheme of proposed process for low lactose milk.*

The major equipment for the overall process consist of storage tanks, feed tanks, bioreactor for MBR system, fermenter, cell disrupter, mixing equipments, membranes, pumps and heat exchangers. The details in sizing, specifying and costing for these items can be seen in Appendix 3.2.

7.5.2 Preliminary cost estimation

7.5.2.1 Fixed capital investment

Total purchase cost of major equipment (PCE) (see Table A.3, Appendix 3) = £ 439,854

Total physical plant cost (PPC) = PCE x (1+sum of factors for estimation (f_1-f_9))

(factors for estimation are referred in Table 6.1, page 251 in Chemical Engineering Vol. 6, 3rd by Coulson *et al.*, 1999)

Then PPC = 439,854 x 3.4 = £ 1,495,504

Fixed capital = PPC x (1+sum of factors for estimation (f_{10}-f_{12}))

= 1,495,504x1.45= £2,168,481

7.5.2.2 Direct production cost

The direct production cost for the process can be calculated from the sum of variable costs and fixed costs. The variable costs depend on the amount of product produced while fixed costs do not rely on production rate. The percentage of variable costs and fixed costs are presented in Figure 7.2 and Figure 7.3 respectively (see details in Appendix 3, Table A3.2 and A.3.3).

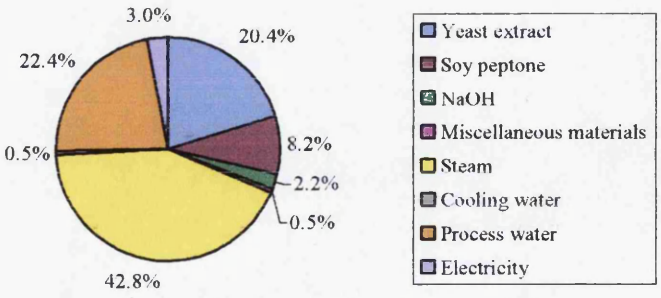


***Figure 7.2** The cost percentages associated with variable costs*

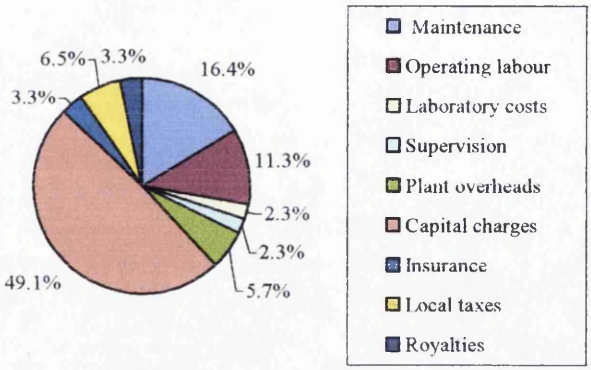


***Figure** 7.3 The cost percentages associated with fixed costs*

Direct production cost = Variable costs + Fixed costs = £220,723 +£662,937 = £883,660

The total amount of product per year = 6000 m^3

Based on this design, the cost of low lactose milk product then increases from normal cost about £0.15/litre. The cost can be slightly decreased when increasing the operating time to produce more quantity of product per day. In reality, the variable costs from raw materials used for enzyme production might be less than this estimation because one batch of the enzyme can be used approximately up to 15-20 days according to its half-life stability if the operating condition is properly set (while on this design, one batch of the enzyme is planed to used per day). The potential to reduce the amount of lactose less than this point is probably carried out if the proportion of the enzyme used in MBR system is increased.

7.5.3 Important potential studies for process validation

To ensure process capability on producing a product of the required quality, further studies are required on long term process stability, the use of bio-products and enzyme β-galactosidase, the acceptability of product, the composition, the nutritional quality, consumer acceptability and safety (microbial quantity) need to be considered.

Selecting appropriate process equipments such as type and size of membrane, pump and ancillary system with good installation are able to assist the satisfactory on long term process stability. In addition, adjusting a suitable condition, sterile process, controlling the temperature, time for operation and cleaning the process after use well also help to stabilize the enzyme and product quality.

Milk is a complex biological fluid and consists of water, milk fat, milk protein, carbohydrates and nutrient. Most high molecular weight components in milk will be retained in the system after raw milk separation step. Although these contents would be expected to remain the same level with raw milk after reconstitution, some components that contribute to nutrient contents after the process will be changed. In general, milk contains several essential nutrients (these required by the human) such as calcium, vitamin D, protein (methionine and lysine), potassium, vitaminA, vitaminB_{12}, riboflavin, niacin and phosphorus. During the process, the physicochemical properties in milk will be influenced especially the product after MBR has to be diluted with water. The nutrient contents will therefore be affected and the altered composition of low lactose milk obtained after reconstitution will have an impact on its physical appearance, flavor and texture. Once formulated, the acceptability in final product from consumers has to be evaluated in terms of nutritional value, physical properties and organoleptic properties. Extra nutrients might be added to improve the quality further.

The retentate from raw milk separation process and permeate from MBR will be treated with heat at high temperature for a short time after reconstitution step. Although the hydrolysis reaction in MBR is operated in septic condition (hygienic), heat treatment is still applied with the permeate product to secure it from contamination. Two-types of pasteurization, high temperature/short time (HTST) and ultra-high temperature (UHT) have been used. For HTST process, milk is hold at 72 °C for 15 seconds (Fox and Mcsweeney, 1998). This is considered adequate to kill almost all yeast, mold, common spoilage bacteria and common pathogenic heat-resistance organisms. The shelf life from HTST process is about two to three weeks. To extend the longer life product up to two to six months, UHT can be applied instead. The process involves heating milk at a temperature 130-140 °C for 3-5 seconds and there are some minor changes for chemical in the product (Fox and Mcsweeney, 1998).

7.6 Recommendations of future work

Based on the work in this study, the following topics should be investigated further:

This study shows high cell concentration can be produced with suitable concentration of the medium. The effect of broth composition on enzyme production should be investigated and some supplemented medium such as whey and skimmed milk probably encourage

high productivity especially using the permeate solution after it has passed these supplemented medium through the microfiltration membrane to eliminate large molecules, then it will be easier to harvest the cells.

Different bacterial growth conditions influence the properties of the crude and purified enzyme. Growing at high temperature for example might enhance enzyme activity more effective yield etc. It would also be useful for the treatment of milk product at high temperature to avoid microbial contamination. The application of genetic engineering to produce thermostable β-galactosidase from this strain should be studied although acceptability of such materials is doubtful.

Although the use of crude enzyme on lactose hydrolysis seems to be more economic than the purified form, the presence of other microbial enzyme might interfere hydrolysis reaction and influence final products.

MBR kinetics were poor correlated with model. This needs to be investigated further. For example is the enzyme distributed evenly through the MBR or is the activity concentrated on the membrane. Also the effect of enzyme concentration should be investigated further to confirm the limits of the process in MBR.

Control of microbial contamination in food processing is essential. Using non-sterile materials for lactose reduction in skimmed milk with long term continuous operation influences a high risk of microbial contamination. The ways such as steam sterilization and adding some antimicrobial chemical would develop the process. However, the disadvantages on these methods must be considered. Novalin *et al.* (2005) suggested a combination of sterile filtration and UV irradiation with intensity of 45% of a 25 W lamp for decreasing microbes in the enzyme solution. The microbe number reduced from 10^4 to 10^2 organismsml^{-1} and 10% less enzyme activity was detected.

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Appendix 1 Calibration Curves for Analytical Methods

A 1.1 Molar Extinction Coefficient (ε)

The simplicity of molar extinction coefficient assay was carried out in 0.1 M phosphate buffer with different pH at a nitrophenyl-galactoside concentration of 0.1 mM and the results were calculated in comparison with absorbance coefficient of the product nitrophenol at pH 7.6 (13,300 l $mol^{-1}cm^{-1}$, John, 2002).

pH	average absorbance at 420nm	ε
4	0.084	1117
4.5	0.092	1217
5	0.094	1250
5.5	0.121	1603
6	0.208	2760
6.5	0.418	5553
7	0.830	11039
7.5	1.253	13300
8	1.582	21041

Table A 1.1 *The molar extinction coefficient of nitrophenyl-galactoside at various pH.*

A 1.2 Calibration curve of BSA from Folin assay

The concentration curve of BSA from 0 to 250 μg/ml and its absorbance at 750 nm with Folin assay in a 4 ml cuvette was employed as standard curve in the calculation of protein concentration.

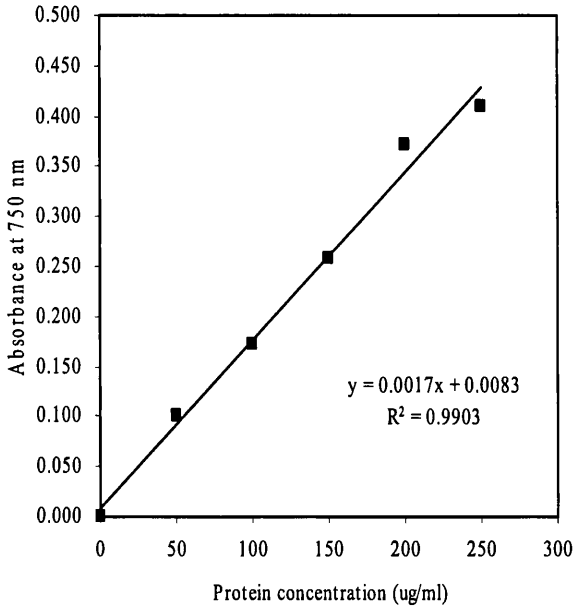

Figure A1.1 *The standard curve of BSA concentration from Folin assay.*

A 1.3 Calibration curve of BSA from Bradford assay

The concentration curve of BSA from 0 to 1000 μg/ml and its absorbance at 595 nm with Bradford assay in a 4 ml cuvette was employed as standard curve in the calculation of protein concentration.

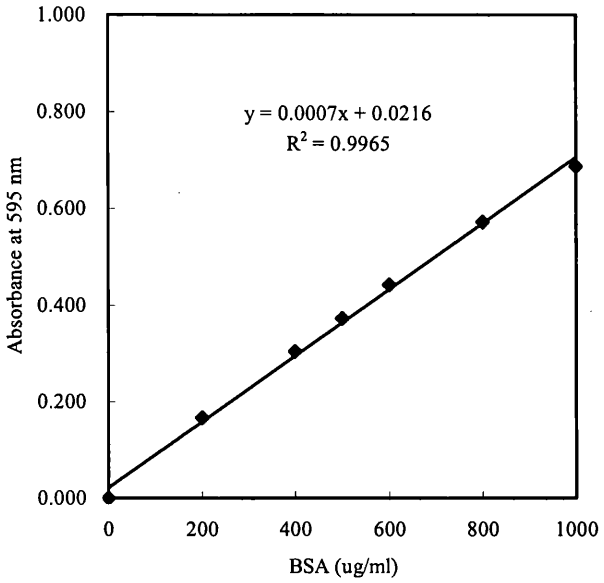

Figure A 1.2 *The standard curve of BSA concentration from Bradford assay.*

A 1.4 Standard curve of glucose concentration from GOPOD assay

The concentration curve of glucose from 0 to 1 mg/ml and its absorbance at 510 nm with GOPOD assay in a 4 ml cuvette was employed as standard curve in the calculation of glucose concentration.

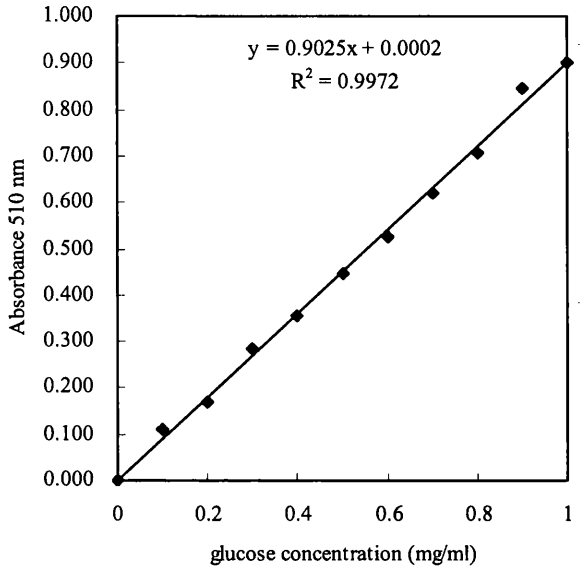

Figure A 1.3 *The standard curve of glucose concentration from GOPOD assay.*

A 1.5 Standard curve of lactose concentration from HPLC

The relationship between concentration of lactose from 0 to 4 g/l and its area count from HPLC was employed as standard curve in the calculation of lactose concentration.

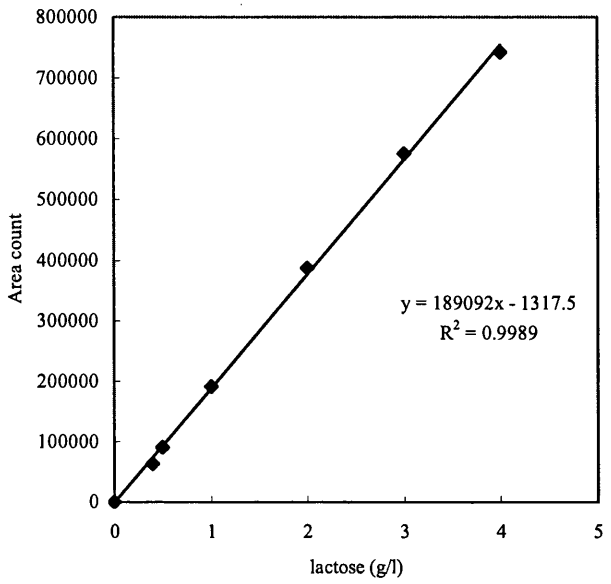

Figure A 1.4 *The standard curve of lactose concentration from HPLC.*

Appendix 2 Computer Programming for Mathematical Model

Five mathematical models were investigated for predicting the reaction of lactose hydrolysis in batch bioreactor. The programme for each model was analyzed by using nonlinear regression analysis with SCIENTIST software and written as following:

Kinetic equation for Model I

$$r = \frac{V_{max} S}{K_m + S}$$

```
// Michaelis-Menten kinetics (Model I)
IndVars: T
DepVars: G, C
Params: VMAX, KM
//Starting values
CL0=55.51
C=CL0-G
G'=VMAX*(CL0-G)/ (KM+(CL0-G))
// Initial conditions
T=0
G=0
***
```

Kinetic equation for Model II

$$r = \frac{V_{max} S}{K_m\left(1+\frac{P}{K_i}\right)+S}$$

```
// Michaelis-Menten kinetics with competitive inhibition (Model II)
IndVars: T
DepVars: G, C
Params: VMAX, KM, KI
//Starting values
CL0=55.51
C=CL0-G
G'=VMAX*(CL0-G)/ (KM*(1+G/KI)+(CL0-G))
// Initial conditions
T=0
G=0
***
```

Kinetic equation for Model III

$$r = \frac{V_{max} S}{K_m\left(1+\frac{P}{K_i}\right)+S\left(1+\frac{P}{K_i}\right)}$$

// Michaelis-Menten kinetics with noncompetitive inhibition (Model III)

IndVars: T

DepVars: G, C

Params: VMAX, KM, KI

//Starting values

CL0=55.51

C=CL0-G

G'=VMAX*(CL0-G)/ ((KM+ (CL0-G))*(1+G/KI))

// Initial conditions

T=0

G=0

***

Kinetic equation for Model IV

$$r = \frac{V_{max} S}{K_m\left(1+\frac{P}{K_i}+\frac{P}{K_i'}\right)+S\left(1+\frac{P}{K_i}+\frac{P}{K_i'}\right)}$$

// Michaelis-Menten kinetics with both noncompetitive inhibition products (Model IV)

IndVars: T

DepVars: G, C

Params: VMAX, KM, KI, KI'

//Starting values

CL0=55.51

C=CL0-G

G'=VMAX*(CL0-G)/ ((KM*(1+G/KI+G/ KI'))+((CL0-G)*(1+G/KI+G/ KI')))

// Initial conditions

T=0

G=0

***

Kinetic equation for Model IV

$$r = \frac{V_{max\,f}\left(S - \frac{P}{K_{eq}}\right)}{K_m\left(1 + \frac{P}{K_p}\right) + S}$$

// Reversible reaction-Haldane kinetic equation (Model V)

IndVars: T

DepVars: G, C

Params: VMAX, KM, KP, KED

//Starting values

CL0=55.51

C=CL0-G

G'=VMAX*((CL0-G)-(G/KED))/ (KM*(1+G/KP)+(CL0-G))

// Initial conditions

T=0

G=0

***

The model for lactose hydrolysis in continuous membrane reactor was written as below;

Kinetic equation for continuous MBR

$$\frac{dP}{dt} = -D_P P + r \quad \text{when} \quad r = \frac{V_{max\,f}\left(S - \frac{P}{K_{eq}}\right)}{K_m\left(1 + \frac{P}{K_p}\right) + S}$$

// Reversible reaction-Haldane kinetic equation with continuous MBR

IndVars: T

DepVars: G, C

Params: VMAX, KM, KP, KED, DP

//Starting values

CL0=55.51

C=CL0-G

G'=-(G*DP) + (VMAX*((CL0-G)-(G/KED))/(KM*(1+G/KP)+(CL0-G)))

// Initial conditions

T=0

G=0

***

Appendix 3 Preliminary estimates on sizing and cost for proposed process

3.1 Calculation in sizing and costing equipments

Section 1: Milk separation

1. Sizing of feed tank for skimmed milk

Type of tank: vertical cylindrical tank with conical base and flat roof

Material: stainless steel Type 304

Volume capacity: 10 m^3

Volume of tank = volume of cylinder + volume of conical

$$V = \left(\frac{\pi d^2 L}{4}\right) + \left(\frac{\pi r^2 L}{3}\right)$$

Volume of cylinder = $\left(\frac{\pi d^2 L}{4}\right)$ = 10 m^3 (for tank design, assuming the ration of height to diameter of cylinder is 1:1, then d = L)

d = L = 2.33

Volume of conical = $\left(\frac{\pi r^2 L}{3}\right)$ (the height is assumed to be equal to the diameter of the conical)

$$= \left(\frac{\pi d^3}{12}\right) = \left(\frac{\pi (2.33)^3}{12}\right) = 3.31 \text{ m}^3$$

Then, the volume of liquid in cylinder part = 10-3.31 = 6.69 m^3 so space on the top left = 2.33-1.57= 0.76 m. The total height of the feed tank = 4.66m.

Total area of tank = 13.31 m^3

Purchased equipment cost can be calculated as follow;

$$Ce = C S^n$$

Where Ce = the purchased equipment costs

S = the characteristic size parameter

C = the cost constant

n = the index for that type of equipment

Note: the data are given in the table 6.2, page 258 (Chemical Engineering Vol. 6, 3rd by Coulson *et al.,* 1999) and for stainless steel material the equation need to multiply by the factor of 2.5

Thus, the purchase cost for the feed tank for skimmed milk

$= C S^n \times 2.5 = 1450 \text{ x } (13.31)^{0.6} \text{x} 2.5 = £17{,}132.27$

2. Sizing of storage tank for permeate UF skimmed milk solution

Type of tank: vertical cylindrical tank with flat base and cone roof

Material: stainless steel Type 304

Volume of liquid (V_L): 9 m^3

Volume of tank = volume of cylinder + volume of conical

$$V = \left(\frac{\pi d^2 L}{4}\right) + \left(\frac{\pi r^2 L}{3}\right)$$

Volume of cylinder = $\left(\frac{\pi d^2 L}{4}\right) = 9 \text{ m}^3$

then

$$d = L = 2.25$$

Top space for gas vapour = 0.3 V_L

then $\left(\frac{\pi d^2 L_p}{4}\right) = 0.3(9) \text{ m}^3$

$$L_p = 0.68\text{m}$$

Total height of the cylinder = $L + Lp = 2.93$ m

Volume of conical = $\left(\frac{\pi r^2 L}{3}\right) = \left(\frac{\pi (2.25)^3}{12}\right) = 2.98\text{m}^3$

Therefore, total volume of storage tank for permeate UF solution

$= 11.65 + 2.98 \text{ m}^3 = 14.63 \text{ m}^3$

Thus, the purchase cost for storage tank for permeate UF solution

$= C S^n \times 2.5 = 1400 \text{ x } (14.63)^{0.55} \text{ x} 2.5 = £15{,}309.2$

3. Ultrafiltration membrane

Base on the experiment, the UF with surface area 0.18 m^2 was used.

The volume is scaled up 1000 times for proposed process. Then the surface area of membrane should be required about 180 m^2

The hollow fibre ultrafiltration module from Koch membrane manufacturer is selected with its dimension 6″x72″ (0.15m x 1.83m) and surface area = 12.7 m^2 for one module.
Then the number of hollow fibre membrane used are = 180/12.7 = 14.2 modules

4. Pump for 10000 l milk

Select membrane size 6″x72″ (0.15m x 1.83m) and diameter of fibre inside 2 mm

Then surface area of each fibre = $2\pi rh = 2\pi$ x (0.001) x 1.83 = 0.012 m^2

Require membrane surface area = 180 m^2, so the number of fibre in membrane tube = 180/0.012 = 15000 fibre tubes

Assume the velocity of fluid is 1 m/s, the volume in fibre is π x $(0.001)^2$ x 1 = 3.14 x 10^6

Then the volume passes membrane in 1 sec = $3.14x10^{-6}x15000 = 4.7 \times 10^{-2}$ m^3/s

Pressure drop in membrane is calculated by;

$$\Delta P_f = 8f\left(\frac{L}{d_i}\right)\frac{\rho u^2}{2}$$

where ΔP_f = pressure drop, N/m^2

f = friction factor

L = pipe length, m

d_i = pipe inside diameter, m

ρ = fluid density, kg/m^3

u = fluid velocity, m/s

And $\mathrm{Re} = \frac{\rho u d_i}{\mu}$ where μ = fluid viscosity, Ns/m^2

Then $\mathrm{Re} = \frac{1029x1x0.002}{2.1x10^{-3}} = 980$ when milk density = 1029 kg/m^3

milk viscosity = $2.1x10^{-3}$ Ns/m^2

from relationship between Re and f in pipe, f = 0.08

Substitute values, therefore

$$\Delta P_f = 8x0.08x\left(\frac{1.83}{0.002}\right)\frac{1029x(1)^2}{2}$$

$$= 301{,}291 \text{ N/m}^2$$

The power required for pumping can be calculated from:

$$power = \frac{\Delta P Q_P X 100}{\eta_P}$$

where ΔP = pressure differential across the pump, N/m^2

Q_P = flow rate, m^3/s

η_P = pump efficiency, per cent

then $$power = \frac{301291 x 4.7 x 10^{-2} x 100}{65} = 21.79 \text{ kW}$$

5. Heat exchanger for milk separation

Rate of heat transfer can be defined as;

$$Q^{\wedge} = M^{\wedge}{}_h C_{ph}(T_{hi} - T_{ho}) = M^{\wedge}{}_c C_{pc}(T_{co} - T_{ci})$$

Where $Q^{\wedge}$ = rate of heat transfer (known as power), W

$M^{\wedge}{}_h$ = mass flow rate of the hot fluid, kg/s

$M^{\wedge}{}_c$ = mass flow rate of the cold fluid, kg/s

C_{ph} = the heat capacity of the hot fluid, J/kg ^{0}C

C_{pc} = the heat capacity of the cold fluid, J/kg ^{0}C

T_{hi} = the inlet temperature of the hot fluid, ^{0}C

T_{ho} = the outlet temperature of the hot fluid, ^{0}C

T_{ci} = the inlet temperature of the cold fluid, ^{0}C

T_{co} = the outlet temperature of the cold fluid, ^{0}C

Milk enters the exchanger at a flow rate = 169.2 m^3/h, and assume flow rate of cooling water at 10 °C = 150 m^3/h. The temperature in the system is maintained at 25 °C.

then

$$M^{\wedge}{}_h = 169.2 \frac{m^3}{h} x \frac{1h}{3600s} x \frac{1000kg}{1m^3} = 47 kg/s$$

$$M^{\wedge}{}_c = 150 \frac{m^3}{h} x \frac{1h}{3600s} x \frac{1000kg}{1m^3} = 41.67 kg/s$$

C_{pc} of water = $4.19 x 10^3$ J/kg °C

$$C_{ph} \text{ (milk)} = 0.94 \frac{cal}{gl} x \frac{4.187J}{1cal} x \frac{1000g}{1kg} = 3.94 x 10^3 J/kg^0C$$

Therefore heat transfer can be calculated as follow;

$$Q^{\wedge} = M^{\wedge}{}_h C_{ph}(T_{hi} - T_{ho}) = 47x3.94x10^3 x(40-25) = 2777 \text{ kW}$$

Final temperature of cooling water;

$$T_{co} = T_{ci} + \frac{Q^{\wedge}}{M^{\wedge}{}_c C_{pc}} = 10 + \frac{2.78x10^6}{41.67x4.19x10^3} = 25.9^0 C$$

From $Q^{\wedge} = UA\Delta T_m$ and $\Delta T_m = F_t x \Delta T_{lm}$

Where U = heat transfer coefficient, W/m^2 °C, assume to be 1,400 W/m^2 °C

A = heat-transfer area, m^2

ΔT_m = the mean temperature difference, °C

$$\Delta T_{lm} = (40\text{-}25.9)\text{-}(25\text{-}10)/\ln(40\text{-}25.9/25\text{-}10) = 14.52$$

R= (40-25)/ (25.9-10) = 0.94, S = (25.9-10)/ (40-10) = 0.53; then F_t obtained from graph in Figure 12.19, page 657 (Chemical Engineering Vol. 6, 3rd by Coulson *et al.*, 1999) 0.88

$$A = \frac{2777x10^3}{1400x0.88x14.52} = 155.24 \text{ m}^2$$

Purchased cost can be calculated as = bare cost from Figure 6.3a, page 253 (Chemical Engineering Vol. 6, 3rd by Coulson *et al.*, 1999) x Type factor x Pressure factor
Selected materials for shell is carbon steel and tube is stainless steel, Bare cost = £60,000,
Type factor= fixed tube sheet = 0.8, Pressure factor in range 1-10 bar = 1.0.
Then cost = 60,000x0.8x1 = £48,000

Section 2: Enzyme preparation

1. Cell cultivation

1.1 Sizing of feed tank for washing cells in MF system

Type of tank: vertical cylindrical tank with conical base and flat roof
Material: stainless steel Type 304
Volume capacity: 2.5 m^3
Volume of tank = volume of cylinder + volume of conical

$$V = \left(\frac{\pi d^2 L}{4}\right) + \left(\frac{\pi r^2 L}{3}\right)$$

Volume of cylinder = $\left(\frac{\pi d^2 L}{4}\right)$ = 2.5 m^3 (for tank design, assuming the ration of height to diameter of cylinder is 1:1, then d = L)

d = L = 1.47m

Volume of conical = $\left(\frac{\pi r^2 L}{3}\right)$ (the height is assumed to be equal to the diameter of the conical)

$$= \left(\frac{\pi d^3}{12}\right) = \left(\frac{\pi (1.47)^3}{12}\right) = 0.83 m^3$$

Then, the volume of liquid in cylinder part = 2.5-0.83 = 1.67 m^3 so space on the top left = 1.47-0.98=0.49 m. The total height of the feed tank = 2.94 m.

Total volume of tank = 3.33 m^3

Thus, the purchase cost for feed tank for washing cells in MF system

= $CS^n \times 2.5$ = 1450 x $(3.33)^{0.6}$x2.5 =£7,460.61

1.2 Microfiltration membrane for washing cells

Base on the experiment, the MF with surface area 1.8 m^2 used for 100 l cell suspension.

The volume is scaled up 2500 l for proposed process. Then the surface area of membrane should be required about 45 m^2.

The hollow fibre crossflow microfiltration module from Koch membrane manufacturer is selected with its dimension 6″ 2/3x43″ (0.17m x 1.09m) and surface area = 9.8 m^2 for one module.

Then the number of hollow fibre membrane required are = 45/9.8 = 4.6 modules

1.3 Pump for washing 2500 l cell suspension in MF system

Select membrane size 6″2/3x43″ (0.17m x 1.09m) and diameter of fibre inside 1.3 mm

Then surface area of each fibre = $2\pi rh = 2\pi \times (6.5 \times 10^{-4}) \times 1.09 = 4.45 \times 10^{-3}$ m^2

Require membrane surface area = 45 m^2, so the number of fibre in membrane tube = $45/4.45 \times 10^{-3}$ = 10104.6 fibre tubes

Assume the velocity of fluid is 1 m/s, the volume in fibre is $\pi \times (6.5 \times 10^{-4})^2 \times 1 = 1.33 \times 10^{-6}$

Then the volume passes membrane in 1 sec = $1.33 \times 10^{-6} \times 10105 = 1.34 \times 10^{-2}$ m^3/s

Then $\text{Re} = \frac{1040x1x0.001}{1.2x10^{-3}} = 866.7$

(when assume density = 1040 kg/m^3 and viscosity = 1.2x10^{-3} Ns/m^2)

from relationship between Re and f in pipe, f = 0.08

Substitute values, therefore

$$\Delta P_f = 8x0.08x\left(\frac{1.09}{0.001}\right)\frac{1040x(1)^2}{2}$$

$$= 362{,}752 \text{ N/m}^2$$

The power required for pumping can be calculated as follow;

$$power = \frac{362752x1.34x10^{-2}x100}{65} = 7.48 \text{ kW}$$

1.4 Heat exchanger for washing cells

Cell suspension enters the exchanger at a flow rate = 48.2 m^3/h, and assume flow rate of cooling water at 10 ^{0}C = 60 m^3/h. The temperature in the system is maintained at 25 °C.

then

$$M\hat{}_h = 48.2\frac{m^3}{h}x\frac{1h}{3600s}x\frac{1000kg}{1m^3} = 13.39kg/s$$

$$M\hat{}_c = 60\frac{m^3}{h}x\frac{1h}{3600s}x\frac{1000kg}{1m^3} = 16.67kg/s$$

C_{pc} of water = 4.19x10^3 J/kg °C

C_{ph} assume to be equal to C_{pc}

Therefore heat transfer can be calculated as follow;

$$Q\hat{} = M\hat{}_h C_{ph}(T_{hi} - T_{ho}) = 13.39x4.19x10^3x(40-25) = 1122 \text{ kW}$$

Final temperature of cooling water;

$$T_{co} = T_{ci} + \frac{Q\hat{}}{M\hat{}_c C_{pc}} = 10 + \frac{1.12x10^6}{16.67x4.19x10^3} = 26.03^0 C$$

From $Q\hat{} = UA\Delta T_m$ and $\Delta T_m = Fx\Delta T_{lm}$

Where U = heat transfer coefficient, W/m^2 °C, assume to be 1,400 W/m^2 °C

A = heat-transfer area, m^2

ΔT_m = the mean temperature difference, °C

$$\Delta T_{lm} = (40\text{-}26.03)\text{-}(25\text{-}10)/\ln(40\text{-}26.03/25\text{-}10) = 14.31$$

R= (40-25)/ (26.03-10) = 0.94, S = (26.03-10)/ (40-10) = 0.53; then F_t obtained from graph in Figure 12.19, page 657 (Chemical Engineering Vol. 6, 3rd by Coulson *et al.*, 1999) 0.88

$$A = \frac{1122x10^3}{1400x0.88x14.31} = 63.64 \text{ m}^2$$

Purchased cost can be calculated as = bare cost from Figure 6.3a, page 253 (Chemical Engineering Vol. 6, 3rd by Coulson *et al.*, 1999) x Type factor x Pressure factor
Selected materials for shell is carbon steel and tube is stainless steel, Bare cost = £32,000,
Type factor= fixed tube sheet = 0.8, Pressure factor in range 1-10 bar = 1.0.
Then cost = 32,000x0.8x1 = £25,600

2. Enzyme separation

2.1 Sizing of feed tank for enzyme separation from cell debris in MF system

Type of tank: vertical cylindrical tank with conical base and flat roof
Material: stainless steel Type 304
Volume capacity: 0.25 m^3
Volume of tank = volume of cylinder + volume of conical

$$V = \left(\frac{\pi d^2 L}{4}\right) + \left(\frac{\pi r^2 L}{3}\right)$$

Volume of cylinder = $\left(\frac{\pi d^2 L}{4}\right)$ = 0.25 m^3 (for tank design, assuming the ration of height to diameter of cylinder is 1:1, then d = L)

d = L = 0.68 m

Volume of conical = $\left(\frac{\pi r^2 L}{3}\right)$ (the height is assumed to be equal to the diameter of the conical)

$$= \left(\frac{\pi d^3}{12}\right) = \left(\frac{\pi (0.68)^3}{12}\right) = 0.08 m^3$$

Then, the volume of liquid in cylinder part = 0.25-0.08 = 0.17 m^3 so space on the top left = 0.68-0.47 = 0.21m. The total height of the feed tank = 1.36 m.

Total volume of tank = 0.33 m^3

Thus, the purchase cost for enzyme separation from cell debris in MF system

= $C S^n \times 2.5$ = 1450 x $(0.33)^{0.6}$x2.5 =£1,863.87

2.2 Microfiltration membrane for enzyme separation

Base on the experiment, the MF with surface area 1.8 m^2 used for 100 l cell suspension.

The volume is scaled up 250 l for proposed process. Then the surface area of membrane should be required about 4.5 m^2

The hollow fibre crossflow microfiltration module from Koch membrane manufacturer is selected with its dimension 6″x43″ (0.17m x 1.09m) and surface area = 6.3 m^2 for one module.

Then the number of hollow fibre membrane required are = 4.5/6.3 = 0.71 modules

2.3Pump for 250 l enzyme separation in MF system

Select membrane size 6″x43″ (0.15m x 1.09m) and diameter of fibre inside 1.3 mm

Then surface area of each fibre = $2\pi rh = 2\pi$ x $(6.5x10^{-4})$ x 1.09 = $4.45x10^{-3}$ m^2

Require membrane surface area = 4.5 m^2, so the number of fibre in membrane tube = $4.5/4.45x10^{-3}$ = 1011.2 fibre tubes

Assume the velocity of fluid is 1 m/s, the volume in fibre is $\pi x (6.5x10^{-4})^2$ x1 = 1.33 x 10^{-6}

Then the volume passes membrane in 1 sec = $1.33x10^{-6}$x1011 = 1.34 x 10^{-3} m^3/s

Then $$\mathrm{Re} = \frac{1000x1x0.001}{1.0x10^{-3}} = 1000$$

(when assume density = 1000 kg/m^3 and viscosity = $1.0x10^{-3}$ Ns/m^2)

from relationship between Re and f in pipe, f = 0.08

Substitute values, therefore

$$\Delta P_f = 8x0.08x\left(\frac{1.09}{0.001}\right)\frac{1000x(1)^2}{2}$$

$$= 348{,}800 \text{ N/m}^2$$

The power required for pumping can be calculated as follow;

$$power = \frac{348800x1.34x10^{-3}x100}{65} = 0.72 \text{ kW}$$

2.4 Heat exchanger for enzyme separation

Cell suspension enters the exchanger at a flow rate = 4.8 m^3/h, and assume flow rate of cooling water at 10 °C = 10 m^3/h. The temperature in the system is maintained at 25 °C.

then

$$M\hat{}_h = 4.8\frac{m^3}{h} x \frac{1h}{3600s} x \frac{1000kg}{1m^3} = 1.33kg/s$$

$$M\hat{}_c = 10\frac{m^3}{h} x \frac{1h}{3600s} x \frac{1000kg}{1m^3} = 2.78kg/s$$

C_{pc} of water = $4.19x10^3$ J/kg °C

C_{ph} assume to be equal to C_{pc}

Therefore heat transfer can be calculated as follow;

$$Q\hat{} = M\hat{}_h C_{ph}(T_{hi} - T_{ho}) = 1.33x4.19x10^3 x(40-25) = 83.6 \text{ kW}$$

Final temperature of cooling water;

$$T_{co} = T_{ci} + \frac{Q\hat{}}{M\hat{}_c C_{pc}} = 10 + \frac{8.36x10^4}{2.78x4.19x10^3} = 17.18^0 C$$

From $Q\hat{} = UA\Delta T_m$ and $\Delta T_m = F_t x \Delta T_{lm}$

Where U = heat transfer coefficient, W/m^2 °C, assume to be 1,400 W/m^2 °C

A = heat-transfer area, m^2

ΔT_m = the mean temperature difference, °C

$$\Delta T_{lm} = (40\text{-}17.18)\text{-}(25\text{-}10)/\ln(40\text{-}17.18/25\text{-}10) = 18.62$$

R= (40-25)/ (17.18-10) = 2.09, S = (17.18-10)/ (40-10) = 0.24; then F_t obtained from graph in Figure 12.19, page 657 (Chemical Engineering Vol. 6, 3rd by Coulson *et al.,* 1999) 0.88

$$A = \frac{83.6x10^3}{1400x0.95x18.62} = 3.38 \text{ m}^2$$

Purchased cost can be calculated as = bare cost from Figure 6.3a, page 253 (Chemical Engineering Vol. 6, 3rd by Coulson *et al.,* 1999) x Type factor x Pressure factor

Selected materials for shell is carbon steel and tube is stainless steel, Bare cost = £4,000,
Type factor= fixed tube sheet = 0.8, Pressure factor in range 1-10 bar = 1.0.
Then cost = 4,000x0.8x1 = £3,200

2.5 Sizing of storage tank for enzyme permeate from MF system

Type of tank: vertical cylindrical tank with flat base and cone roof
Material: stainless steel Type 304
Volume of liquid (V_L): 0.25 m^3
Volume of tank = volume of cylinder + volume of conical

$$V = \left(\frac{\pi d^2 L}{4}\right) + \left(\frac{\pi r^2 L}{3}\right)$$

Volume of cylinder = $\left(\frac{\pi d^2 L}{4}\right)$ = 0.25 m^3

then

$$d = L = 0.68$$

Top space for gas vapour = 0.3 V_L

then $\left(\frac{\pi d^2 L_p}{4}\right) = 0.3(0.25)\ m^3$

$$L_p = 0.21\text{m}$$

Total height of the cylinder = $L + Lp = 0.89$ m

Volume of conical = $\left(\frac{\pi r^2 L}{3}\right) = \left(\frac{\pi(0.68)^3}{12}\right) = 0.08\text{m}^3$

Therefore, total volume of storage tank for enzyme permeate from MF system
= 0.32+0.08m^3 = 0.40m^3
Thus, the purchase cost of storage tank for enzyme permeate from MF system
= $CS^n \times 2.5$ = 1400 x $(0.4)^{0.55}$x2.5 =£2,114.47

Section 3: Lactose hydrolysis

1. Enzyme reactor

Type of reactor: vertical cylindrical reactor and headplate on the top
Material: stainless steel Type 304
Volume capacity: 2.5 m^3
Volume of tank = volume of cylinder

$$V = \left(\frac{\pi d^2 L}{4}\right)$$

Volume of cylinder = $\left(\frac{\pi d^2 L}{4}\right)$ = 2.5 m^3 (for reactor design, assuming the ration of height to diameter of cylinder is 3:1, then 3d = L)

d = 1.02 m

L= 3.06 m

Top space for gas vapour = 0.3 V_L

then $\left(\frac{\pi d^2 L_p}{4}\right)$ = 0.3(2.5) m^3

L_p = 0.92m

Total height of the cylinder = $L + Lp = 3.97$ m

Then, the total volume of liquid in reactor = 3.25 m^3 so space on the top= 0.92m.

Thus, the purchase cost for reactor = $C S^n \times 2.5$ = 9300 x $(3.25)^{0.4}$x2.5 =£37,254.29

2. Sizing of storage tank for product permeate from MBR system

Type of tank: vertical cylindrical tank with flat base and cone roof

Material: stainless steel Type 304

Volume of liquid (V_L): 10 m^3

Volume of tank = volume of cylinder + volume of conical

$$V = \left(\frac{\pi d^2 L}{4}\right) + \left(\frac{\pi r^2 L}{3}\right)$$

Volume of cylinder = $\left(\frac{\pi d^2 L}{4}\right)$ = 10 m^3

then

d = L = 2.33

Top space for gas vapour = 0.3 V_L

then $\left(\frac{\pi d^2 L_p}{4}\right)$ = 0.3(10) m^3

L_p = 0.70m

Total height of the cylinder = $L + Lp = 3.03$ m

Volume of conical = $\left(\frac{\pi r^2 L}{3}\right) = \left(\frac{\pi(2.33)^3}{12}\right) = 3.31\text{m}^3$

Therefore, total volume of storage tank for product permeate from MBR system
$= 12.92+3.31\text{m}^3 = 16.23\ \text{m}^3$
Thus, the purchase cost of storage tank for product permeate from MBR system
$= C S^n \times 2.5 = 1400 \text{ x } (16.23)^{0.55}\text{x}2.5 = £16{,}208.52$

3. Ultrafiltration membrane

Base on the experiment, the UF with surface area 0.18 m^2 was used.
The volume is scaled up 1000 times for proposed process. Then the surface area of membrane should be required about 180 m^2
The hollow fibre ultrafiltration module from Koch membrane manufacturer is selected with its dimension 6″x72″ (0.15m x 1.83m) and surface area = 12.7 m^2 for one module.
Then the number of hollow fibre membrane used are = 180/12.7 = 14.2 modules

4. Pump for MBR system

Select membrane size 6″x72″ (0.15m x 1.83m) and diameter of fibre inside 2 mm
Then surface area of each fibre = $2\pi rh = 2\pi \text{ x } (0.001) \text{ x } 1.83 = 0.012\ \text{m}^2$
Require membrane surface area = 180 m^2, so the number of fibre in membrane tube = 180/0.012 = 15000 fibre tubes
Assume the velocity of fluid is 1 m/s, the volume in fibre is $= \pi \text{ x } (0.001)^2 \text{ x } 1$
$= 3.14 \text{ x } 10^{-6}$
The volume passes membrane in 1 sec = $3.14\text{x}10^{-6}\text{x}15000 = 4.7 \text{ x } 10^{-2}\ \text{m}^3/\text{s}$

Then $\text{Re} = \frac{1000x1x0.002}{1.0x10^{-3}} = 2000$

(when assume density = 1000 kg/m^3 and viscosity = $1.0\text{x}10^{-3}\ \text{Ns/m}^2$)
from relationship between Re and f in pipe, f = 0.039
Substitute values, therefore

$$\Delta P_f = 8x0.039x\left(\frac{1.83}{0.002}\right)\frac{1000x(1)^2}{2}$$
$$= 142{,}740\ \text{N/m}^2$$

The power required for pumping can be calculated as follow;

$$power = \frac{142740x4.7x10^{-2}x100}{65} = 10.32\ \text{kW}$$

5. Heat exchanger for MBR system

Fluid enters the exchanger at a flow rate = 169.2 m^3/h, and assume flow rate of cooling water at 10 °C = 150 m^3/h. The temperature in the system is maintained at 25 °C.

then

$$M\hat{}_h = 169.2\frac{m^3}{h} x \frac{1h}{3600s} x \frac{1000kg}{1m^3} = 47kg/s$$

$$M\hat{}_c = 150\frac{m^3}{h} x \frac{1h}{3600s} x \frac{1000kg}{1m^3} = 41.67kg/s$$

C_{pc} of water = 4.19x10^3 J/kg °C

C_{ph} assume to be equal to C_{pc}

Therefore heat transfer can be calculated as follow;

$$Q\hat{} = M\hat{}_h C_{ph}(T_{hi} - T_{ho}) = 47x4.19x10^3 x(40-25) = 2954 \text{ kW}$$

Final temperature of cooling water;

$$T_{co} = T_{ci} + \frac{Q\hat{}}{M\hat{}_c C_{pc}} = 10 + \frac{2.95x10^6}{47x4.19x10^3} = 24.98^0 C$$

From $Q\hat{} = UA\Delta T_m$ and $\Delta T_m = F_t x \Delta T_{lm}$

Where U = heat transfer coefficient, W/m^2 °C, assume to be 1,400 W/m^2 °C

A = heat-transfer area, m^2

ΔT_m = the mean temperature difference, °C

$$\Delta T_{lm} = (40\text{-}24.98)\text{-}(25\text{-}10)/\ln(40\text{-}24.98/25\text{-}10) = 15.04$$

R= (40-25)/ (24.98-10) = 1.00, S = (24.98-10)/ (40-10) = 0.50; then F_t obtained from graph in Figure 12.19, page 657 (Chemical Engineering Vol. 6, 3rd by Coulson *et al.*, 1999) 0.88

$$A = \frac{2954x10^3}{1400x0.81x15.04} = 173.2 \text{ m}^2$$

Purchased cost can be calculated as = bare cost from Figure 6.3a, page 253 (Chemical Engineering Vol. 6, 3rd by Coulson *et al.*, 1999) x Type factor x Pressure factor

Selected materials for shell is carbon steel and tube is stainless steel, Bare cost = £65,000, Type factor= fixed tube sheet = 0.8, Pressure factor in range 1-10 bar = 1.0.

Then cost = 65,000x0.8x1 = £52,000

Section 4: Milk reconstitution

1. Sizing of mixing tank with water (for 9000 l)

Type of tank: vertical cylindrical tank with flat base and six flat blade turbines

Material: stainless steel Type 304

Volume of liquid (V_L): 9 m^3

Volume of tank = volume of cylinder

Volume of cylinder = $\left(\frac{\pi d^2 L}{4}\right) = 9\ m^3$

then

$$d = L = 2.25$$

Top space = 0.3 V_L

then $\left(\frac{\pi d^2 L_p}{4}\right) = 0.3(9)\ m^3$

$$L_p = 0.68m$$

Total height of the mixing tank= $L + Lp = 2.93$ m

Diameter of the impeller = $d_i = \frac{d_t}{3} = \frac{2.25}{3} = 0.75$ m

The height of impeller from the base = diameter of the impeller = 0.75 m

Width of baffle = $W_b = \frac{d_t}{10} = \frac{2.25}{10} = 0.23$ m

Power requirement for mixing

$$P = N'_p \rho N_i^3 D_i^5$$

when N'p = constant value of the power number in the turbulent regime = 5

ρ = fluid density = 1000 kg/m^3

N_i = stirrer speed; assume to be 4 s^{-1}

D_i = impeller diameter = 0.75m

Then $P = (5) \times 1000 \times (4)^3 \times (0.75)^5 = 75938\ kg.m^2s^{-3} = 75.9$ kW

Total volume of tank = 9 m^3

Thus, the purchase cost of mixing tank with water

$= C S^n \times 2.5 = 1400 \times (9)^{0.55} \times 2.5 =$£11,719.29

2. Sizing of mixing tank after reconstitution (for 10000 l)

Type of tank: vertical cylindrical tank with flat base and six flat blade turbines

Material: stainless steel Type 304

Volume of liquid (V_L): 10 m^3

Volume of tank = volume of cylinder

Volume of cylinder = $\left(\frac{\pi d^2 L}{4}\right) = 10\text{m}^3$

then

$$d = L = 2.33$$

Top space = 0.3 V_L

then $\left(\frac{\pi d^2 L_p}{4}\right) = 0.3(10)\ \text{m}^3$

$$L_p = 0.7\ \text{m}$$

Total height of the mixing tank= $L + Lp = 3.03$ m

Diameter of the impeller = $d_i = \frac{d_t}{3} = \frac{2.33}{3} = 0.78$ m

The height of impeller from the base = diameter of the impeller = 0.78m

Width of baffle = $W_b = \frac{d_t}{10} = \frac{2.33}{10} = 0.23$ m

Power requirement for mixing

$$P = N'_p \rho N_i^3 D_i^5$$

when N'p = constant value of the power number in the turbulent regime = 5

ρ = fluid density = 1029 kg/m^3

N_i = stirrer speed; assume to be 4 s^{-1}

D_i = impeller diameter = 0.78m

Then P = (5) x 1029 x $(4)^3$x $(0.78)^5$ = 95069 kg.m^{2}s^{-3} = 95.1 kW

Total volume of tank = 10 m^3

Thus, the purchase cost of mixing tank after reconstitution

= $C S^n \times 2.5$ = 1400 x $(10)^{0.55}$x2.5 =£12,418.47

3.2 Conclusion in sizing, specifying and costing equipments

Storage tank

The storage of liquid is universally used vertical cylindrical tanks with flat base and conical roof and kept at atmospheric pressure. To ease operating for sterilization, cleaning and preventing from rust, the stainless steel Type 304 is selected as the material for the storage tank. Consideration of tank thickness is necessary to ensure that the storage tank is sufficiently safe to withstand the loads. The minimum thickness of the tank wall must be resisting the hydrostatic pressure. The minimum thickness of different diameter tanks

including a corrosion allowance is concluded in page 811 in Chemical Engineering Vol. 6, 3rd by Coulson *et al.*, 1999.

The geometry of the storage tanks will be as follow:

Storage tank for permeate UF solution in step (1)

Volume of the tank = 14.63 m^3

Volumetric liquid hold up in the storage tank = 9 m^3

Diameter of the storage tank = 2.25 m

Liquid depth = 2.25 m

Height of the storage tank = 2.93 m

Minimum wall thickness = 9 mm

Cost of storage tank = £15,309.20

Storage tank for enzyme permeate from MF system in step (2)

Volume of the tank = 0.4 m^3

Volumetric liquid hold up in the storage tank = 0.25 m^3

Diameter of the storage tank = 0.68 m

Liquid depth = 0.68 m

Height of the storage tank = 0.89 m

Minimum wall thickness = 5 mm

Cost of storage tank = £2,114.47

Storage tank of product in step (3)

Volume of the tank = 16.23 m^3

Volumetric liquid hold up in the storage tank = 10 m^3

Diameter of the storage tank = 2.33 m

Liquid depth = 2.33 m

Height of the storage tank = 3.03 m

Minimum wall thickness = 9 mm

Cost of storage tank = £16,208.52

Feed tank

The feed tanks are operated with membrane units. The tank is designed in vertical cylindrical shape with conical base and flat roof. The material use for the tank is made of stainless steel Type 304. The minimum thickness of tank is mentioned in page 811 in Chemical Engineering Vol. 6, 3rd by Coulson *et al.*, 1999.

The geometry of feeding tanks will be as follow:

Feed tank for skimmed milk in step (1)

Volume of the tank = 13.31 m^3

Volumetric liquid hold up in the tank = 10 m^3

Diameter of the tank = 2.33 m

Liquid depth = 3.9 m
Height of the tank = 4.66 m
Minimum wall thickness = 9 mm
Cost of feed tank = £17,132.27

Feed tank for washing cells using MF system in step (2)

Volume of the tank = 3.33 m^3
Volumetric liquid hold up in the tank = 2.5 m^3
Diameter of the tank = 1.47 m
Liquid depth = 2.45 m
Height of the tank = 2.94 m
Minimum wall thickness = 12 mm
Cost of feed tank = £7,460.61

Feed tank for enzyme separation from cell debris using MF system in step (2)

Volume of the tank = 0.33 m^3
Volumetric liquid hold up in the tank = 0.25 m^3
Diameter of the tank = 0.68 m
Liquid depth = 1.15 m
Height of the tank = 1.36 m
Minimum wall thickness = 5 mm
Cost of feed tank = £1,863.87

Reactor for MBR system

Bubble column reactor is chosen for the MBR system since the shear level is significantly lower than in stirred vessels and sterile nitrogen gas is sparged and recycled into the reactor to assist the mixing and stabilize the enzyme. The structure of reactor is generally cylindrical vessel with height greater than twice the diameter. The volumetric liquid hold up in the tank is about 2.5 m^3. The material use for reactor construction in this scale should be made of corrosion-resistant stainless steel for facility in cleaning and sterilisation. The headplate equipped with ports such as feed port, temperature sensor and gas is also made of stainless steel. The geometry of reactor will be as follow:

Volume of the reactor = 3.25 m^3
Volumetric liquid hold up in the reactor = 2.5 m^3
Diameter of the reactor = 1.02 m
Liquid depth = 3.06 m
Height of the reactor = 3.97 m
Cost of storage tank = £37,254.29

Mixing equipment

Two mixing tank with volume capacity 0.9 m^3 (for product dilution with water) and 1 m^3 (for reconstitution) are considered. The material of the construction for the mixing tank is stainless steel type 304. The shape of tank is vertical cylindrical tank with flat base. The impeller and baffles are mounted in the tank. The ratio of tank diameter to impeller diameter is normally about 3:1. The type of impeller use depends on viscosity of the liquid and sensitivity of the system to mechanical shear. Flat-blade turbine, known as the Rushton turbine, is suitable for low-to-medium viscosity liquid and usually operated at high rotational speeds. The flow pattern of this impeller is radial-flow characteristic. The baffles are installed to reduce vortexing and swirling of the liquid. Four equally-spaced baffles with the width 1/10-1/12 of the tank diameter are usually sufficient.

The geometry of the mixing equipment will be as follow:

Mixing equipment for product dilution with water in step (4)
Volumetric liquid hold up in the tank = 9 m^3
Diameter of the tank= 2.25m
Liquid depth = 2.25m
Height of tank = 2.93m
Diameter of the impeller = 0.75m
Height of impeller from the base= 0.75m
Width of the baffle = 0.23m
Power motor = 75.9 kW
Liquid density = 1000 kg/m^3
Cost of mixing tank = £11,719.29
Mixing equipment for reconstitution in step (4)
Volumetric liquid hold up in the tank = 10 m^3
Diameter of the tank= 2.33m
Liquid depth = 2.33m
Height of tank = 3.03m
Diameter of the impeller = 0.78m
Height of impeller from the base= 0.78m
Width of the baffle = 0.23m
Power motor = 95.1 kW
Liquid density = 1029 kg/m^3
Cost of mixing tank = £12,418.47

Membrane

The selection of membrane for the proposed process is considered on the basis of experimental data. The characteristic of membrane used in each step will be as follow:

Membrane system for milk separation in step(1) and lactose hydrolysis in MBR in step (3)

Type: UF membrane in hollow fibre configuration with 50 kDa MWCO

Surface area required = 180 m^2

Use hollow fibre UF membrane (Koch membrane manufacturer) dimension = 6″x72″ (0.15m x 1.83m) and surface area = 12.7 m^2 for one module
Diameter of fibre inside membrane = 2 mm
Number of hollow fibre ultrafiltration module = 15
Cost of membrane per surface area is about £200
Total membrane area= 12.7x15 =190.5 m^2
Then cost of UF membrane for both step = 200x381 = £76,200

Membrane system for washing cells and enzyme separation in step (2)

Type: MF membrane in hollow fibre configuration with pore size 0.2 micron

- *For washing cells*

Surface area required = 45 m^2
Use hollow fibre MF membrane (Koch membrane manufacturer) dimension = 6″ 2/3x43″ (0.17m x 1.09m) and surface area = 9.8 m^2 for one module.
Diameter of fibre inside membrane = 1.33 mm
Number of hollow fibre microfiltration module = 5
Cost of membrane per surface area is about £200
Total membrane area= 9.8x5 = 49 m^2
Then cost of MF membrane = 200x49 = £9,800

- *For enzyme separation*

Surface area required = 4.5 m^2
Use hollow fibre MF membrane (Koch membrane manufacturer) dimension = 6″x43″ (0.15m x 1.09m) and surface area = 6.3 m^2 for one module.
Diameter of fibre inside membrane = 1.33 mm
Number of hollow fibre microfiltration module = 1
Total membrane area= 6.3x1 = 6.3 m^2
Then cost of UF membrane for both step = 200x6.3 = £1,260

Pump

The liquid is pumped from tank through pipes and membrane system and circulated back to the tank by centrifugal pump. Generally, the energy required by pump depends on the height of the liquid through the system, pressure used for delivery, the length and diameter of pipe, flow rate and physical properties of liquid. The velocity of liquid in pipe is typically about 1-3 m/s and allowable pressure drop is 0.5 kPa/m. In this case, the power required to drive liquid through the pipes is considered to be less than power used to pump liquid pass through the membrane system.

Pump for 10000 l milk separation in step (1)

Fluid density = 1029 kg/m^3
Fluid viscosity = $2.1x10^{-3}$ Ns/m^2
Flow rate of fluid = 169.2 m^3/h
Pressure drop in the system = 301,291 N/m^2
Power required for pumping = 21.8 kW
According to above information, pump series SHE 80-200/220 (from Lowara pump) is selected. Its specifications are maximum delivery up to 210 m^3/h, Motor: 400v 3ph 50Hz 22kW 2900rpm and the cost is £3,188.01

Pump for washing 2500 l cell suspension in MF system in step (2)

Fluid density = 1040 kg/m^3
Fluid viscosity = $1.2x10^{-3}$ Ns/m^2
Flow rate of fluid = 48.2 m^3/h
Pressure drop in the system = 362,752 N/m^2
Power required for pumping = 7.48 kW
According to above information, pump series SHE 50-160/75 (from Lowara pump) is selected. Its specifications are maximum delivery up to 84 m^3/h, Motor: 400v 3ph 50Hz 7.5kW 2900rpm and the cost is £1,378.28

Pump for 250 l enzyme separation in MF system in step (2)

Fluid density = 1000 kg/m^3
Fluid viscosity = $1x10^{-3}$ Ns/m^2
Flow rate of fluid = 4.8 m^3/h
Pressure drop in the system = 348,800 N/m^2
Power required for pumping = 0.72 kW
According to above information, pump series SHE 32-125/07/A (from Lowara pump) is selected. Its specifications are maximum delivery up to 18 m^3/h, Motor: 400v 3ph 50Hz 0.75kW 2900rpm and the cost is £683.15

Pump for MBR system in step (3)

Fluid density = 1000 kg/m^3

Fluid viscosity = $1x10^{-3}$ Ns/m^2

Flow rate of fluid = 169.2 m^3/h

Pressure drop in the system = 142,740 N/m^2

Power required for pumping = 10.32 kW

According to above information, pump series SHE 80-160/110 (from Lowara pump) is selected. Its specifications are maximum delivery up to 180 m^3/h, Motor: 400v 3ph 50Hz 11kW 2900rpm and the cost is £2,064.42

Heat exchanger

Shell and tube heat exchanger is designed for the process. The material for shell is made of carbon steel and tube is from stainless steel. Operating pressure in system is in range 1-10 bar. Then the pressure factor is equal to 1. The cost of heat exchanger is estimated from Figure 6.3a, page 253 (Chemical Engineering Vol. 6, 3rd by Coulson *et al.,* 1999). The inlet cooling water temperature is assumed to be 10 °C and heat transfer coefficient is about 1400 W/m^2 °C. The temperature of fluid during operating in the system is maintained at 25 °C.

Heat exchanger for milk separation in step (1)

Fluid enters the exchanger at a flow rate = 169.2 m^3

Flow rate of cooling water = 150 m^3/h

Rate of heat transfer = 2777 kW

Final temperature of cooling water = 25.9 °C

Heat transfer area = 155.24 m^2

Cost of heat exchanger = £48,000

Heat exchanger for washing cells in step (2)

Fluid enters the exchanger at a flow rate = 48.2 m^3

Flow rate of cooling water = 60 m^3/h

Rate of heat transfer = 1122 kW

Final temperature of cooling water = 26.03 °C

Heat transfer area = 63.64 m^2

Cost of heat exchanger = £25,600

Heat exchanger for enzyme separation in step (2)

Fluid enters the exchanger at a flow rate = 4.8 m^3

Flow rate of cooling water = 10 m^3/h

Rate of heat transfer = 83.6 kW

Final temperature of cooling water = 17.18 °C

Heat transfer area = 3.38 m^2

Cost of heat exchanger = £3,200

Heat exchanger for MBR system in step (3)

Fluid enters the exchanger at a flow rate = 169.2 m^3

Flow rate of cooling water = 150 m^3/h

Rate of heat transfer = 2954 kW

Final temperature of cooling water = 24.98 °C

Heat transfer area = 173.2 m^2

Cost of heat exchanger = £52,000

Table A3.1 *The cost of major equipment items for process production.*

Equipment items	Cost (pounds)
Section1:Milk separation	
feed tank 10,000 l	17,132.27
Storage tank for permeate UF solution	15,309.20
pump for milk separation	3,188.01
Heat exchanger	48,000
Ultrafiltration membrane	38,100
Section2:Enzyme preparation	
Fermentor	75,000
feed tank for washing cells in MF system 2500 l	7,460.61
pump for washing cells	1,378.28
Microfiltration membrane for washing cells	9,800
Heat exchanger	25,600
Cell disrupter	20,000
feed tank for enzyme separation from cell debris in MF system	1,863.87
pump for enzyme separation	683.15
Microfiltration membrane for enzyme separation	1,260
Heat exchanger	3,200
storage tank for enzyme permeate from MF system	2,114.47
Section3:Lactose hydrolysis	
Enzyme reactor	37,254.29
storage tank for product permeate from MBR system	16,208.52
Ultrafiltration membrane	38,100
pump for MBR system	2,064.42
Heat exchanger	52,000
Section4:Milk reconstitution	
mixing tank with water	11,719.29
mixing tank after reconstitution	12,418.47
Total equipment costs (£)	439,854.85

Table A3.2 *Summary of variable costs*

Variable costs	**unit cost**	**amount use per year**	**cost per year**
Raw materials			
Yeast extract	5000 £/t	9	45,000
Soy peptone	2000£/t	9	18,000
NaOH (50% solution)	120 £/t	40	4,800
		Total cost (£/year)	*67,800*
Miscellaneous material			*1,084.24*
Utilities			
Steam	7 £/t	13,500	94,500
Cooling water	0.015£/t	75,000	1,125
Process water	0.6 £/t	82,500	49,500
Electricity	0.012£/MJ	516,510	6,713.28
		Total cost (£/year)	*151,838.28*
Total variable costs(£/year)			*220,722.52*

Table A3.3 *Summary of fixed costs*

Fixed costs	Cost (pounds)
Maintenance	108,424.2
Operating labour	75,000
Laboratory costs	15,000
Supervision	15,000
Plant overheads	37,500
Capital charges	325,272.7
Insurance	21,684.8
Local taxes	43,369.7
Royalties	21,684.8
total fixed costs (£)	662,936.2