STUDIES ON THE IMMOBILIZED KLUYVEROMYCES MARXIANUS FOR LACTOSE HYDROLYSIS

A thesis submitted towards the partial fulfilment of the requirements for the degree of Master of Technology in Food Technology and Biochemical Engineering course affiliated to Faculty of Engineering and Technology, Jadavpur University

Submitted by
Urmi Sarkar
EXAMINATION ROLL NO.: M4FTB22001
REGISTRATION NO.: 156622 of 2020-21

Under the guidance of
Dr. Debabrata Bera
Associate Professor, Department of Food Technology and Biochemical Engineering
Faculty of Engineering and Technology
Jadavpur University
Kolkata – 700032, India
2022

Department of Food Technology and Biochemical Engineering
Faculty of Engineering and Technology
Jadavpur University
Kolkata - 700032

CERTIFICATE OF RECOMMENDATION

I hereby recommend the thesis entitled "***STUDIES ON THE IMMOBILIZED KLUYVEROMYCES MARXIANUS FOR LACTOSE HYDROLYSIS***" carried out under my supervision by Urmi Sarkar of Registration No. 156622 of 2020-21. The thesis has been evaluated by me and found satisfactory. It is therefore, being accepted in partial fulfilment of the requirement for awarding the degree of Master of Technology in Food Technology and Biochemical Engineering course affiliated to Faculty of Engineering and Technology, Jadavpur University.

Dean
Faculty of Engineering and Technology
Jadavpur University

Prof. Prashanta Kumar Biswas
HOD
Department of Food Technology and Biochemical Engineering
Jadavpur University

Dr. Debabrata Bera
(Thesis Supervisor)
Associate Professor
Department of Food Technology and Biochemical Engineering
Jadavpur University

Department of Food Technology and Biochemical Engineering
Faculty of Engineering and Technology
Jadavpur University
Kolkata - 700032

CERTIFICATE OF APPROVAL

The foregoing thesis is hereby approved as a creditable study in **Master of Technology in Food Technology and Biochemical Engineering** and presented in a manner satisfactory to warrant its acceptance as a prerequisite to the degree for which it has been submitted. It is understood that by this approval the undersigned do not necessarily endorse or approve any statement made, opinion expressed or conclusion drawn therein but approve the thesis only for the purpose for which it is submitted.

Dr. Debabrata Bera

(Thesis Supervisor)

Associate Professor

Department of Food Technology

and Biochemical Engineering

Jadavpur University

Department of Food Technology and Biochemical Engineering
Faculty of Engineering and Technology
Jadavpur University
Kolkata - 700032

DECLARATION OF ORIGINALITY AND COMPLIANCE OF ACADEMIC ETHICS

I hereby declare that this thesis contains literature survey and original research work by the undersigned candidate, as part of my Master of Technology in Food Technology and Biochemical Engineering.

All information in this document have been obtained and presented in accordance with academic rules and ethical conduct.

I also declare that, as required by these rules and conduct, I have fully cited and referred all material and results that are not original to this work.

Name: Urmi Sarkar

Examination Roll No.: M4FTB22001

Thesis Title: "*STUDIES ON THE IMMOBILIZED KLUYVEROMYCES MARXIANUS FOR LACTOSE HYDROLYSIS"*

Signature with date:

URMI SARKAR

ACKNOWLEDGEMENT

I owe a deep sense of gratitude to my respected thesis supervisor Dr. Debabrata Bera, Associate Professor, Department of Food Technology and Biochemical Engineering, Jadavpur University, for his esteemed guidance, valuable suggestions, constant encouragement and affection at every stage of the entire tenure of the project without which I could not have finished the work. It has been my proud privilege to work under his guidance.

I would also like to express deep felt gratefulness to our Head of Department, Prof. Prashanta Kumar Biswas for giving me this opportunity to work in this project.

I would like to thank my senior Ms. Sreemoyee Chakraborty for her kind help, guidance and support throughout my work. I would also like to thank all the non-teaching staff, all my batch mates, and lab mates for their kind cooperation.

Last, but not the least, I wish to express my profound gratitude and my deep feelings for my family who have been the constant source of my energy, inspiration and determination for going ahead with my academic pursuit.

ABSTRACT

Kluyveromyces marxianus was immobilized in calcium alginate beads for studying the hydrolysis of lactose using intracellular β – galactosidase produced by the microorganism. The effect of alginate concentration, bead diameter, pH, temperature, substrate concentration and biomass concnetration on the percentage of lactose conversion was studied. Alginate concentration was found to be inversely proportional to lactose conversion. Highest lactose conversion was observed at 3% (w/v) sodium alginate concentration. Lactose conversion increased with decreasing bead size and the highest conversion was observed at 2.94 mm. Lactose conversion was highest at pH 4.4 and 25^0c temperature. It was observed that with increasing substrate concentration, conversion of lactose first increased but then decreased and reached a maximum value indicating the behaviour of enzymatic reaction rate according to Michaelis-Menten kinetics. With increasing biomass concentration, lactose conversion first increased and then decreased. Highest lactose conversion was observed at 188.89 mg/ml biomass concentration. A packed bed column was operated under different volumetric flow rates using the immobilized cell system and 4% (w/v) lactose solution as the substrate. An external mass transfer model was predicted for the packed bed using the colburn factor $J_D = KRe^{-(1-n)}$ and considering first order reaction rate kinetics. The mass transfer model $J_D = 0.06362\ (Re)^{-0.02}$ was found to correlate with the actual experiment. The model was valid for Reynold's number ranging from 0.09 to 0.4 for this experiment. It was observed that with increasing flow rate, the rate of external mass transfer also increased. Damkohler number (Da) was calculated and it was observed that for all flow rates it was less than 1. It was deduced that the diffusion rate was much greater and thus the overall reaction was limited by the enzymatic reaction. Pressure drop across packed bed operating at different volumetric flow rates was calculated using the Ergun equation. It was observed that with increasing flow rate the pressure drop across the packed bed also increased. Pressure drop across the packed bed for volumetric flow rates 0.90 ml/min, 1.71 ml/min, and 3.30 ml/min were 7.40 Pa, 14.08 Pa, and 27.23 Pa respectively. Friction factor for different flow rates were calculated. It was observed that the friction factor decreased with increasing flow rate. A graph of log f_P vs log Re_P was plotted and a straight-line curve with a negative slope and R^2 square value equal to 1 was observed. The graph represented that the system existed in the laminar flow regime, which correlated with the experimental data.

TABLE OF CONTENTS

LIST OF ABBREVIATIONS

EC – Enzyme Commission

SH – Sulfhydryl group

O_2 – Oxygen

S^- - Sulphur anion

OH^- - Hydroxyl ion

% - Percentage

w/v – Weight per volume

BOD – Biological oxygen demand

COD – Chemical oxygen demand

ppm – parts per million

µm – Micrometer

GI – Glycemic index

USD – United States Dollar

US – United States

^{0}c – Degree centigrade

LAB – Lactic acid bacteria

GRAS – Generally recognized as safe

mM – Millimole per litre

M – Molarity

ml – Millilitre

min – Minute

hr – hour

K_m – Michaelis-Menten constant

V_{max} – Maximum velocity of an enzymatic reaction

ml/hr – Millilitre/hour

w/w – Weight per weight

g/L – Gram per litre

g – Gram

psig – Pound per square inch (guage)

rpm – Revolutions per minute

mm – Millimetre

mg/ml – Milligram per millilitre

cm – Centimetre

nm – Nanometer

UV – Ultraviolet

Vis – Visible

Conc. – Concentration

eqn. – Equation

Pa – Pascal

LIST OF FIGURES AND TABLES

Chapter 1
Introduction and Literature review

1.1. Introduction

β – galactosidase (EC. 3.2.1.23) or beta – D – galactoside galactohydrolase is a hydrolytic enzyme that hydrolyzes the β – 1,4 glycosidic linkage present in milk sugar lactose, and produces glucose and galactose. It is commonly known as lactase. It also catalyzes transgalactosylation reactions producing galactooligosaccharides (GOS) from lactose. Hydrolysis of lactose enables it to be applied for the production of lactose-free milk and dairy products, which are suitable for people suffering from lactose malabsorption and intolerance. Lactose is highly hygroscopic, less soluble, and less sweet than its monomeric sugars, glucose, and galactose. Lactose is easily crystallized and is responsible for the sandy and gritty texture in ice cream, frozen milks, and frozen desserts. Furthermore, lactose is a serious environmental pollutant owing to its high biological and chemical oxygen demand values. Hydrolysis of lactose improves its sweetness, solubility, and reduces the problems of crystallization in frozen milk products. Lactose hydrolysis enables the conversion of whey into valuable products, thereby overcoming the problems of whey disposal (Shukla & Wierzbicki, 1975).

Lactose can be hydrolyzed either chemically or enzymatically. Chemical hydrolysis of lactose is done using either cation exchange resins or mineral acids. Chemical hydrolysis is carried out under severe reaction conditions (temperature – 100 to 150^0c and pH – 1.0 – 2.0). Temperatures in the range of 50 – 70^0c require pH < 1.0 for efficient hydrolysis. Chemical hydrolysis is cheaper, rapid, and simple but comes with some undesirable features. Some of the disadvantages of chemical lactose hydrolysis include – (1) milk protein denaturation due to severe reaction conditions, (2) reaction between amino acids from protein hydrolysis and sugars from lactose hydrolysis, resulting in Maillard browning; which requires an additional decolourization step, (3) salts present in whey can cause deactivation of acids, thereby requiring a demineralization step, (4) expensive plant setup to prevent corrosive conditions, (5) undesirable by-product formation. Enzymatic hydrolysis is carried out using β – galactosidase enzyme as the catalyst. Enzymatic hydrolysis is preferred over chemical hydrolysis because undesirable by-product formation is minimized due to mild reaction conditions. Furthermore, enzymes are highly specific and highly active at low concentrations (Abdelrahim, 1989).

The active site of the enzyme molecule has one sulfhydryl (-SH) group and one imidazole group. The hydrolysis reaction takes place via SN_2 mechanism. The imidazole group acts as a nucleophile and attacks the C1 carbon to remove the galactosyl group present in lactose. The – SH group acting as an acid, donates a proton to the galactosidic O_2 atom. An intermediate formation of a galactosyl – enzyme complex takes place. The S^- anion accepts a proton either from a water molecule (lactose hydrolysis) or from another mono or di – saccharide (transgalactosylation), assisting in the attack of OH^- at the C1 carbon; thus, forming the final products. The anomeric carbon does not undergo inversion at any step of the enzyme-catalyzed reaction, so the beta configuration of the products retains at the anomeric carbon (Shukla & Wierzbicki, 1975).

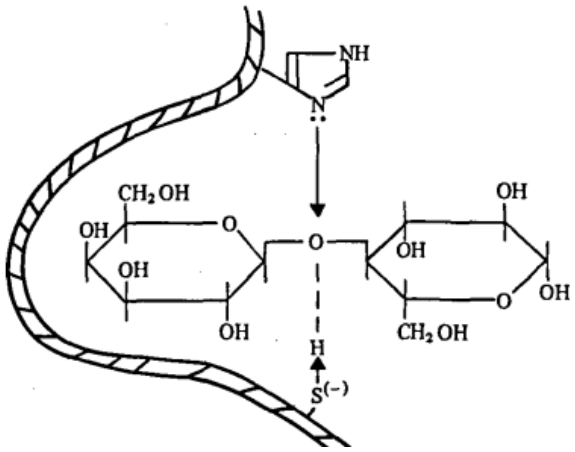


Figure 1 – Active site of β – galactosidase enzyme and lactose molecule

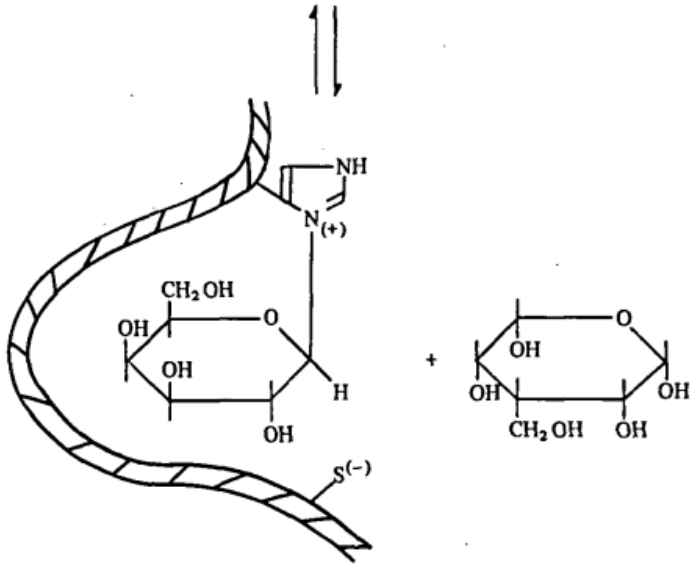


Figure 2 – β – galactosidase – galactose complex and glucose

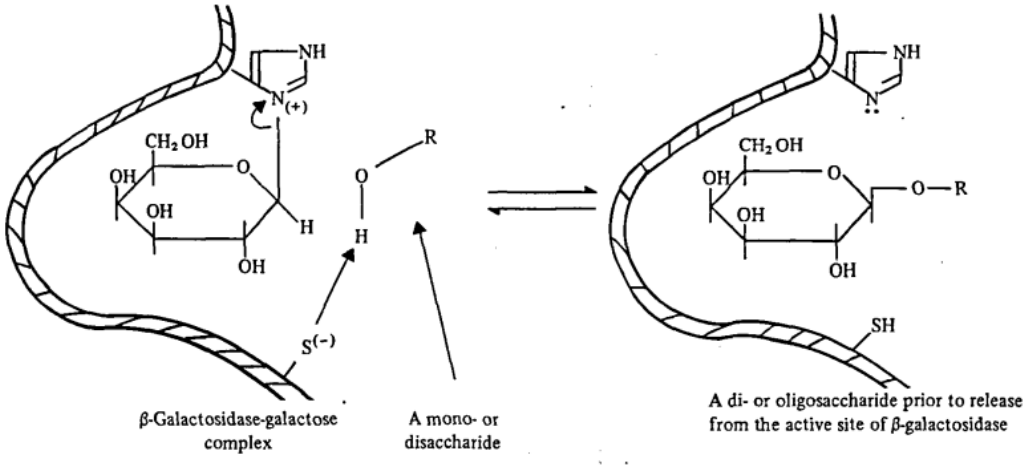


Figure 3 – Transfer of galactosyl group from the active site of β – galactosidase

1.2. Significance of β – galactosidase

The main carbohydrate present in milk and whey is lactose. It is made up of two sugars, glucose and galactose, joined by a β - 1,4 glycosidic linkage. Lactose content in mammalian milk ranges from 3-8% (w/v) whereas 70-80% in cheese whey. Usage of lactose is limited owing to its high hygroscopic nature, less sweetness, lower solubility, poor digestibility, easy crystallization, and high biological oxygen demand (Anisha, 2017).

Around 75% of the world population suffers from lactose malabsorption where lactose remains undigested and passes to the large intestine, and finally urine due to either deficiency of lactase in the villi of the jejunum (middle portion of the small intestine) or its decreased activity. Lactose malabsorption leads to lactose intolerance characterized by osmotic diarrhea from tissue dehydration, poor absorption of calcium due to high acidity, fermentative diarrhea from microbial fermentation in the intestine, watery explosive diarrhea, cramps in the abdomen, bloated feeling, flatulence, and nausea (Misselwitz et al., 2013; Shukla & Wierzbicki, 1975). Studies have shown that the incidence of lactose intolerance in South Indians (66.6%) is much more than that in North Indians (27.4%) (Tandon et al., 1981). Deficiency of lactase can be of three types – primary, secondary, and congenital. Decreased production of the enzyme along villi of the small intestine is defined as primary lactase deficiency (common among 2-20 years of aged individuals). Secondary deficiency is defined as lactase deficiency due to certain intestinal pathologies. Congenital deficiency, although rare in newborns, is a genetic disorder resulting in a reduced amount or complete absence of the enzyme (Saqib et al., 2017). Thus, lactose hydrolyzed dairy products are suitable for individuals suffering from lactose malabsorption and intolerance.

Whey is a major by-product of the dairy industry consisting of whey protein, water-soluble vitamins and minerals, and lactose. Whey protein recovered by ultrafiltration is an important nutrient supplement in the food industry. However, the whey permeate consists of a huge amount of lactose. Due to a high BOD value (30,000 – 50,000 ppm) and a COD value (60,000 – 80,000 ppm), whey streams are toxic to the environment. When whey is disposed into streams and lakes, it favours the growth of bacteria and algae; which in turn uses up the dissolved oxygen, thereby creating an anaerobic condition that has an adverse effect on the aquatic life. With cheese production increasing gradually, yearly whey production is approximately more than 145 million tons (6 million tons of lactose). Thus, whey disposal is a major concern due to its high volume and high organic matter (Das et al., 2015). In 2013, the global production of whey was 180 million tonnes of which 40% is either used as feed/ fertilizer or disposed of as waste (Vera et al., 2019). India produces 1.5 million tons of paneer and 2 million tons of channa, with 75-85% of that being whey (Gupte & Nair, 2010). β-D-galactosidase can be used to overcome the difficulties of whey disposal. Hydrolysis of whey lactose (about 4.5-5%) can convert it into a sweetener which can be further used in bakeries, confectioneries, beverages, and canned fruit syrups (Anisha, 2017). Hydrolysis of whey permeates (after removal of the protein) using β – galactosidase from *Pseudoalteromonas haloplanktis* for the production of D-tagatose, a natural healthy non-nutritive sweetener having the potential of being used in low GI food products has been reported (Van de Voorde

et al., 2014). Whey lactose can be utilized as feed for alcohol fermentation using yeast. β-D-galactosidase from Kluyveromyces marxianus has been used to produce ethanol from whey (Ariyanti & Hadiyanto, 2013).

Lactose crystallization becomes a problem in the dairy industry when lactose crystals exceed 30 μm in length. Lactose crystallization results in a sandy, gritty or mealy texture and deposit formation in dairy products like ice cream, frozen desserts, sweetened condensed milk, dried whey, condensed whey, unsweetened condensed milk, dry milk, and frozen milk. This problem can be overcome by adding lactose hydrolysis (Shukla & Wierzbicki, 1975). Skryplonek et al (2017) have reported a lactose-free frozen yogurt with enhanced sweetness and improved texture, viscosity, and brightness. Improved texture resulted from lactose hydrolysis to monosaccharides which reduces the freezing point of the mix. Increased acidity of lactose-free frozen yogurt indicates that lactose hydrolysis enhances the fermentation process.

In 2020, the global market size for lactose-free products was estimated to be USD 12.1 billion. The market is expected to grow and reach USD 18.4 billion by 2025 at a CAGR (compound annual growth rate) of 8.7%. Approximately 90% of adults in East Asia suffer from lactose intolerance. 30 million adults in the US start showing symptoms by the age of 20 years. In African Americans, the symptoms start at the age of 2 years. The increase in the market share for lactose hydrolyzed products has been attributed to the awareness of consumers about lactose intolerance. The same reason has been attributed to the growing demand for lactose–hydrolyzed products in the US, Europe, and the Asia Pacific region. The Asia Pacific region has been projected to hold the largest market share of lactose–free products with China being the dominant country. Lactose-free milk has been projected to hold the largest share in the lactose-free product market. Dairy products with reduced sugar or no added sugar have been projected to be the fastest growing segment among all other lactose-free products (Lactose-Free Products Market, 2020).

Lactose hydrolysis using β – galactosidase can solve many of the technological, health, and environment-related issues with lactose utilization. Lactase-treated milk can increase cheese ripening. Yogurt preparation using lactose hydrolyzed milk is faster and sweeter. Lactase-treated milk and milk products can be used by lactose-intolerant people. Lactase can solve problems of low solubility, less sweetness, and disposal of whey (Panesar et al., 2006). Due to consumer demand, the market share for lactose hydrolyzed products is increasing thereby encouraging the use of β – galactosidase enzyme.

1.3. Sources of β – galactosidase

β – galactosidase can be sourced from varied sources ranging from vegetables, fruits, seeds, and animals to microorganisms. Microorganisms are preferred over other sources due to their shorter generation time, high yield, diversity, ease of handling, cheap substrate, easy availability, and lower production costs. The application of microbial enzymes depends on the type of microbial source. β – galactosidase sourced from bacteria and fungi has optimum pH of 2.5 – 5.4,

making them suitable for acid whey and its permeate. Fungal β – galactosidases have high-temperature stability and can be used at around 50^0c. β – galactosidase sourced from yeasts has optimum pH of 6-7, making them suitable for sweet whey and milk (Panesar et al., 2006; Saqib et al., 2017).

1.3.1. Bacteria

β – galactosidases are extensively sourced from bacteria due to their high stability, ease of fermentation, and high activity. β – galactosidase from *E.coli* has been greatly studied but its usage in food is restricted due to the pathogenic nature of the coliform. LAB consists of a broad range of streptococci, lactococci, and lactobacilli. They are gaining importance as potential sources of β – galactosidase due to their generally recognized as safe (GRAS) status, use as probiotics, and people with lactose intolerance may consume fermented products with hardly any adverse effects. Due to their GRAS status, they do not need extensive purification. *Lactobacillus* sp. and *Bifidobacterium* sp. are the most frequently used probiotics (Anisha, 2017).

Probiotics like *Streptococcus thermophilus, Lactobacillus delbreuckii subsp. bulgaricus, Lactobacillus plantarum, Lactobacillus reuteri, Bifidobacterium longum, Bifidobacterium infantis, Pediococcus acidilactici,* etc. have been reported to produce β – galactosidase. Thermostable β – galactosidases have been reported from *Bacillus stearothermophilus, Thermus* sp., *and Pyrococcus woesei*. Psychrophilic bacteria like *Pseudoalteromonas halpolanktis* and *Arthrobacter psychrolactophilus* have been reported to produce β – galactosidase (Anisha, 2017).

1.3.2. Fungi

Fungal β – galactosidases are generally extracellular and more susceptible to product inhibition by galactose (competitive inhibition). *Aspergillus* sp. is widely used commercially for β – galactosidase production. Fungal sources like *Aspergillus niger, Aspergillus oryzae, Penicillium chrysogenum*, Penicillium expansum, Aspergillus aculeatus etc. have been reported (Husain, 2010).

1.3.3. Yeasts

Yeast β – galactosidases are generally intracellular and inhibited competitively by galactose and non-competitively by glucose (Panesar et al., 2006). *Kluyveromyces* sp. is an important and widely used commercial source for β – galactosidase. *Kluyveromyces lactis* is the most important commercial yeast source as it has a natural dairy habitat. Other important sources include *Kluyveromyces fragilis, Saccharomyces lactis, Kluyveromyces marxianus, Saccharomyces anamensis,* etc. (Husain, 2010; Panesar et al., 2006).

1.3.4. Plants

β – galactosidase is present in the tissues of many fruits and vegetables like tomatoes, persimmons, papaya, strawberries, apples, kidney beans, cowpea, almonds, carrots, radish, peas, (Husain, 2010), coffee, muskmelon, mango, etc. (Saqib et al., 2017).

1.3.5. Animals

β – galactosidase is present in the gut, brain and skin tissue of animals (Shukla & Wierzbicki, 1975).

Table 1 – Some microbial sources producing β - galactosidase

Type of microbial source	***Source***	***Intracellular/ Extracellular***	***Optimum pH***	***Optimum temperature (^{0}c)***	***Reference***
Bacteria	*Streptococcus thermophilus*	Intracellular	7.2	40	Princely et al., 2013
	Lactobacillus reuteri	Intracellular	8.0	50	Nguyen et al., 2006
	Pediococcus acidilactici	Intracellular	6.0	50	Chanalia et al., 2018
	Lactobacillus acidophilus ATCC 4356	Intracellular	6.8	45	Simović et al., 2015
	Bifidobacterium longum BCRC 15708	Intracellular	7.0	37	Hsu et al., 2007; Hsu et al., 2005
Fungi	*Aspergillus nidulans*	Extracellular	5.0	60	Kamran et al., 2018
	Aspergillus oryzae	Extracellular	5.0	50-55	Park et al., 1979
	Aspergillus foetidus	Extracellular	3.5-4.0	60	Borglum & Sternberg, 1972
	Alternaria palmia	Intracellular	5.0-5.5	70	Agrawal et al., 1982
	Aspergillus niger	Extracellular	5.0	50	Martarello et al., 2019
Yeast	*Kluyveromyces fragilis*	Intracellular	6.6	37	Jurado et al., 2002
	Kluyveromyces lactis	Intracellular	6.9-7.3	35	Jurado et al., 2002
	Kluyvermyces marxianus NRRL Y-1196	Intracellular	7.0	50	Ku & Hang, 1992
	Guehomyces pullulans 17-1	Extracellular	4.0	50	Song et al., 2010
	Candida pseudotropicalis B57 and A60	Intracellular	7.5	30	Itoh et al., 1982

1.4. Potential applications of β – galactosidase

β– galactosidase is used in the food industry for producing both lactose hydrolyzed milk and dairy products and transgalactosylated products. Lactose concentration is the most significant variable for favoring either hydrolysis or transgalactosylation reaction. Low lactose concentrations (approx. 300 mM or lower) and high water activity is in favour of lactose hydrolysis. At high lactose concentrations (approx. 980 mM or higher) transgalactosylation is favored because a significant amount of lactose acts as a nucleophile acceptor producing galactosyl lactose. Thus, in milk or whey (lactose content of about 130mM) lactose hydrolysis is dominant over transgalactosylation (Vera et al., 2019).

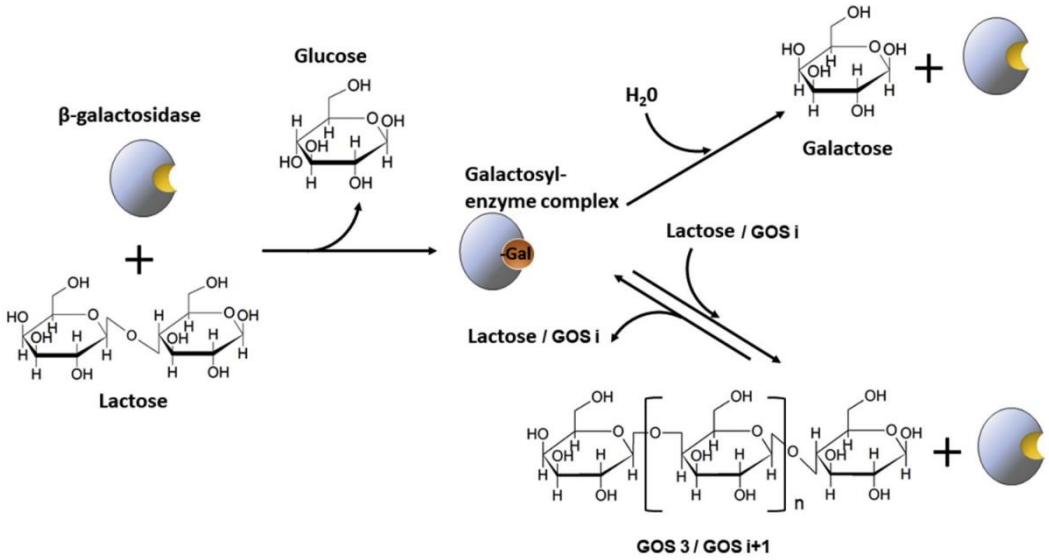


Figure 4 – Synthesis of Galactooligosaccharides at high lactose concentration

From a commercial point of view, immobilized enzymes are preferred over free enzymes for the hydrolysis of lactose due to several advantages. Better stability of enzymes during operations and storage, reusability of enzymes, controlled formation of end products, rapid termination of reactions, easy separation of enzymes from the reaction mixture, and easy adaptability to various engineering designs are some of the benefits that are offered by immobilized enzyme systems. Usage of free enzymes is limited in industries due to problems of instability and loss of enzyme activity during operations and storage, and chances of contaminating the end products (less recovery). Hydrolysis of lactose can be done either in batch mode or continuous mode. Batch mode operations in stirred tank reactors are simple and easy to control but lead to high enzyme and labour costs. Continuous operations using immobilized enzymes offer higher yield and reusability of enzymes (Datta et al., 2013). Similar to enzyme immobilization, whole cells can also be immobilized. The benefit of using immobilized cells over immobilized enzymes is that they can undergo multi-step biosynthetic reactions requiring cofactor which is not possible in case of purified enzymes (Shuler & Kargi, 2001, p. 263).

Hydrolysis of milk and whey/ whey permeate lactose in bioreactors using immobilized β – galactosidase has been extensively studied because it is critical

for industrial production of lactose-free dairy products. Different reactors like membrane reactors, hollow fiber reactors, fluidized bed reactors, and packed bed reactors have been used for the hydrolysis of milk and whey lactose. Enzymes can be either immobilized on the surface or entrapped within the membrane or confined in a defined space by a selective membrane in membrane reactors. Free enzymes can also be used in solutions with the substrate. Thus, the native kinetic properties of the enzyme can be exploited using membrane reactors. Disadvantages of using membrane reactors include fouling of membranes (clogging of membrane pores) and high chances of microbial contamination due to prolonged usage at ambient temperatures. The use of hollow fiber reactors is gaining importance due to their high surface area to volume ratio and high density of enzymes that can fit in a small reactor volume. Fluidized bed reactors are useful when substrates are particulate or viscous as there is less pressure loss and uniform distribution of flow through the axial section of the reactor. Mechanical agitation is not required in these reactors and thus the immobilization support does not get damaged. Packed bed reactors are mostly suited for large-scale industrial lactose hydrolysis because of their less complexity and inexpensive equipment. Other advantages of using packed bed reactors include (i) reduced enzyme loss due to reduced collisions between agitator, enzyme particle, and liquid shearing and (ii) reduced enzyme inhibition due to small difference between product and substrate concentrations inside the reactor (Husain, 2010).

1.4.1. Hydrolysis of milk and whey

Immobilization of β – galactosidase (*Aspergillus oryzae*) in chitosan beads increased its optimal working pH range (6.5 – 7.3); whereas that of the free enzyme was found to be maximum at 7.3. The wide range of optimal pH for the immobilized enzyme was attributed to the fact that there was a pH shift in the microenvironment of the chitosan matrix. On the other hand, the K_m and V_{max} of the immobilized enzyme were 0.011 µmol ml^{-1} and 55.25 µmol ml^{-1} min^{-1} respectively; whereas that for the free enzyme were 0.0086 µmol ml^{-1} and 285.7µmol $ml^{-1}min^{-1}$ respectively. Increase in Km value and decrease in Vmax value for immobilized enzyme were attributed to mass transfer limitations, less accessibility of the enzyme active sites to the substrate, and inability of enzyme to undergo conformational changes due to entrapment inside the chitosan matrix (Wentworth et al., 2004).

Aspergillus oryzae β – galactosidase was immobilized on concanavalin A (Con A) as an inexpensive bio-affinity support. The adsorbed enzyme was then cross-linked using glutaraldehyde. The immobilized enzyme showed higher pH, temperature and storage stability as compared to free enzyme. The reusability of the immobilized enzyme was also studied. Immobilized enzyme retained 78% of its original activity at pH 3.0 as compared to 41% in case of the free enzyme. At 70^0c, immobilized enzyme showed 71% enzyme activity, whereas the free enzyme showed 35% enzyme activity. Galactose inhibition on the activity of the immobilized enzyme was less as compared to the free enzyme. Crosslinked Con A adsorbed enzyme retained 71% initial activity after 7^{th} repeated use. After 2 months of

storage, the crosslinked Con A adsorbed enzyme retained 78% activity while the free enzyme retained 40% activity. Hydrolysis of milk and whey lactose was carried out in batch process at 50^0c. The immobilized enzyme showed 63% milk lactose hydrolysis and 76% whey lactose hydrolysis after 6 hr. The free enzyme showed 51% milk lactose hydrolysis in 6 hr and 71% whey lactose hydrolysis in 9 hr. In continuous operation, in a packed bed reactor, 95% lactose hydrolysis with a flow rate of 20 ml hr^{-1}, after 10 days operation was achieved using the immobilized enzyme system (Ansari & Husain, 2012).

Permeabilized yeast cells (*Kluyveromyces marxianus*) were immobilized in calcium alginate beads and employed for the production of lactose–hydrolyzed skim milk. 87.9% of lactose reduction was observed after 2.5 hrs. The immobilized enzyme system could be reused for up to 8 cycles without any significant decrease in lactose reduction (Panesar et al., 2007). The immobilized cell system was further used for milk lactose hydrolysis in a packed bed reactor. The packed bed reactor was operated for 5 days at a flow rate of 7.0 ml hr^{-1}. 86.4% lactose hydrolysis was observed up to 54 hr operation. The lactose hydrolysis was reduced to 83.3% after 56 hr. After 3 days, the lactose hydrolysis decreased significantly. Lactose reduction decreased to 40.9% after 5 days. The beads remained stable during the 5-day operation (Panesar et al., 2011).

Thermal stability of fungal β – galactosidase immobilized in polyvinyl alcohol gel was greater than that of free enzyme. After 24 hr, 70% activity of the immobilized enzyme was retained at 50^0c and 5% activity was retained at 60^0c. On the other hand, the free enzyme lost all activity at 60^0c after 24 hr. A maximum of 75% lactose conversion was obtained after 5-6hr. Lactose conversion decreased to 50% after 30 runs (Batsalova et al., 1987).

β – galactosidase (Aspergillus oryzae) was immobilized in fibers made up of gelatin and alginate and cross-linked with glutaraldehyde. Stability of the immobilized enzyme was higher than that of the free enzyme at higher pH and higher temperature. At pH 9.0, the immobilized enzyme retained 25% of its initial activity; whereas 11.5% of the initial activity of free enzyme was retained. At 70^0c, the immobilized enzyme retained 27% of its activity; whereas the free enzyme lost all its activity. The immobilized enzyme remained active for 35 days without losing its activity (Tanriseven & Dog˘an, 2002).

Glutaraldehyde was used as a cross-linking agent in calcium alginate – gelatin spheres for the immobilization of commercial β – galactosidase (from *Kluyveromyces lactis*). It was observed that the lactose hydrolysis in cheese whey had decreased to 12% after 12 hrs due to glutaraldehyde. Lactose hydrolysis had increased to 56% with an increase in enzyme concentration and absence of glutaraldehyde. This was attributed to the fact that glutaraldehyde being highly reactive favours cross-linking

between enzyme and support; thereby distorting the active site. Concanavalin A was complexed with the enzyme before immobilization (to prevent leaching of the enzyme from the spheres) and the effect on hydrolysis was observed. With the use of concanavalin A complexed β – galactosidase in absence of glutaraldehyde as a cross-linking agent, maximum lactose hydrolysis of 72% had been obtained after 12 hr (Mörschbächer et al., 2016).

β- galactosidase from *Aspergillus oryzae* was covalently immobilized on carrageenan modified with chitosan. The immobilized enzyme showed wider pH and temperature stability. The optimum pH of the immobilized enzyme shifted from 4.5-5.0 to 5.0-5.5. The optimum temperature of the immobilized enzyme shifted from 50^0c to 45-55^0c. The immobilized enzyme system showed greater operational stability with 97% retention of original activity after 15 uses (Elnashar & Yassin, 2009).

Kluyveromyces lactis whole cells were immobilized in calcium alginate beads and used for milk lactose hydrolysis in a packed bed reactor. It was observed that permeabilization of yeast cells before immobilization increased lactose hydrolysis and ethanol fermentation was also prevented. 99.5% hydrolysis of milk whey lactose was observed at 30^0c for 30 hr (Becerra et al., 2001). *Kluyveromyces lactis* whole cells were immobilized on corn grits using covalent bonds and used for the hydrolysis of milk whey. The immobilized whole cell system lost 10% of its enzyme activity when stored in 15mM phosphate buffer (pH 4.0) at 4^0c. The activity of free cells was completely lost when stored under similar conditions. The lactose hydrolysis was more rapid when the yeast cells were permeabilized with 70% ethanol at 25^0c before immobilization. More than 90% hydrolysis was achieved in a packed reactor. Cell permeabilization also prevented ethanol fermentation, which is necessary in the production of sweet syrup or a microbial substrate from whey (Siso & Doval, 1994).

β – galactosidase from *Penicillium notatum* was immobilized on porous glass modified with glutaraldehyde. The immobilized enzyme showed wider pH and temperature stability as compared to the free enzyme. The optimum pH shifted from pH 4.0 to pH 5.0 for the immobilized enzyme. The optimum temperature shifted from 50^0c to 60^0c for the immobilized enzyme. A packed bed column packed with the immobilized enzyme system was used for the hydrolysis of whey permeate for the production of sweet syrup. It was observed that the immobilized enzyme could be utilized for four 48 hr cycles with an average hydrolysis of 84.5% after each 48 hr cycle. 8% reduction in hydrolysis was observed from 5^{th} to 7^{th} cycle. The hydrolysis was reduced to 65% after the tenth cycle (Szczodrak, 1999).

1.4.2. Galactooligosaccharides

Galactooligosaccharides (GOS) are complex carbohydrates that cannot be easily digested by human beings. Thus, they are used as prebiotics which

greatly stimulate the growth of *Bifidobacteria* and *Lactobacilli* (Davani-Davari et al., 2019). This is beneficial for human health because it activates the immune system against pathogens, reduces risk of colon cancer, reduces the levels of triglycerides and blood cholesterol, improves intestinal motility, and promotes the synthesis of vitamins. Due to their health-promoting properties, they are used as a functional ingredient in many foods. They can substitute synthetic human milk oligosaccharides (HMOs) in infant formulas, as HMOs are very expensive and challenging to produce. They are made up of 2-8 galactose units linked together through either β 1-4 or β 1-6 linkages, and with a terminal glucose unit. GOS can be enzymatically produced using either glycosyltransferases or β – galactosidases. Though glycosyltransferases are highly specific and more efficient catalysts for GOS synthesis than β – galactosidases, the enzymatic process is highly expensive due to the requirement of a nucleotide sugar as a substrate. On the contrary, β – galactosidases can be used to produce GOS from lactose under kinetically controlled conditions. Moreover, β – galactosidases are convenient, robust, cheaper, and frequently used in the food industry. Optimization of important operational parameters like substrate concentration, pH, temperature, water activity, enzyme source, enzyme load, type of immobilization etc. have been done to increase the GOS yield. GOS production using β – galactosidase is generally carried out under high temperatures because the solubility of lactose in water increases with increasing temperature. GOS production is favoured over hydrolysis reaction at high lactose concentrations. Since the yield of GOS is less using β – galactosidase, GOS production is carried out at the highest possible temperature, using supersaturated solutions of lactose. Immobilization of β – galactosidase improves its thermal stability. The immobilized enzyme can also be reused for long-term continuous operations and repeated reaction cycles. It also makes it easier to purify the final product (Vera et al., 2020).

Immobilized β – galactosidase from *Aspergillus oryzae* was used for lactose hydrolysis and production of GOS. It was observed that the amount and type of GOS were directly proportional to the initial lactose concentration. At 500 g/L initial lactose concentration, maximum GOS production (27% w/w of initial lactose) was observed at 50% lactose conversion. It was also observed that GOS production for both free and immobilized enzymes (immobilized on cotton cloth) was identical. However, the thermal stability of the enzyme on immobilization has improved. At 40^0c, the immobilized enzyme had a half-life of 1 year, and at 50^0c, it had a half-life of 48 days. Stable, continuous GOS production with immobilized enzyme was carried out in a packed bed reactor for 2 weeks with no problem. When the lactose concentration in the feed was increased from 200 to 400 g/L, GOS production also increased from 21 to 26% at 40^0c and pH 4.5. Reactor productivities were several times higher than previously reported values. It had been attributed to the fact that the immobilized enzyme could retain its enzyme activity due to larger porosity and larger surface area of the cotton cloth (Albayrak & Yang, 2002).

β – galactosidase immobilized on cotton cloth and free enzyme were used for the production of GOS. The degree of lactose conversion was affected by changes in the pH. It had been observed that the lactose conversion significantly decreased with an increase in pH from 4.5 to 7. However, the immobilized enzyme had shown greater stability within pH range 3 to 7. This enables the immobilized enzyme to be used in both acid and sweet whey. The immobilized enzyme retained 63% of its initial activity after 5 successive reaction cycles. Larger GOS (higher degree of polymerization) was produced less by using the immobilized enzyme system. This was attributed to the steric hindrance due to overlapping of enzymes immobilized on cotton, thereby preventing GOS extension. The yield of GOS using the free enzyme was 32.62% in 3.9 hr at 50^0c, and at an initial lactose concentration of 57.13%. The yield of GOS using the immobilized enzyme was 32.48% in 3.09 hr at 50^0c and an initial lactose concentration of 52.74% (Wang et al., 2021).

Commercial β – galactosidase from *Bacillus circulans* was immobilized on glyoxal agarose beads and used in a packed bed reactor for the continuous production of GOS. The immobilized enzyme retained almost 100% of its activity after 14 successive batch operation runs totalling 280 min at 45^0c. Maximum GOS production was obtained at 50% lactose conversion with an initial 100 g/L lactose concentration and at 45^0c for both free and immobilized enzyme. A continuous packed bed reactor was operated for 213 hr at 45^0c, 100 g/L substrate concentration and a flow rate of 0.2 ml/min. Maximum GOS production was observed at 48.6% lactose conversion similar to that obtained in batch operation. Milk was also used as a feed in the reactor. It was observed that after 8 hr operation, the flow rate decreased. This was attributed to the possible clogging of column and microbial contamination (Rodriguez-Colinas et al., 2016).

Commercial β – galactosidase from *Bacillus circulans* was immobilized via covalent linkage on porous acrylic beads. Half-life of the immobilized enzyme was 90 days at 50^0c. Immobilized enzyme was used in a packed bed reactor for the continuous production of GOS. The enzymatic productivity of GOS using the immobilized system in a packed bed reactor was six times higher than that of free enzyme in a batch reactor. For getting a volumetric productivity in a packed bed reactor similar to that of a batch reactor, the volume of the packed bed reactor was found to be much smaller than the batch reactor. This was found to depend on the dosage of enzyme and reaction time of one batch (Warmerdam et al., 2013).

β – galactosidase from *Aspergillus oryzae* was immobilized via cross-linking to modified glass beads. Immobilized enzyme showed higher temperature and pH stability than free enzyme. the optimum temperature of immobilized enzyme shifted from 50 to 60^0c. The immobilized enzyme retained 51% activity at 70^0c, whereas for the free enzyme it was 10%. Immobilized enzyme in a packed bed reactor was used for continuous production of GOS. The reusability was studied. It was observed that only after 8 reuses, 4.6% of GOS yield was lost. 39.3% of maximum GOS yield

was observed at 56.4% lactose conversion after 2nd cycle of continuous operation (Eskandarloo & Abbasporrad, 2018).

1.5. Methods of immobilization

Immobilization is the method of confining an enzyme or a microbial cell in a defined space. The advantages of using immobilized enzymes include good operational stability, reusability, easy product and enzyme recovery, easy control of reactions, easy terminations of reactions, and easy adaptability to various engineering configurations. Similarly, the advantages of using immobilized microbial cells include availability of high cell density, reutilization of cell system, easy recovery, greater operational stability, protection of cells against hydrodynamic shear and elimination of cell washout problems at high flow rates. Combination of high cell densities along with high flow rates gives higher volumetric productivities. The major disadvantage of using immobilized cells or enzyme system is the presence of diffusional resistance to the transport of substrates and products to the cell or enzyme. In case of immobilization of living cells, cell growth and evolution of gas may lead to mechanical disruption of the immobilized matrix. The benefit of using immobilized cells over immobilized enzymes is that they can undergo multi-step biosynthetic reactions requiring cofactors which is not possible in case of purified enzymes (Damin et al., 2021).

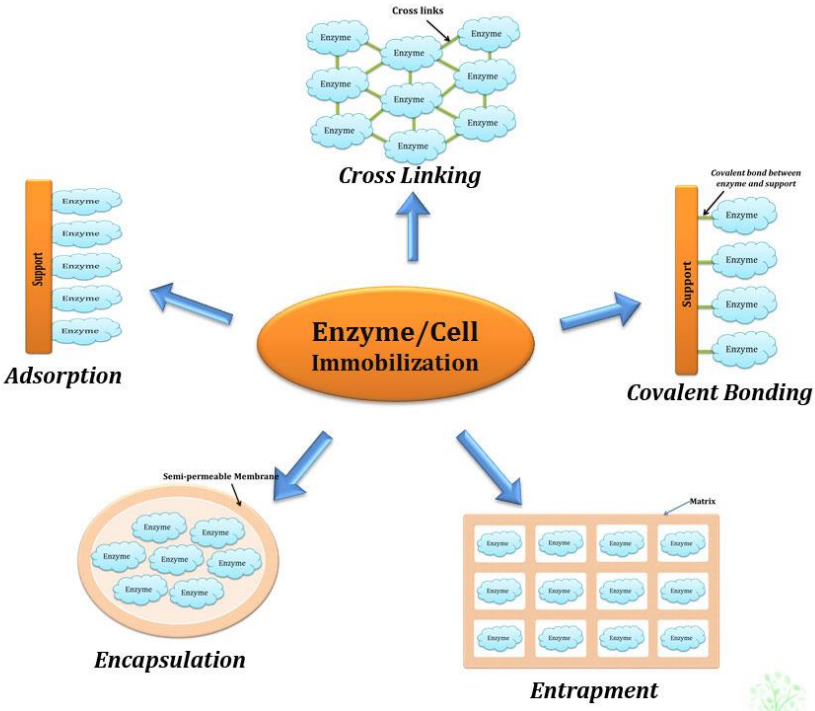


Figure 5 – Different enzyme/cell immobilization methods

1.5.1. Entrapment

Entrapment is the process of trapping enzymes or microbial cells either inside a polymeric porous matrix or a semi-permeable membrane. The polymeric porous matrices can be either naturally occurring polymers or synthetic polymers. Some naturally occurring polymers used for entrapment include alginate, agar, k-carrageenan, chitosan, cellulose, collagen, gelatin, etc. Other synthetic porous matrices used for entrapment

include polyacrylamide, porous metal screens, cellulose triacetate, polystyrene, silica gel, polyurethane, etc. Polyacrylamide was the first gel used for entrapping microbial cells. The gel strength in case of polyacrylamide is directly proportional to the concentration of the gel. Higher gel concentration reduces the pore sizes of the matrix resulting in diffusional resistance to substrates and products. Moreover, polyacrylamide may result in loss of cell or enzyme activity due to the toxic effect of acrylamide and free radical formation during polymerization. Entrapment of cells or enzymes using agar is not preferred much due to poor mechanical strength and use of high temperatures for maintaining agar solution. Polysaccharides like alginate and k-carrageenan are the most widely used porous matrices for entrapment of cells and enzymes. They offer good mechanical strength and mild conditions for immobilization (Bucke & Brown, 1983). The pore size of the gels should be large enough and the immobilized particle should be small enough to overcome the intraparticle diffusional resistance.

Another method of entrapment is microencapsulation and use of macroscopic membranes. Encapsulation is the method of trapping of enzymes inside a hollow sphere surrounded by a semi-permeable membrane. The movement of substrate and products take place through this semi-permeable membrane. Higher number of cells per unit volume of the support can be entrapped inside capsules. Polymers that are used as semi-permeable membranes include polystyrene, polyester, nylon, acrylate, cellulose acetate-ethyl cellulose, polylysine-alginate hydrogel, etc. Membrane selection for encapsulation depend on the type of products and substrates that will diffuse through the membrane in the encapsulated cell or enzyme system (Shuler & Kargi, 2001, p. 265).

Enzyme solutions can be entrapped within thin semi-permeable membranes. The most common configuration is that of a hollow fiber tube which is similar to a shell and tube heat exchanger. The tubes are made of semi-permeable membranes. The enzyme is retained by the membrane, while the products and substrates diffuse through it. In case of cell entrapment, the cells are inoculated and grown on the shell side. Substrates are pumped through the tubes and diffuse through the membrane to reach the cell system. The products diffuse back in the feed stream through the membrane. Polyacrylate, polysulfone, cellulose and nylon are the most commonly used membranes for entrapment (Shuler & Kargi, 2001, p. 80).

1.5.2. Immobilization on support surface

Enzymes and cells can be attached to the surface of an immobilization support either by weak physical forces or by strong covalent forces. Adsorption is the binding of enzymes or cells by weak physical forces like ionic forces, Vander Waals forces and dispersion forces on the support surface. Adsorption offers very less diffusional limitations, since the enzymes and cells are in direct contact with the substrate. Almost full

enzyme activity is retained after adsorption. However, due to the presence of weak binding forces, enzyme washout is a common problem in this kind of immobilization. Cross-linking with glutaraldehyde can overcome this problem, but enzyme denaturation may take place. Simplicity and inexpensiveness make it a useful method of immobilization for microbial cells. However, hydrodynamic shear should be limited to avoid removal of cells from the support. Supports used include carboxymethyl cellulose, sephadex, DEAE cellulose, Sepharose, alumina, porous silica, activated carbon, wood chips, gelatin, chitosan, etc. The supports may be activated for effective immobilization (Shuler & Kargi, 2001, p. 266).

Attachment of enzymes on support surfaces using covalent binding is very common. However, it is not commonly used for cell immobilization. The support surfaces are activated before covalent binding using certain chemical reagents like glutaraldehyde, carbodiimide and cyanogen bromide. These chemicals may be toxic to microbial cells. Titanium and zirconium oxide are used to provide suitable functional groups for covalent binding (Woodward, 1988). Loss of cells during cell growth and limited number of suitable functional groups make this method of immobilization undesirable for microbial cells. Supports with $-NH_2$ groups are treated with glutaraldehyde, with -OH groups treated with cyanogen bromide and with -COOH groups treated with carbodiimide (Datta, 2013).

Enzymes and microbial cells can be cross-linked using glutaraldehyde to form insoluble aggregates. Cross-linking can also be done after adsorption to support surface. Cross-linking may distort the active site of the enzymes and may also affect the metabolic activity of the cells. Cross-linking also offers severe diffusional resistance. Thus, direct cross-linking is not preferable. Various cross-linking agents used for enzymes include 2,2-sulfonic acid, bis-diazo benzidine, glutaraldehyde, etc. (Damin et al., 2021).

A good immobilization support material should be chemically inert and rigid, should have high cell or enzyme loading capacity and should bind the cells or enzymes firmly.

1.5.3. Biological films

Biological films refer to the growth of cells in multiple layers on an inert or a biologically active support surface. In this system, the substrates diffuse through the microbial films and the products diffuse back into the feed stream. The concentration of substrate and products within the microbial films is an important parameter affecting the physiology and metabolism of cells, thereby affecting the final yield. Thickness of the microbial films is an important factor in this type of system. Less biomass concentration in thin films result into less yield. On the other hand, thick biofilms can offer diffusional resistance, thereby creating substrate limiting regions within the films. Therefore, an optimal thickness is required for getting a higher yield. In case of mixed culture, polymer

producing microorganisms influences the stability of the microbial films. Biological films are widely used in the biological treatment of waste water (Shuler & Kargi, 2001, p. 267).

1.6. Aims and Objectives

Increasing incidence of lactose malabsorption and intolerance have influenced consumers to include lactose hydrolyzed milk and other dairy products in their diet. Thus, the increase in the market share for lactose hydrolyzed products has been attributed to the awareness of consumers about lactose intolerance. Moreover, lactose offers some technological and environmental problems which have encouraged the use of lactose hydrolyzing enzymes on a large scale. Using β-galactosidase, the problems of lactose crystallization in frozen milk products, and the problems of whey disposal can be solved. Whey can be converted into useful products.

Kluveromyces sp. are the most widely used sources of β – galactosidase. The aim of the present study to observe the effect of immobilized whole cell producing β – galactosidase on the hydrolysis of lactose. Whole cells of *Kluyveromyces marxianus* producing intracellular β – galactosidase was entrapped in calcium alginate beads and used in the present study.

The objectives of the present study were –

(1) Production and immobilization of *Kluyvermyces marxianus* in calcium alginate matrix.
(2) Studying the effect of different parameters like sodium alginate concentration, bead diameter, pH, temperature, substrate concentration and biomass concentration on lactose conversion.
(3) Continuous operation in a packed bed column using the immobilized cell system under various operating conditions.
(4) Developing an external mass transfer model using Colburn analogy for the packed bed column.
(5) Calculating pressure drop across the packed bed using Ergun equation.
(6) Calculating friction factor for different operating conditions.

Chapter 2
Materials and Methods

2.1. Chemicals

Chemicals used for media preparation included lactose monohydrate, di-potassium hydrogen phosphate (K_2HPO_4), diammonium hydrogen phosphate ($(NH_4)_2HPO_4$), ammonium dihydrogen phosphate ($NH_4H_2PO_4$), magnesium sulfate heptahydrate ($MgSO_4.7H_2O$) procured from Merck, Mumbai. Yeast extract used in media preparation was procured from Nice Chemicals, Kochi. Sodium alginate (procured from Loba Chemie) and calcium chloride dihydrate ($CaCl_2.2H_2O$) (procured from Merck, Mumbai) were used for immobilization of whole cells. Lactose monohydrate ($C_{12}H_{22}O_{11}.H_2O$) solution was used as the substrate for studying lactose hydrolysis. Sodium hydroxide (NaOH) and hydrochloric acid (HCl) procured from Merck, Mumbai were used for pH adjustment. Potassium sodium tartrate tetrahydrate ($C_4H_4O_6KNa.{}_4H_2O$), sodium hydroxide, and 3,5 – dinitrosalicylic acid (DNSA) (all procured from Merck, Mumbai) were used to assay the activity of intracellular β – galactosidase enzyme entrapped in calcium alginate beads.

2.2. Composition of maintenance media and fermentation media

The maintenance media used had the following composition per liter – Yeast extract – 0.2% w/v; Beef extract – 0.1% w/v; NaCl – 0.5% w/v; Agar – 3% w/v; pH – 6.8 – 7.0. The media was sterilized using an autoclave at 121^0c at 15 psig for 15 mins. The micro-organism was preserved on agar slants at 4^0c.

The media used for fermentation had the following composition per liter – Lactose monohydrate – 3% w/v; K_2HPO_4 – 0.2% w/v; Yeast extract – 0.1% w/v; $(NH_4)_2HPO_4$ – 0.1% w/v; $NH_4H_2PO_4$ – 0.1% w/v $MgSO_4.7H_2O$ – 0.02% w/v; pH – 6.0. The media was sterilized using an autoclave at 121^0c at 15 psig for 15 mins. K_2HPO_4 was sterilized separately and added aseptically just before inoculation.

2.3. Preparation of inoculum and production of micro-organisms

Kluyveromyces marxianus was used for this study. For inoculum preparation, a loopful of microorganisms was transferred aseptically from slant culture to 50 ml sterile fermentation media, taken in a 250 ml Erlenmeyer flask. It was incubated at 29^0c for 24 hr in a rotary shaker incubator at 120 rpm.

For experimental production of the microorganism, 50ml of the sterile fermentation media was taken in a 250 ml Erlenmeyer flask and inoculated with 10% (v/v) inoculum. It was incubated at 29^0c for 24 hr in a rotary shaker incubator at 120 rpm.

2.4. Whole cell recovery

Recovery of whole microbial cells was done by centrifuging the fermentation broth in a Remi R-8C centrifuge for 15 min at 4500 rpm. After centrifugation, the supernatant was discarded and cell mass was recovered.

2.5. Whole cell immobilization

A suspension of whole cells was prepared using 1.5 ml distilled water. 3 ml of 3% (w/v) sodium alginate solution was added to the cell suspension and thoroughly mixed. The mixture was then extruded dropwise in a 4% (w/v) $CaCl_2$ solution using a hypodermic syringe. The spherical beads thus formed were kept for hardening at 4^0c for 1 hr. after hardening, the beads were thoroughly washed with distilled water to remove any excess $CaCl_2$ or un-immobilized cells. The beads were stored immersed in distilled water in the refrigerator for further use.

2.6. Diameter of immobilized beads

For calculating the diameter of immobilized beads, a 100 ml measuring cylinder was taken and filled with water up to the 20 ml mark. Beads were then added to the

measuring cylinder and a rise in the volume of water was noted. The difference in the volume was taken to be the total volume occupied by the beads. The volume of one bead was calculated and consequently, the diameter of the bead was calculated.

2.7. Effect of different parameters

Different processing parameters like concentration of sodium alginate, diameter of beads, pH, temperature, concentration of substrate, and biomass concentration were taken for studying their effect on the hydrolysis of lactose by entrapped intracellular enzyme in calcium alginate beads. 4% w/v lactose solution was used as a substrate for studying lactose hydrolysis.

2.7.1. Alginate concentration

Various concentrations of sodium alginate such as 3% w/v, 3.75% w/v to 4.5% w/v were taken to study the effect of different alginate concentrations on lactose hydrolysis. The beads were first brought down to room temperature and then filtered before use. Incubation of beads was done in 50 ml substrate at 29^0c for 30min, under shake flask conditions at 120 rpm. In order to study the effect on lactose hydrolysis, the filtrate was assayed after the beads were filtered. Alginate concentration displaying the highest conversion of lactose was used for further study.

2.7.2. Bead diameter

Different hypodermic syringes producing beads of varying diameters – 2.94 mm, 3.11 mm, and 3.95 mm were used to study the effect of different bead diameters on lactose hydrolysis. The beads were first brought down to room temperature and then filtered before use. Incubation of beads was done in 50 ml substrate at 29^0c for 30 min, under shake flask conditions at 120 rpm. In order to study the effect on lactose hydrolysis, the filtrate was assayed after the beads were filtered. Bead diameter showing the highest conversion of lactose was used for further study.

2.7.3. Effect of pH

Different pH such as 4.05, 4.4, 5, 5.43, and 6.0 were taken to study the effect of different pH on lactose hydrolysis. 0.1 N HCl and 0.1 N NaOH were used for pH adjustment. The beads were first brought down to room temperature and then filtered before use. Incubation of beads was done in 50 ml substrate at 29^0c for 30min, under shake flask conditions at 120 rpm. In order to study the effect on lactose hydrolysis, the filtrate was assayed after the beads were filtered.

2.7.4. Effect of temperature

Different temperatures such as 25^0, 29^0 and 37^0c were taken to study the effect of different temperatures on lactose hydrolysis. The beads were first brought down to room temperature and then filtered before use. Incubation of beads was done in 50 ml substrate at different incubation temperatures for 30 min, under shake flask conditions at 120 rpm. In order to study the effect on lactose hydrolysis, the filtrate was assayed after the beads were filtered. The temperature showing the highest lactose conversion was used for further study.

2.7.5. Concentration of substrate

Different lactose concentrations like 0.01 M, 0.03 M, 0.06 M, and 0.2 M were taken to study the effect of increasing concentration on the conversion of lactose.. The beads were first brought down to room temperature and then filtered before use. Incubation of beads was done in 50 ml substrate at 29^0c for

30 min, under shake flask conditions at 120 rpm. In order to study the effect on lactose hydrolysis, the filtrate was assayed after the beads were filtered.

2.7.6. Biomass concentration

Different biomass concentrations like 84.45 mg/ml, 188.89 mg/ml, and 337.78 mg/ml were taken for studying the effect of biomass concentration on lactose conversion. The beads were first brought down to room temperature and then filtered before use. 4% (w/v) substrate concentration was used in this experiment, similar to other experiments except in 2.7.5. Incubation of beads was done in 50 ml substrate at 29^0c for 30 min, under shake flask conditions at 120 rpm. In order to study the effect on lactose hydrolysis, the filtrate was assayed after the beads were filtered. Biomass concentration showing the highest lactose conversion was used for further study.

2.8. Packed bed column

A glass column of 1.95 cm internal diameter was used. Glass wool was inserted to seal the outlet such that a bed of immobilized beads can be formed. A pinch valve was used to regulate the flow of substrate. The packed bed column was operated at different flow rates – 0.90, 1.71, and 3.30 cm^{-3} min^{-1}. The height of the packed bed was 13.2 cm. The column was run for 5 hr at room temperature with sample collection at the outlet at definite time intervals. The first sample was collected at an interval of 5 min. The next samples were collected at an interval of 10 min up to the completion of 1 hr. After 1 hr of column run, the samples were collected at an interval of 30 min up to the completion of 5 hr. The samples were assayed for determining the amount of lactose conversion at specific time intervals. The concentration of substrate remaining after conversion was also calculated.

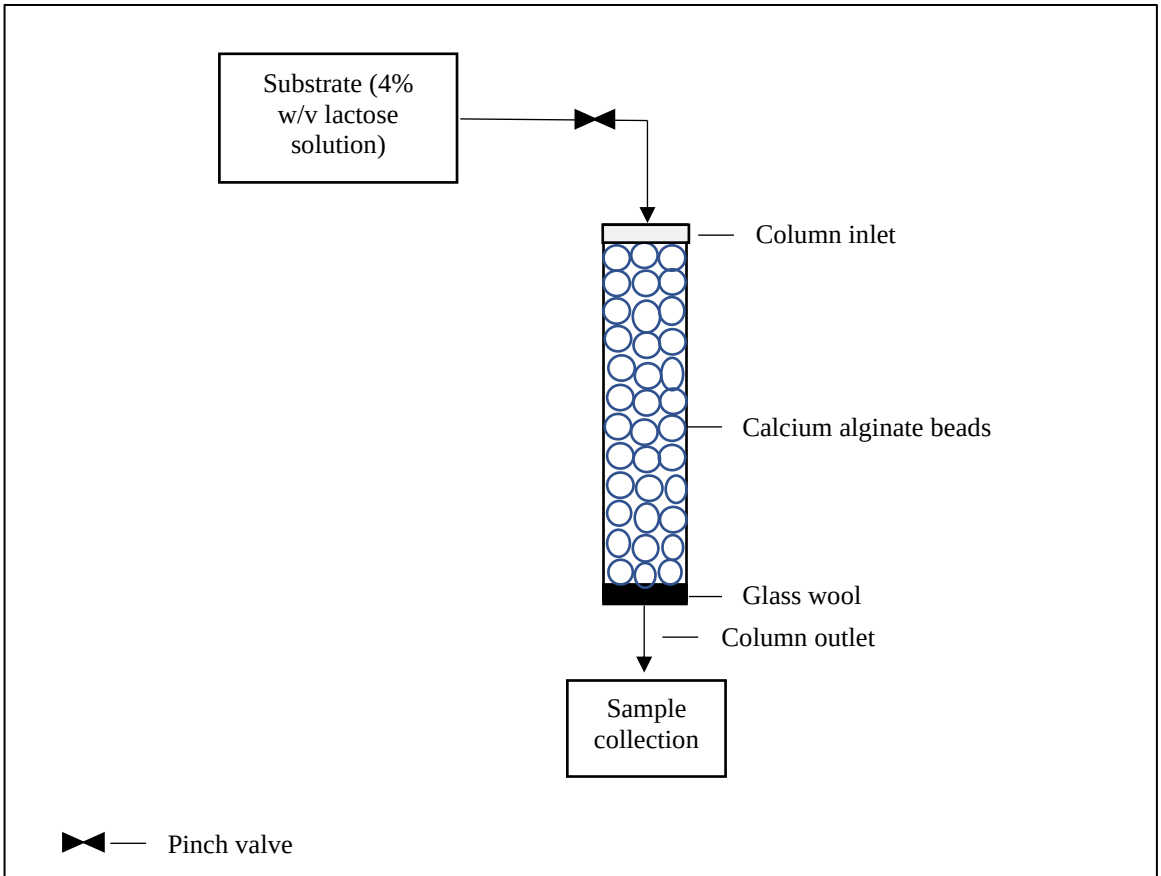


Figure 1. Schematic diagram of experimental set up for packed bed column with calcium alginate beads

2.9. Void fraction of packed bed

The void fraction of packed bed is represented by ε. A 10 ml measuring cylinder was taken and beads were poured into it. It was filled with water using a 10 ml pipette such that to fill the gaps in between the beads. The void volume was noted. It was further filled with water and the total volume in the measuring cylinder was noted. The total volume occupied by the beads was calculated by subtracting the pipette reading from the total volume in the measuring cylinder. The void fraction was then calculated as,

ε = Volume occupied by fluid (void)/ Total volume of bead and void

2.10. External mass transfer model

For establishing an external mass transfer model in a packed bed reactor using an immobilized cell system, some assumptions were made (Shuler & Kargi, 2001, p. 86)

(1) Uniform distribution of enzyme throughout the spherical particles
(2) First order rate of reaction taking place
(3) Michaelis Menten equation used to describe the reaction kinetics
(4) Absence of any partitioning between substrate in the interior and exterior of the immobilized particle
(5) Steady state condition

The biochemical rate of reaction in a packed bed column consisting of immobilized cells was assumed to follow first-order reaction kinetics. Thus, the observed rate of reaction can be expressed as follows,

$$-r_L = - k_L.C_L \qquad \text{(i)}$$

where r_L represents the rate of reaction (mg cm^{-3} min^{-1}), k_L represents the observed reaction rate constant (min^{-1}) and C_L represents the concentration of substrate in bulk (mg cm^{-3}). The values of k_L were experimentally obtained by operating the packed bed reactor at different volumetric flow rates.

When there is a fluid flow in a packed bed reactor, the velocity of the fluid near the surface of the packed particles is very low. This forms a stationary film of fluid around the surface of the particles. The diffusion of the substrate from this stationary film to the surface of the immobilized particle occurs mainly by molecular diffusion. Since this diffusion rate may be very slow, the observed reaction rate can be significantly decreased by this external diffusion rate. The external rate of mass transfer is directly proportional to the driving force and the external available surface area for mass transfer. The external rate of mass transfer can be given as follows,

$$r_E = k_E.Am.(C_L-C_S) \qquad \text{(ii)}$$

where, r_E represents the rate of external mass transfer (mg cm^{-3} min^{-1}), k_E represents the external mass transfer coefficient (cm min^{-1}), A_m represents the surface area to volume ratio for mass transfer (cm^2 cm^{-3}) and (C_L-C_S) represents the driving force for mass transfer with C_L being the substrate concentration in bulk liquid (mg cm^{-3}) and C_S being the substrate concentration at the surface of the immobilized cells (mg cm^{-3}).

A_m for a packed bed can be calculated as follows,

$$A_m= 6(1-\varepsilon)/d_P \qquad \text{(iii)}$$

where, ε represents the void fraction of the packed bed and d_P represents the diameter of the spherical particle (cm). ε of the packed bed is obtained from 2.9 and d_P is obtained from 2.6.

The biochemical rate of reaction for the immobilized cell system can also be written as,

$$r_L = k_S.C_S \qquad \text{(iv)}$$

where, k_S represents the surface first order reaction rate constant (min^{-1}), which includes both internal mass transfer and the intrinsic reaction. The effective internal mass transfer coefficient can be assumed constant at very low substrate concentration. Since the substrate concentration used in this experiment is very low, so the eqn.(iv) is valid throughout (Cheng et al., 2011).

The reaction was assumed to be in a steady state condition. At steady state condition, the rate of external mass transfer and the overall rate of biochemical reaction are equal. Therefore, by equating eqn. (ii) and eqn. (iv) and then rearranging gives,

$$C_S = (k_E.A_m.C_L)/(k_S + k_E.A_m) \qquad \text{(v)}$$

Putting eqn.(v) into eqn.(iv), and equating with eqn.(i),

$$k_L = (k_S.k_E.A_m)/(k_S + k_E.A_m) \qquad \text{(vi)}$$

or,

$$1/k_L = 1/k_S + 1/(k_E.A_m) \qquad \text{(vii)}$$

The value of k_S is constant for this experiment since it represents the intrinsic reaction rate constant. However, the external mass transfer coefficient k_E varies with diameter of reactor, flow rate of fluid and properties of fluid. A dimensionless group can be used for correlating the external mass transfer coefficient with these operational parameters and fluid properties. The dimensionless group can be given as,

$$J_D = (k_E.\rho/G).(\mu/\rho.D_f)^{2/3} \qquad \text{(viii)}$$

where, J_D represents the Colburn factor, G represents the mass flux or the effective mass flow rate through the packed bed (g cm^{-2} min^{-1}), ρ, μ and D_f represents the density (g cm^{-3}), viscosity (g cm^{-1} min^{-1}) and diffusivity (cm^2 min^{-1}) of the fluid respectively.

G can be calculated as following,

$$G = (F.\rho)/(A_c.\varepsilon) \qquad \text{(ix)}$$

where, F represents the volumetric flow rate (cm^3 min^{-1}) of fluid through the packed bed and Ac represents the cross-sectional area of the column (cm^2).

Correlations for mass transfer rates vary in dependence of J_D on Re as follows,

$$J_D = K.Re^{-(1-n)} \qquad \text{(x)}$$

where, K is a constant, Re represents the Reynold's number and the value of n depends on mass transfer conditions and varies from 0.1 to 1.0 depending on the flow characteristics. This range has covered all the values of n in eqn.(x) which have been proposed in the chemical engineering literature (Nath & Chand, 1996).

Reynold's number in a packed bed column can be calculated as follows,

$$Re = (d_P.\ \rho.\ U_0)/\mu.(1-\varepsilon) \qquad \text{(xi)}$$

where, d_P, ρ, μ, and ε represent the same as previously used. U_0 represents superficial fluid velocity through the packed bed and can be expressed as $U_0 = F/A_c$, with F and A_c representing as previously used.

Equating eqn.(viii) and eqn.(x) k_E can be calculated as follows,

$$k_E = (K/\rho).\{\mu/(\rho\ .\ D_f)\}^{-2/3}.(d_P/\mu)^{n-1}.G^n \qquad \text{(xii)}$$

or,

$$k_E = A.G^n \qquad \text{(xiii)}$$

where,

$$A = (K/\rho).\{\mu/(\rho.D_f)\}^{-2/3}.(d_P/\mu)^{n-1} \quad \text{(xiv)}$$

Putting eqn.(xiii) into eqn.(vii),

$$1/k_L = (1/A.A_m) \, . \, (1/G^n) + 1/k_S \quad \text{(xv)}$$

A plot of $1/k_L$ versus $1/G^n$ for different values of n gives a straight line with slope $(1/A.A_m)$ and intercept $(1/k_S)$. The values of A and k_S were calculated from the graph. Thevalue of k_E was then calculated using eqn.(xiii). The value of n was selected based on the best fit line with highest R^2 value close to 1. After calculating k_S, the value of constant K was calculated using eqn.(xii). Then J_D was calculated using eqn.(viii). From the values of k_E and A_m, the rate of external mass transfer (r_E) and the concentration of substrate at the surface (C_S) of the immobilized particle were calculated. Experimental Reynold's number was calculated using eqn.(xi). An external mass transfer model was predicted using constant K and eqn.(x). J_D was experimentally found using eqn.(x) by substituting the Reynold's number obtained from eqn.(xi). The mass transfer correlation was verified by plotting calculated J_D vs experimental J_D.

2.10.1. Residence time in packed bed column

The residence time in the packed bed column for different flow rates can be calculated using the following formula,

$$\tau = \{V_p \, (1- \varepsilon)\}/ F \quad \text{(xvi)}$$

where, V_p is the volume of the packed bed (cm^3). ε is the void fraction calculated in 2.9. F represents the volumetric flow rate through the packed bed ($cm^3 \, min^{-1}$).

2.10.2. Damkohler Number

Damkohler number is characterized as follows,

$$Da = \frac{\text{Maximum rate of reaction}}{\text{Maximum rate of diffusion}} \quad \text{(xvii)}$$

Michaelis-Menten kinetics can be used to describe the kinetics of the enzymatic reaction. According to the assumption made in point (4) in 2.10, substrate concentration at the surface and bulk are the same. Thus, eqn.(iv) can also be written as,

$$r_L = (r_{max}.C_S)/(K_m + C_S) \quad \text{(xviii)}$$

When the rate of the enzymatic reaction is maximum, $(Km + C_S)$ can be approximately taken as C_S. Therefore, r_L is equal to r_{max} when the rate of reaction is maximum.

Thus, r_{max} can be written as follows from eqn.(iv),

$$r_{max} = k_S \, . \, C_S \quad \text{(xix)}$$

Maximum rate of diffusion takes place when the substrate concentration at the surface is zero. Thus, from eqn.(ii) maximum rate of diffusion can be written as,

$$r_E = k_E \, . \, A_m \, . \, C_L \quad \text{(xx)}$$

Thus, the Damkohler number can be expressed as follows,

$$Da = r_{max}/ (k_E \, . \, A_m \, . \, C_L) \quad \text{(xxi)}$$

2.11. Pressure drop across packed bed column

Pressure drop across the packed bed column was calculated by using the Ergun equation. It is the most commonly used equation for determining pressure gradient across a fixed bed or a fluidized bed (Bayram et al., 2018). The Ergun equation is given as follows,

$$\Delta P/L = \{150.(1-\varepsilon)^2.\mu.U_0)\}/(\Phi^2.\ \varepsilon^3.d_P^2) + \{1.75.(1-\varepsilon).\rho.U_0^2\}/(\varepsilon^3.\Phi.d_P) \quad \text{(xxii)}$$

where, ΔP represents the pressure drop (dyne cm^{-2}), L represents the height of the packed bed (cm), ρ and μ represent same as that in 2.10, i.e., the fluid density (g cm^{-3}) and viscosity (g cm^{-1} min^{-1}), Φ represents the sphericity of the packed bed particles, U_0 represents the superficial velocity of fluid through the packed bed (cm min^{-1}), and ε represents the same as that in 2.9, i.e., the void fraction of the packed bed.

Here, the superficial velocity of fluid flowing through the packed bed column is given as,

$$U_0 = F/A_c \quad \text{(xxiii)}$$

where, F represents same as that in 2.10, i.e., the volumetric flow rate of fluid through the packed bed (cm^3 min^{-1}) and A_c represents same as that in 2.10, i.e., the cross-sectional area of the column or the packed bed (cm^2).

2.12. Friction factor calculation

The friction factor can be described by Ergun as follows,

$$f_P = (\Delta P/H)\ .\ (d_P/\rho\ U_0^2)\ .\ \varepsilon^3/\ (1-\varepsilon) \quad \text{(xxiv)}$$

where, f_P is the friction factor and all other symbols represent the same as in 2.11.

From eqn.(xxii) and (xxiv) the friction factor can also be represented as follows,

$$f_P = (150/Re_P) + 1.75 \quad \text{(xxv)}$$

where Re_P represents the bed particle Reynold's number.

The bed particle Reynold's number can be expressed as follows,

$$Re_P = (d_P\ \rho\ U_0)/\{\mu\ (1-\varepsilon)\} \quad \text{(xxvi)}$$

where, all the symbols represent same as that in 2.11.

2.13. Assay method for β – galactosidase

2.13.1. DNSA assay method

3,5 – dinitrosalicylic acid (DNSA) oxidizes the free aldehyde (aldose) or ketone (ketose) functional group present in reducing sugars and gets reduced to 3 – amino – 5 – nitrosalicylic acid under alkaline conditions. The final product is an aromatic reddish-brown complex that has a maximum absorbance at 540 nm. The intensity of the colour is proportional to the amount of reducing sugar present in an unknown sample.

2.13.2. Preparation of Standard Curve for Glucose

Glucose stock solution was prepared by adding 250 mg of glucose to 100 ml of distilled water. The working solution was prepared by diluting the stock solution by 10 times. 2 M NaOH was prepared. 60% (w/v) Rochelle salt solution was prepared. DNS (3,5 – dinitrosalicylic acid) reagent was prepared by adding 1 g DNSA to 20 ml 2 M NaOH and volume made up to 100 ml by Rochelle salt solution. 1.5 ml, 2.0 ml, 2.5 ml, and 3.0 ml glucose working solution were taken in test tubes. Distilled water was added to each test tube to make the volume up to 3.0 ml. 1.0 ml DNS reagent was added to each test tube and the test tubes were kept in a boiling water bath for 5mins. Then the test tubes were kept in a cold-water bath till the temperature dropped down to room temperature. Absorbance values were taken at 540 nm in a UV – Vis spectrophotometer and a standard curve was constructed. The standard curve was further used for studying the lactose hydrolysis.

Chapter 3
Results and Discussion

3.1. Standard Curve for Glucose

A standard curve for glucose was constructed using UV – Vis spectrophotometer at 540 nm. The lactose conversion percentage was calculated using the standard curve given in Figure 1. The absorbance values for different amounts of glucose is given in Table 1.

Table 1 – Standard curve for Glucose

Sample volume (ml)	*Amount of Glucose (mg)*	*Absorbance*
1.5	0.375	0.024
2	0.500	0.075
2.5	0.625	0.140
3	0.750	0.189

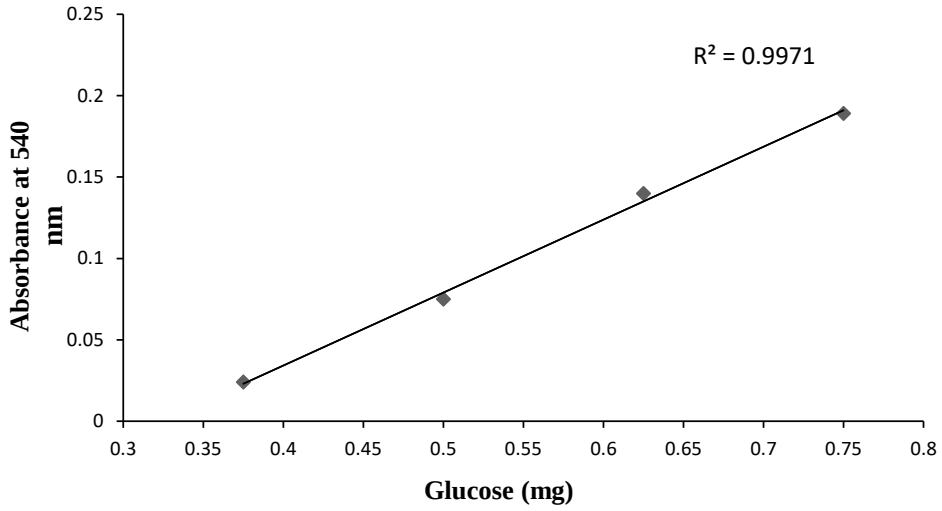


Figure 1 – Standard curve for absorbance at 540 nm vs glucose (mg)

3.2. Effect of alginate concentration and bead diameter

3.2.1. Alginate concentration

Sodium alginate is a polysaccharide having two monomers; 1,4 linked β – D – manuronic acid and 1,4 linked α – L – guluronic acid. When sodium alginate is mixed with calcium ions, it forms a stable and rigid gel by cross-linking. There is an inverse relationship between the pore size of the gel and the concentration of alginate. With increasing concentration, there is an increase in the cross-linking of gel which reduces the pore size. It has been observed that with an increasing concentration of sodium alginate, enzyme activity decreased in immobilized enzyme systems (Das et al., 2015; Dey et al., 2003; Longo et al., 1992). Varying concentrations of sodium alginate were thus selected for varying the degree of cross-linking; affecting the pore size of the beads. It was observed that by decreasing the concentration of sodium alginate from 4.5% w/v to 3% w/v, the lactose conversion % increased. This may be due to the fact that higher alginate concentration resulted in smaller pore size which offered resistance to the diffusion of substrate and product molecules to and from the beads respectively. Alginate concentration lower than 3% w/v would result in a larger

pore size which might lead to enzyme leakage, thereby decreasing the enzyme activity.

Table 2 – Lactose conversion% with different alginate concentrations

Alginate concentration (% w/v)	*Lactose conversion %*
3	71.38
3.75	65.48
4.5	60.32

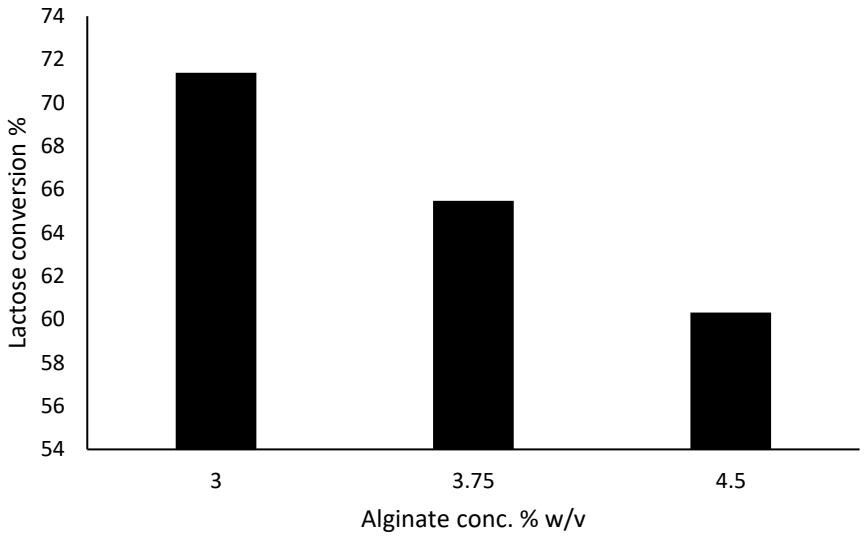


Figure 2 – Lactose conversion % vs Alginate concentration (% w/v)

3.2.2. Bead diameter

It has been observed in immobilized enzyme systems that enzyme activity decreases with increasing bead size. This may be due to the fact that an increase in bead size offers greater resistance to the transfer of the substrate from a bulk liquid phase to the active site of the enzyme present inside the gel matrix (Won et al., 2005; Knezevic et al., 2002). Similar observations were made when the bead diameter was varied using different hypodermic syringes in this experiment. The lactose conversion % increased when the bead diameter was decreased from 3.95 mm to 2.95 mm. Similar conversion % was observed for a bead diameter of 3.11 mm and 3.95 mm. Lactose conversion% at different bead diameters is given in Table 3.

Table 3 – Lactose conversion% at different bead diameters (mm)

Bead diameter (mm)	*Lactose conversion %*
2.94	66
3.11	63
3.95	63

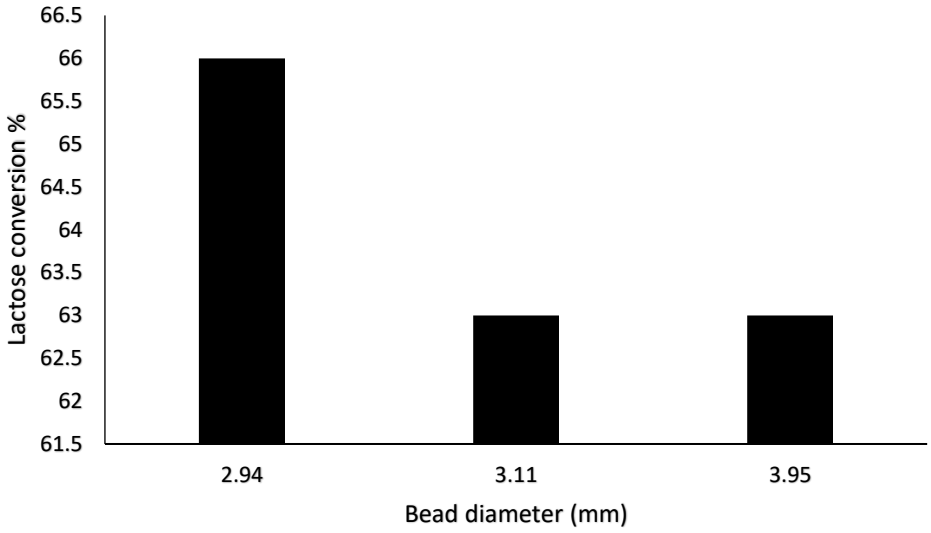


Figure 3 – Lactose conversion% vs Bead diameter (mm)

3.3. Effect of pH

Enzyme–catalyzed reactions are affected by pH. Enzymes are proteins and changes in pH may create alterations in the molecular structure of enzymes, which in turn affects the activity of the enzyme. Different pH was used for studying the effect of pH on the enzymatic hydrolysis of lactose. Maximum lactose conversion was observed at a substrate pH of 4.4. β – galactosidase from yeasts generally have a neutral optimum pH between 6-7. Immobilization can create a shift in the optimum pH as a result of change in the microenvironment of the enzyme. Maximum lactose conversion at pH 4.4 makes it suitable for application in acid whey (pH 3.6 – 4.5).

Table 4 – Lactose conversion % at different pH

pH	*Lactose conversion %*
4.05	71.29
4.40	76.77
5	71.61
5.43	65
6.00	60.32

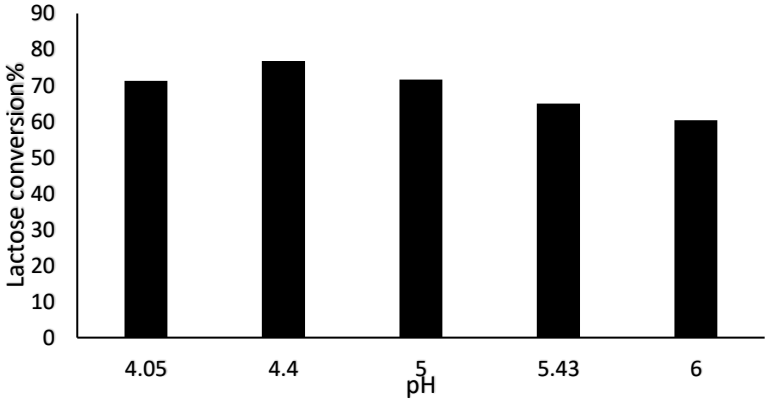


Figure 4 – Lactose conversion% vs pH

3.4. Effect of temperature

Enzyme–catalyzed reactions are dependent on temperature. There is an optimum temperature for enzymatic reactions, above or below which the enzymatic activity decreases. Above optimum temperature, the enzymes may denature resulting in a lowered enzymatic activity. The enzymatic reaction was carried out at different incubation temperatures. The highest lactose conversion was observed at 25^0c. Immobilization can create a shift in the optimum temperature of enzymes.

Table 5 – Lactose conversion% at different incubation temperatures

Temperature (^{0}c)	*Lactose conversion %*
25	76.6
29	68.6
37	66.95

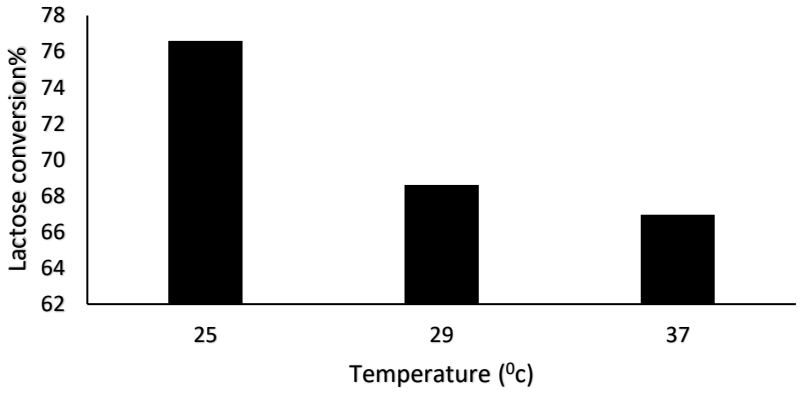


Figure 5 – Lactose conversion% vs temperature (^{0}c)

3.5. Effect of substrate concentration

When the concentration of the substrate is increased from a very low level, the rate of the enzyme-catalyzed reaction also increases and shows a linear rise initially. As the concentration of the substrate keeps increasing, the rate of the enzyme-catalyzed reaction starts reducing slowly and ultimately reaches a maximum value which corresponds to the maximum velocity of the enzymatic reaction. Beyond this maximum value, the rate of the reaction will not increase with any increase in the substrate concentration. The reason behind this phenomenon is the lack of active sites of the enzyme with increasing substrate molecules since the enzyme concentration is constant. When the reaction reaches the maximum value, the enzyme is said to be saturated since at this point no active sites are left for binding of the substrate molecules (Das, 2005). Similar trend was observed in this experiment when different concentrations of lactose was taken. With an initial low substrate concentration, the conversion of lactose increased linearly which represents the rate of the reaction. With a further increase in the substrate concentration, the conversion of lactose became almost constant, indicating that the rate of reaction had reached a maximum value. The percentage of lactose conversion at different substrate concentrations is given in Table 6.

Table 6 – Lactose conversion% at different substrate concentration (M)

Substrate concentration (M)	*Lactose conversion%*
0	0
0.01	66.35
0.03	80.4
0.06	76.31
0.2	78.48

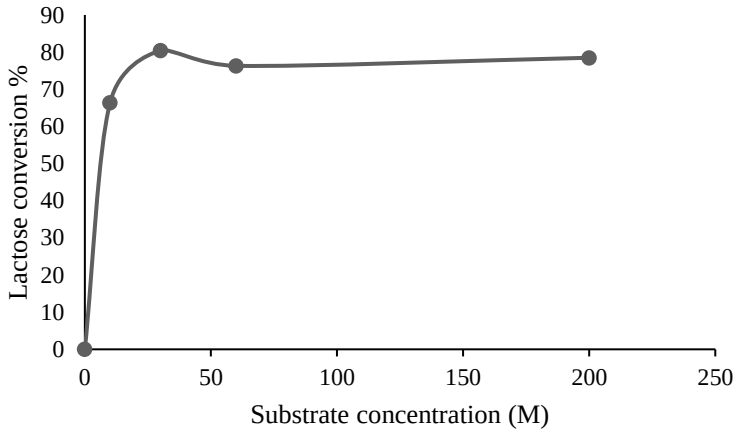


Figure 6 – Lactose conversion% vs substrate concentration (M)

3.6. Effect of biomass concentration

Different biomass concentrations were taken to study the effect of biomass concentration on the activity of the immobilized enzyme. It was observed that the lactose conversion% increased with increasing biomass concentration from 84.45 mg/ml to 188.89 mg/ml. This increase may be attributed to the higher availability of enzyme with increased biomass (Panesar et al., 2011). However, a subsequent increase in biomass concentration had resulted in a decrease in the lactose conversion%. This may be attributed to the agglomeration of cells which resulted in a decreased available surface area for the diffusion of the substrate, thereby reducing the lactose conversion. Lactose conversion% at different biomass concentrations is given in Table 7.

Table 7 – Lactose conversion% at different biomass concentration (g/ml)

Biomass concentration (mg/ml)	*Lactose conversion%*
84.45	75.88
188.89	100
337.78	82.84

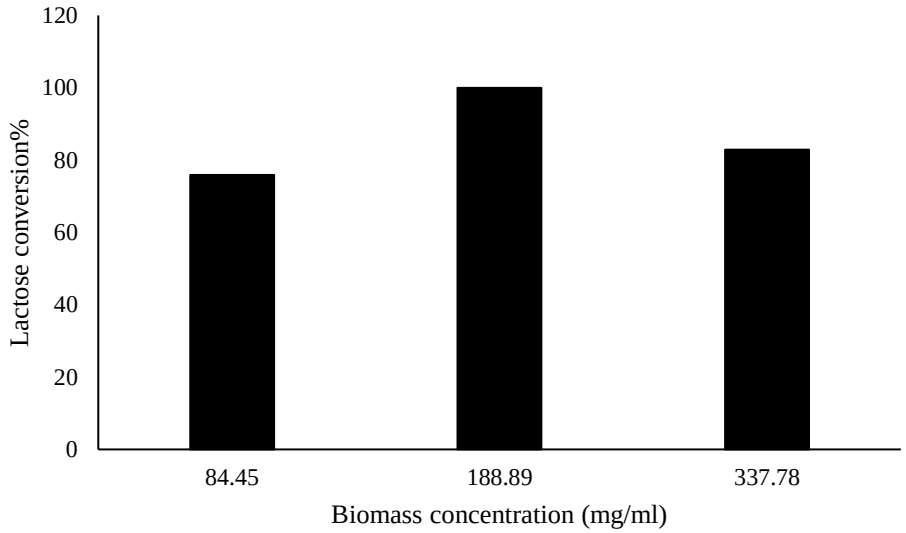


Figure 7 – Lactose conversion% vs biomass concentration (mg/ml)

3.7. External mass transfer model

In case of enzymes immobilized on the internal surfaces of a porous matrix, the substrate has to get transported from the bulk liquid to the surface of the matrix. From the matrix surface, the substrate has to get diffused through the pores and reach the surface of the catalyst. Thus, in an immobilized system substrate diffusion and enzymatic reaction occurs simultaneously. Therefore, an external mass transfer model is required. The packed bed column was operated at different volumetric flow rates. Samples were collected at definite time intervals. The samples were assayed for lactose conversion. The remaining substrate concentration was also calculated. Using eqn.(i), a plot of dC_L/dt vs C_L was constructed, where t (min) represents the time intervals in which the samples were collected. The value of observed reaction rate constant (k_L) was experimentally found from the slope of the straight line. The residence time in the packed bed column at different flow rates was calculated using eqn.(xvi). The values of k_L and residence time for different volumetric flow rates are given in Table 8. It was observed that the value of k_L decreased with increasing flow rate. This may be attributed to the fact that the residence time in the packed bed decreases with increasing flow rate. This effects the diffusion of substrate molecules from the bulk liquid to the surface of the immobilized particles. This is turn reduces the observed rate of biochemical reaction.

Table 8 – k_L and residence time at different flow rates

Packed bed height (cm)	***F (cm^3 min^{-1})***	***k_L (min^{-1})***	***Residence time (min)***
13.20	0.90	0.01	32.41
	1.71	0.0048	17.06
	3.30	0.0039	8.84

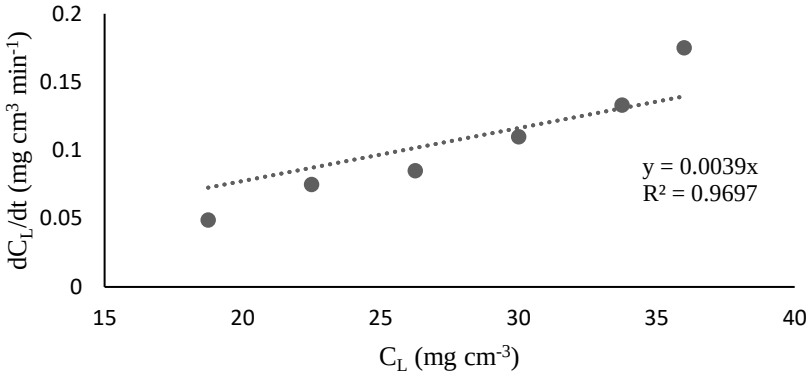


Figure 8 – dC_L/dt vs C_L at F = 0.90 cm^3 min^{-1}

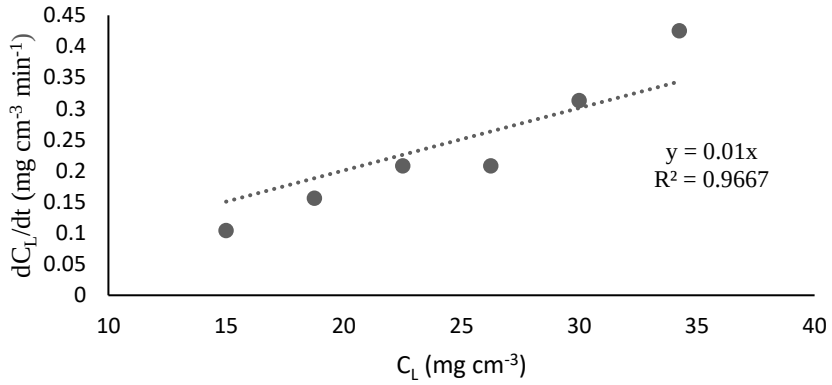


Figure 9 – dC_L/dt vs C_L at F = 1.71 cm^3 min^{-1}

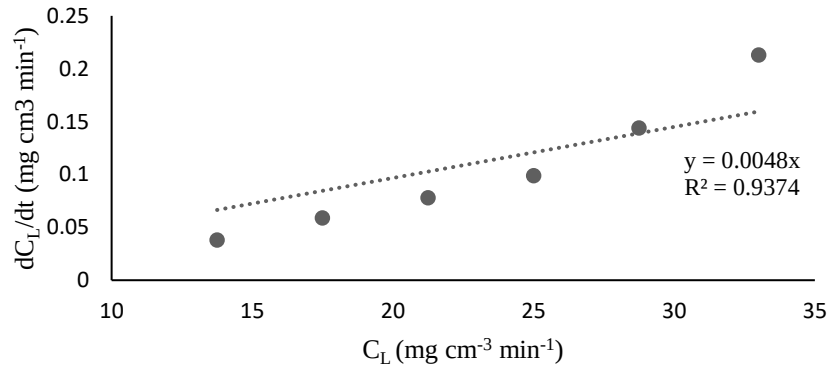


Figure 10 – dC_L/dt vs C_L at F = 3.30 cm^3 min^{-1}

The properties of fluid like viscosity (μ), density (ρ) and diffusivity (D_f) as observed in eqn.(viii), (ix), (xi), (xii) and (xiv) are obtained from previously published literature (Mcdonald & Turcotte, 1948; Morison & Mackay, 2001; Gabardo et al., 2011).

$\mu = 1.239$ g cm^{-1} min^{-1}

$\rho = 1.01214$ g cm^{-3}

$D_f = 309.6$ x 10-6 cm^2 min^{-1}

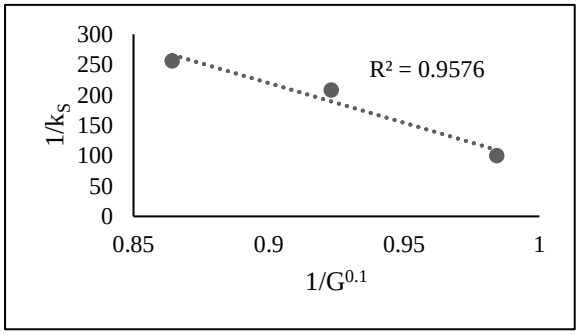
The void fraction (ε) of the packed bed was 0.26. Using eqn.(xv), a plot of $1/k_L$ vs $1/G^n$ was constructed with different values of n ranging between 0<n<1. The values of mass flux (G) at different volumetric flow rates were calculated using eqn.(ix). The plot gives a straight line with slope $1/AA_m$ and intercept $1/k_S$. The magnitude of $1/AA_m$ and $1/k_S$ were taken. The value of n was selected based on the best fit line with the highest R^2 value close to 1. It was observed that the best fit line was obtained using n value of 0.98. It was observed that for all values of n, $1/k_S$ showed an increasing trend with decreasing $1/G^n$. The values of $1/k_S$, G, $1/G^n$, for n values ranging from 0.1 to 0.9, 0.95, 0.96, 0.97, 0.98, and 0.99 at different volumetric flow rates are given in Table 9.1 & Table 9.2.

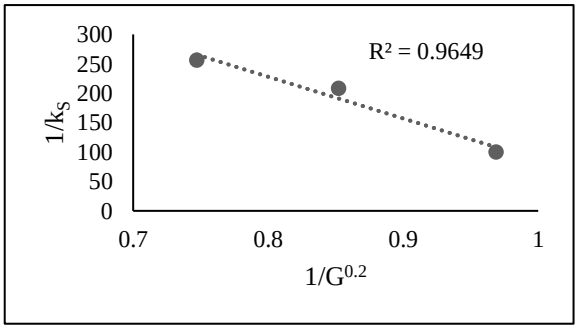
Table 9.1 – Various parameters for lactose conversion in a packed bed column for n = 0.1 to 0.7; unit of $1/G^n$ is $g^{-n}\ cm^{2n}\ min^n$

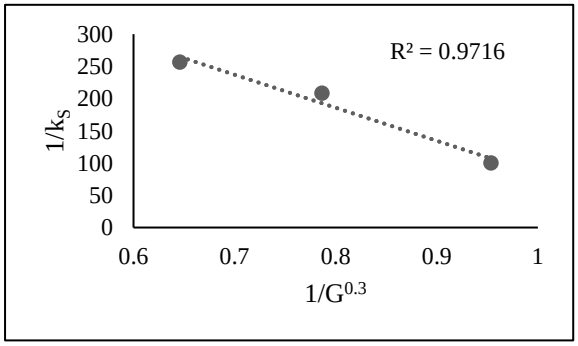
F ($cm^3\ min^{-1}$)	*G ($g\ cm^{-2}\ min^{-1}$)*	*$1/k_S$ (min)*	*$1/G^{0.1}$*	*$1/G^{0.2}$*	*$1/G^{0.3}$*	*$1/G^{0.4}$*	*$1/G^{0.5}$*	*$1/G^{0.6}$*	*$1/G^{0.7}$*
0.90	1.1718	100.00	0.9843	0.9688	0.9536	0.9386	0.9238	0.9093	0.8950
1.71	2.2263	208.33	0.9231	0.8521	0.7865	0.7260	0.6702	0.6187	0.5711
3.30	4.2965	256.41	0.8643	0.7471	0.6458	0.5582	0.4824	0.4170	0.3604

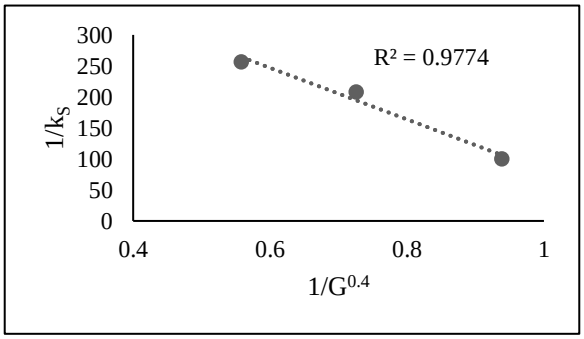
Table 9.2 – Various parameters for lactose conversion in a packed bed column for n = 0.8 to 0.9, 0.95, 0.96, 0.97, 0.98, and 0.99; unit of $1/G^n$ is $g^{-n}\ cm^{2n}\ min^n$

F ($cm^3\ min^{-1}$)	*G ($g\ cm^{-2}\ min^{-1}$)*	*$1/k_S$ (min)*	*$1/G^{0.8}$*	*$1/G^{0.9}$*	*$1/G^{0.95}$*	*$1/G^{0.96}$*	*$1/G^{0.97}$*	*$1/G^{0.98}$*	*$1/G^{0.99}$*
0.90	1.1718	100.00	0.8809	0.8671	0.8602	0.8588	0.8575	0.8561	0.8548
1.71	2.2263	208.33	0.5271	0.4866	0.4675	0.4638	0.4601	0.4564	0.4528
3.30	4.2965	256.41	0.3115	0.2693	0.2503	0.2467	0.2432	0.2396	0.2362









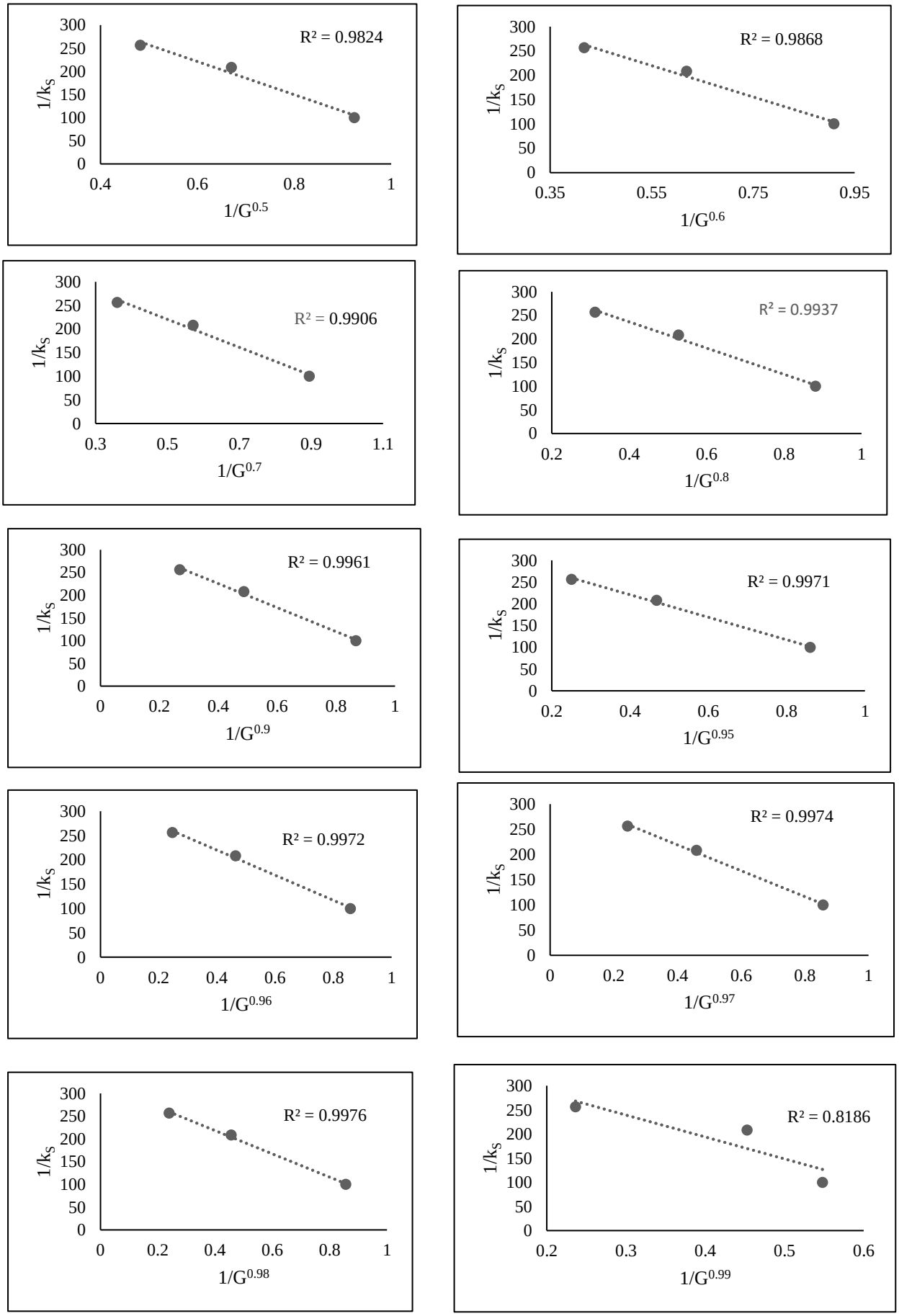


Figure 11 – Plot of $1/k_S$ vs $1/G^n$ for different values of n

3.7.1. Calculation of external available surface area for mass transfer (A_m)

From eqn.(iii),

$$A_m = \{6(1-\varepsilon)\}/ d_P$$

The diameter (d_P) of a spherical immobilized particle was 0.294 cm. The void fraction (ε) of the packed bed was 0.26. Therefore from eqn.(iii),

$$A_m = 15.102 \text{ cm}^2 \text{ cm}^{-3}$$

3.7.2. Calculation of experimental Reynold's number

Reynold's number for fluid flow in packed bed was experimentally found using eqn.(xi). It was observed that the Reynold's number increased with increasing flow rate. This is attributed to the fact that the Reynold's number increases as the fluid moves from laminar regime towards the transient regime. Reynold's number at different volumetric flow rates are given in Table 10.

Table 10 – Experimental Reynold's number for different volumetric flow rates

F (cm^3 min^{-1})	*Experimental Reynold's Number*
0.90	0.0977
1.71	0.1856
3.30	0.3583

3.7.3. Calculation of external mass transfer coefficient and rate of external mass transfer

The magnitude of slope and intercept from the plot of $1/k_S$ vs $1/G^n$ for n = 0.98 were used to calculate A and k_S. The value of k_E was calculated using eqn.(xiii). Using eqn.(vi), the value of k_L was also calculated. From eqn.(xii), the value of constant K was calculated. The average value of K was used for predicting a mass transfer model as shown in eqn.(x). It was observed that with increasing flow rate the external mass transfer coefficient also increased. This may be attributed to the fact that as the fluid moves from the laminar regime towards the transient regime, diffusion of substrate molecules increases. As a result, the rate of mass transfer also increases. The values of k_S, k_E, k_L, A and K at different fluid flow
rates are given in Table 11.

Table 11 – Values of experimental and calculated k_L and values of k_S, k_E, A and K for different fluid flow rates and n=0.98

F (cm^3 min^{-1})	*Experimental k_L (min^{-1})*	*Calculated k_L(min^{-1})*	*k_S (min^{-1})*	*k_E (cm min^{-1})x 10^{-4}*	*A (g^{-n} cm^{2n+1} $min^{-(1-n)}$)*	*K*
0.90	0.01	0.0019	0.0031	3.02		0.06358
1.71	0.0048	0.0023	0.0031	5.67	25.88×10^{-5}	0.06364
3.30	0.0039	0.0026	0.0031	10.8		0.06364
					Average	0.06362

The concentration of substrate at the surface of immobilized beads (C_S) was calculated using eqn.(v). The rate of external mass transfer (r_E) was then

calculated from eqn.(ii). It was observed that with increasing volumetric flow rate the external mass transfer also increased. This may be attributed to the fact that as the fluid moves from the laminar regime towards the transient regime, diffusion of substrate molecules increases. As a result, the rate of mass transfer also increases. The values of r_E, and (C-C_S) at different volumetric flow rates are given in Table 12. It was observed that the difference between the bulk substrate concentration and concentration of substrate on the surface of the immobilized particles decreases as the volumetric flow rate increases. This is due to increase in rate of mass transfer with
increasing flow rate. Diffusion of substrate molecules take place rapidly from the bulk liquid to the surface of immobilized cells, thereby reducing the difference between the two.

Table 12– Values of r_E and C-C_S at different volumetric flow rates

F (cm^3 min^{-1})	*r_E (mg cm^{-3} min^{-1})*	*C-C_S (mg cm^{-3})*
0.90	0.07411	16.25
1.71	0.09145	10.68
3.30	0.10471	6.42

3.7.4. Mass transfer correlation

An external mass transfer model was predicted using eqn.(x). The predicted external mass transfer model is given as follows,

$$J_D = 0.06362\ (Re)^{-0.02}$$

The value of J_D was calculated using eqn.(viii). J_D was also experimentally found using the predicted mass transfer model by substituting the Reynold's number obtained in Table 10. The correlation was verified by plotting a straight line for calculated J_D vs experimental J_D. The values of experimental and calculated J_D are given in Table 13. It was observed that the R^2 value for the best fit line is 0.9995 which is very close to 1. This represents that the predicted mass transfer model correlates with the actual experiment. This model is valid for Reynold's number ranging from 0.09 to 0.4 for this experiment.

Table 1 3 – Values of experimental J_D and calculated J_D

Experimental J_D	*Calculated J_D*
0.066649	0.065225
0.065799	0.064455
0.064939	0.063616

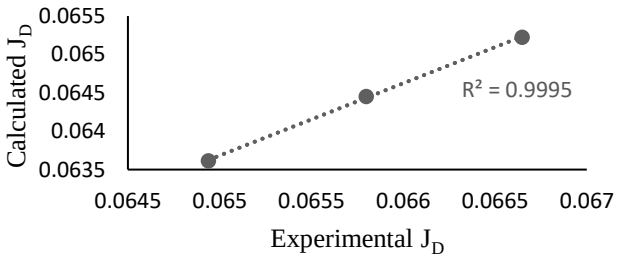


Figure 12 – Calculated J_D vs Experimental J_D

3.7.5. Damkohler Number (Da)

Immobilized enzymes face diffusional resistances. Damkohler number is used to determine whether the diffusional resistance significantly effects the enzymatic reaction. If the Damkohler number is >> 1, the reaction rate is much greater and the overall reaction is limited by the mass transfer resistance. If the Damkohler number is <<1, mass transfer or diffusion is much greater and the overall reaction is limited by the enzymatic reaction (Bhalia et al., 1999). The Damkohler number was calculated for different volumetric flow rates using eqn.(xxi). It was observed that with increasing flow rate, Da decreased. It was also observed that with increasing flow rate, there was an increase in the rate of mass transfer. This can be correlated with Da. The decrease in the Damkohler number was attributed to the increase in mass transfer with increasing flow rate. It was also observed that for each flow rate, Da <<1. This represents that the diffusion rate is much greater and the overall reaction is limited by the enzymatic reaction. The Da values for different flow rates are given in Table 14.

Table 14 – Da values for different volumetric flow rates

F (cm^3 min^{-1})	*Damkohler number (Da)*
0.90	0.4036
1.71	0.2654
3.30	0.1596

3.8. Pressure drop across packed bed column

Pressure drop across a packed bed column occurs due to friction between the fluid and the packed bed. In order to pump a fluid through a packed bed, the pumping power should be known. The pumping power is directly proportional to the pressure drop across a packed bed column. The greater the pressure drop, the larger will be the power consumption required for maintaining the desired flow of fluid. The pressure drop across the packed bed column operating at different volumetric flow rates was calculated using the Ergun equation as given in eqn.(xxii). It was observed that with increasing flow rate, the pressure drop also increased. The values for pressure drop at different volumetric flow rates are given in Table 14.

Table 15 – Pressure drop at different volumetric flow rates

F (cm3 min-1)	*Pressure drop (Pa)*
0.90	7.40
1.71	14.08
3.30	27.23

3.8.1. Friction factor calculation

The friction factor was calculated using eqn.(xxvi). For a packed bed, Reynold's number less than 1 corresponds to laminar flow. The Reynold's number was experimentally found in the range from 0.09 to 0.4. It was observed that with increasing flow rate, there was a decrease in the friction factor. This was attributed to the fact that with increasing flow rate, there was an increase in the Reynold's number. Usually, friction factor decreases with increasing Reynolds's number. A graph of log f_P vs log Re_P was plotted in Figure 14. It was a straight-line curve with a negative slope with R^2 square value equal to 1.

The graph showed that the system existed in the laminar flow regime, which correlated with the experimental data. The values of friction factor and Reynold's number for different flow rates are given in Table 16. Values of log f_P and log Re_P are given in Table 17.

Table 16 – Friction factor for different flow rates

F (cm³ min⁻¹)	***f_P***	***Experimental Reynold's number***
0.90	1537.06	0.0977
1.71	809.94	0.1856
3.30	420.39	0.3583

Table 17 – log f_P vs log Re_P

log Re_P	***log f_P***
-1.0101	3.1867
-0.7314	2.9085
-0.4458	2.6237

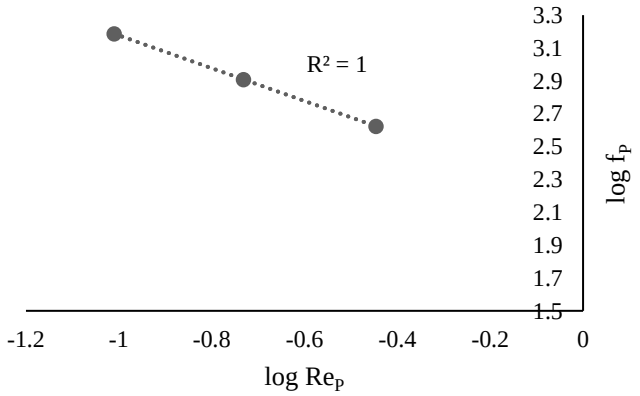


Figure 13 – Logf_P vs log Re_P

Chapter 5

Conclusion

Conclusion

Whole cells of *Kluyveromyces marxianus* was immobilized in calcium alginate beads. It was observed that parameters like alginate concentration and bead size were inversely proportional to the conversion percentage of lactose. Maximum lactose conversion was observed at 3% (w/v) alginate concentration and 2.94 mm bead diameter. Decreasing alginate concentration increased the pore size and decreasing bead size increased the available surface area to volume ratio, which all increased the lactose conversion percentage. Maximum conversion of lactose was observed at pH 4.4 and 25^0c temperature. Low pH value enables the immobilized cell system to be used in acid whey (pH 3.6-4.5). By using different substrate concentrations, it was observed the immobilized enzyme system followed Michaelis-Menten kinetics. The immobilized cell system was used in a packed bed column for continuous operation of lactose hydrolysis under various operating conditions. In case of an immobilized enzyme system, the substrate first needs to be transported from the bulk solution to the surface of the immobilized particle. The substrate forms a stationary layer around the surface from which it diffuses to the surface of the catalyst. Thus, an external mass transfer coefficient needs to be developed for an immobilized enzyme system. Furthermore, the diameter of the reactor, type of reactor, and operating conditions are affected by the external mass transfer in immobilized enzyme systems. An external mass transfer model was developed using the Chilton-Colburn factor $J_D = Re^{-(1-n)}$. The predicted mass transfer model is $J_D = 0.06362\ Re^{-0.02}$. It was observed that the predicted mass transfer model correlated with the experimental data. Damkohler number was calculated for the system. For each operating condition, the Damkohler number was less than 1, which indicates that the diffusion of substrate was much greater and the overall system was limited by the enzymatic reaction. The pressure drop across a packed bed is important for determining the power consumption required for pumping a fluid across the packed bed. The pressure drop across the packed bed was calculated using the Ergun equation. It was observed that with increasing substrate flow rate, the pressure drop also increased indicating more power consumption at higher volumetric flow rates. There was a decrease in the friction factor with increasing Reynold's number. A graph of log f_P vs Re_P showed that the system existed in the laminar flow regime, which correlated with the experimental data.

REFERENCES

Abdelrahim, K. A. (1989). Production and characterization of b-galactosidase from psychrotrophic Bacillus subtilis.

Agrawal, S., Sonawat, H. M., & Dutta, S. M. (1982). Thermostable beta-galactosidase from fungi. *Journal of dairy science*, *65*(5), 866-870.

Albayrak, N., & Yang, S. T. (2002). Production of galacto-oligosaccharides from lactose by Aspergillus oryzae β-galactosidase immobilized on cotton cloth. *Biotechnology and Bioengineering*, *77*(1), 8-19.

Anisha, G. S. (2017). β-galactosidases. In *Current developments in biotechnology and bioengineering* (pp. 395-421). Elsevier.

Ansari, S. A., & Husain, Q. (2012). Lactose hydrolysis from milk/whey in batch and continuous processes by concanavalin A-Celite 545 immobilized Aspergillus oryzae β galactosidase. *Food and Bioproducts processing*, *90*(2), 351-359.

Ariyanti, D., & Hadiyanto, H. (2013). Ethanol production from whey by Kluyveromyces marxianus in batch fermentation system: kinetics parameters estimation. *Bulletin of Chemical Reaction Engineering & Catalysis*, *7*(3), 179.

Batsalova, K., Kunchev, K., Popova, Y., Kozhukharova, A., & Kirova, N. (1987). Hydrolysis of lactose by β-galactosidase immobilized in polyvinylalcohol. *Applied microbiology and biotechnology*, *26*(3), 227-230.

Becerra, M., Baroli, B., Fadda, A. M., Méndez, J. B., & Siso, M. G. (2001). Lactose bioconversion by calcium-alginate immobilization of Kluyveromyces lactis cells. *Enzyme and Microbial Technology*, *29*(8-9), 506-512.

Bhalia, S., Naldu, A. D., & Kamaruddin, A. I. (1999). Modeling and Simulation of an Enzymatic Reactor for Hydrolysis of Palm Oil. *Artificial Cells, Blood Substitutes, and Biotechnology*, *27*(5-6), 435-440.

Borglum, G. B., & Sternberg, M. Z. (1972). Properties of a fungal lactase. *Journal of Food Science*, *37*(4), 619-623.

Bucke, C., & Brown, D.E. (1983). Immobilized cells. *Philosophical Transactions of the Royal Society of London. B, Biological Sciences*, *300*(1100), 369-389.

Carević, M., Vukašinović-Sekulić, M., Ćorović, M., Rogniaux, H., Ropartz, D., Veličković, D., & Bezbradica, D. (2018). Evaluation of β-galactosidase from Lactobacillus acidophilus as biocatalyst for galacto-oligosaccharides synthesis: Product structural characterization and enzyme immobilization. *Journal of bioscience and bioengineering*, *126*(6), 697-704.

Chanalia, P., Gandhi, D., Attri, P., & Dhanda, S. (2018). Purification and characterization of β-galactosidase from probiotic Pediococcus acidilactici and its use in milk lactose hydrolysis and galactooligosaccharide synthesis. *Bioorganic chemistry*, *77*, 176-189.

Cheng, C. W., Abdulla, R., Sridhar, R. R., & Ravindra, P. (2011). Determination of external mass transfer model for hydrolysis of Jatropha oil using immobilized lipase in recirculated packed-bed reactor. *Advances in Chemical Engineering and Science*, *1*(04), 289.

Damin, B. I. S., Kovalski, F. C., Fischer, J., Piccin, J. S., & Dettmer, A. (2021). Challenges and perspectives of the β-galactosidase enzyme. *Applied Microbiology and Biotechnology*, *105*(13), 5281-5298.

Das, B., Roy, A. P., Bhattacharjee, S., Chakraborty, S., & Bhattacharjee, C. (2015). Lactose hydrolysis by β-galactosidase enzyme: optimization using response surface methodology. *Ecotoxicology and Environmental Safety*, *121*, 244-252.

Das, B., Roy, A. P., Bhattacharjee, S., Chakraborty, S., & Bhattacharjee, C. (2015). Lactose hydrolysis by β-galactosidase enzyme: optimization using response surface methodology. *Ecotoxicology and Environmental Safety*, *121*, 244-252.

Datta, S., Christena, L. R., & Rajaram, Y. R. S. (2013). Enzyme immobilization: an overview on techniques and support materials. *3 Biotech*, *3*(1), 1-9.

Davani-Davari, D., Negahdaripour, M., Karimzadeh, I., Seifan, M., Mohkam, M., Masoumi, S. J., ... & Ghasemi, Y. (2019). Prebiotics: definition, types, sources, mechanisms, and clinical applications. *Foods*, *8*(3), 92.

Dey, G., Bhupinder, S., & Banerjee, R. (2003). Immobilization of alpha-amylase produced by Bacillus circulans GRS 313. *Brazilian Archives of Biology and Technology*, *46*, 167-176.

Elnashar, M. M., & Yassin, M. A. (2009). Lactose hydrolysis by β-galactosidase covalently immobilized to thermally stable biopolymers. *Applied biochemistry and biotechnology*, *159*(2), 426-437.

Eskandarloo, H., & Abbaspourrad, A. (2018). Production of galacto-oligosaccharides from whey permeate using β-galactosidase immobilized on functionalized glass beads. *Food chemistry*, *251*, 115-124.

Goncalves, J. A., & Castillo, F. J. (1982). Partial purification and characterization of β-D-galactosidase from Kluyveromyces marxianus. *Journal of dairy science*, *65*(11), 2088-2094.

Gupte, A. M., & Nair, J. S. (2010). β-galactosidase production and ethanol fermentation from whey using Kluyveromyces marxianus NCIM 3551. *Journal of Scientific & Industrial Research*, *69*, 855-859.

Hsu, C. A., Lee, S. L., & Chou, C. C. (2007). Enzymatic production of galactooligosaccharides by β-galactosidase from Bifidobacterium longum BCRC 15708. *Journal of Agricultural and Food Chemistry*, *55*(6), 2225-2230.

Hsu, C. A., Yu, R. C., & Chou, C. C. (2005). Production of β-galactosidase by Bifidobacteria as influenced by various culture conditions. *International Journal of Food Microbiology*, *104*(2), 197-206.

Husain, Q. (2010). β Galactosidases and their potential applications: a review. *Critical reviews in biotechnology*, *30*(1), 41-62.

Itoh, T., Suzuki, M., & Adachi, S. (1982). Production and characterization of β-galactosidase from lactose-fermenting yeasts. *Agricultural and Biological Chemistry*, *46*(4), 899-904.

Kamran, A., Bibi, Z., Aman, A., & Ul Qader, S. A. (2019). Purification and catalytic behavior optimization of lactose degrading β-galactosidase from Aspergillus nidulans. *Journal of food science and technology*, *56*(1), 167-176.

Knezevic, Z., Bobic, S., Milutinovic, A., Obradovic, B., Mojovic, L., & Bugarski, B. (2002). Alginate-immobilized lipase by electrostatic extrusion for the purpose of palm oil hydrolysis in lecithin/isooctane system. *Process Biochemistry*, *38*(3), 313-318.

Ku, M. A., & Hang, Y. D. (1992). Production of yeast lactase from sauerkraut brine. *Biotechnology letters*, *14*(10), 925-928.

Lactose-Free Products Market. (2020, June). MARKETSANDMARKETS. Retrieved August 2022, from https://www.marketsandmarkets.com/Market-Reports/lactose-free-products-market-4457397.html

Longo, M. A., Novella, I. S., García, L. A., & Díaz, M. (1992). Diffusion of proteases in calcium alginate beads. *Enzyme and microbial technology*, *14*(7), 586-590.

Martarello, R. D. A., Cunha, L., Cardoso, S. L., de Freitas, M. M., Silveira, D., Fonseca-Bazzo, Y. M., ... & Magalhães, P. O. (2019). Optimization and partial purification of beta-galactosidase production by Aspergillus niger isolated from Brazilian soils using soybean residue. *AMB Express*, *9*(1), 1-13.

Misselwitz, B., Pohl, D., Frühauf, H., Fried, M., Vavricka, S. R., & Fox, M. (2013). Lactose malabsorption and intolerance: pathogenesis, diagnosis and treatment. *United European gastroenterology journal*, *1*(3), 151-159.

Mörschbächer, A. P., Volpato, G., & Souza, C. F. V. D. (2016). Kluyveromyces lactis β-galactosidase immobilization in calcium alginate spheres and gelatin for hydrolysis of cheese whey lactose. *Ciência Rural*, *46*, 921-926.

Nath, S., & Chand, S. (1996). Mass transfer and biochemical reaction in immobilized cell packed bed reactors: correlation of experiment with theory. *Journal of Chemical Technology & Biotechnology: International Research in Process, Environmental AND Clean Technology*, *66*(3), 286-292.

Nguyen, T. H., Splechtna, B., Steinböck, M., Kneifel, W., Lettner, H. P., Kulbe, K. D., & Haltrich, D. (2006). Purification and characterization of two novel β-galactosidases from Lactobacillus reuteri. *Journal of Agricultural and Food Chemistry*, *54*(14), 4989-4998.

Panesar, P. S., Kumari, S., & Panesar, R. (2010). Potential applications of immobilized β-galactosidase in food processing industries. *Enzyme research*, *2010*.

Panesar, P. S., Panesar, R., Singh, R. S., Kennedy, J. F., & Kumar, H. (2006). Microbial production, immobilization and applications of β-D-galactosidase. *Journal of Chemical Technology & Biotechnology: International Research in Process, Environmental & Clean Technology*, *81*(4), 530-543.

Panesar, R., Panesar, P. S., Singh, R. S., & Bera, M. B. (2007). Applicability Of Alginate Entrapped Yeast Cells For The Production Of Lactose-Hydrolyzed Milk. *Journal of Food Process Engineering*, *30*(4), 472-484.

Panesar, R., Panesar, P. S., Singh, R. S., & Kennedy, J. F. (2011). Hydrolysis of milk lactose in a packed bed reactor system using immobilized yeast cells. *Journal of Chemical Technology & Biotechnology*, *86*(1), 42-46.

Park, Y. K., De Santi, M. S. S., & Pastore, G. M. (1979). Production and characterization of β-galactosidase from Aspergillus oryzae. *Journal of food science*, *44*(1), 100-103.

Princely, S., Basha, N. S., Kirubakaran, J. J., & Dhanaraju, M. D. (2013). Biochemical characterization, partial purification, and production of an intracellular beta-galactosidase from

Streptococcus thermophilus grown in whey. *European Journal of Experimental Biology*, *3*(2), 242-251.

Rodriguez-Colinas, B., de Abreu, M. A., Fernandez-Arrojo, L., de Beer, R., Poveda, A., Jimenez-Barbero, J., ... & Plou, F. J. (2011). Production of galacto-oligosaccharides by the β-galactosidase from Kluyveromyces lactis: comparative analysis of permeabilized cells versus soluble enzyme. *Journal of agricultural and food chemistry*, *59*(19), 10477-10484.

Saqib, S., Akram, A., Halim, S. A., & Tassaduq, R. (2017). Sources of β-galactosidase and its applications in food industry. *3 Biotech*, *7*(1), 1-7.

Shukla, T. P., & Wierzbicki, L. E. (1975). Beta-galactosidase technology: A solution to the lactose problem. *Critical Reviews in Food Science & Nutrition*, *5*(3), 325-356.

Shuler, M. L., & Kargi, F. (2001). *Bioprocess Engineering: Basic Concepts* (2nd ed.). NJ: Prentice Hall.

Simović, M., Vukašinović-Sekulić, M., Grbavčić, S., Stojanović, M., Mihailović, M., Dimitrijević, A., & Bezbradica, D. (2015). Optimization of (-galactosidase production from lactic acid bacteria. *Hemijska industrija*, *69*(3), 305-312.

Siso, M. G., & Doval, S. S. (1994). Kluyveromyces lactis immobilization on corn grits for milk whey lactose hydrolysis. *Enzyme and microbial technology*, *16*(4), 303-310.

Skryplonek, K., Gomes, D., Viegas, J., Pereira, C., & Henriques, M. (2017). Lactose-free frozen yogurt: Production and characteristics. *Acta Scientiarum Polonorum Technologia Alimentaria*, *16*(2), 171-179.

Song, C., Liu, G. L., Xu, J. L., & Chi, Z. M. (2010). Purification and characterization of extracellular β-galactosidase from the psychrotolerant yeast Guehomyces pullulans 17-1 isolated from sea sediment in Antarctica. *Process Biochemistry*, *45*(6), 954-960.

Szczodrak, J. (2000). Hydrolysis of lactose in whey permeate by immobilized β-galactosidase from Kluyveromyces fragilis. *Journal of Molecular Catalysis B: Enzymatic*, *10*(6), 631-637.

Tandon, R. K., Joshi, Y. K., Singh, D. S., Narendranathan, M., Balakrishnan, V., & Lal, K. (1981). Lactose intolerance in North and South Indians. *The American journal of clinical nutrition*, *34*(5), 943-946.

Tanriseven, A., & Doğan, Ş. (2002). A novel method for the immobilization of β-galactosidase. *Process Biochemistry*, *38*(1), 27-30.

Vera, C., Guerrero, C., Aburto, C., Cordova, A., & Illanes, A. (2020). Conventional and non-conventional applications of β-galactosidases. *Biochimica et Biophysica Acta (BBA)-Proteins and Proteomics*, *1868*(1), 140271.

Wang, G., Wang, H., Chen, Y., Pei, X., Sun, W., Liu, L., ... & Wang, M. (2021). Optimization and comparison of the production of galactooligosaccharides using free or immobilized Aspergillus oryzae β-galactosidase, followed by purification using silica gel. *Food Chemistry*, *362*, 130195.

Warmerdam, A., Benjamins, E., de Leeuw, T. F., Broekhuis, T. A., Boom, R. M., & Janssen, A. E. (2014). Galacto-oligosaccharide production with immobilized β-galactosidase in a packed-bed reactor vs. free β-galactosidase in a batch reactor. *Food and Bioproducts Processing*, *92*(4), 383-392.

Wentworth, D. S., Skonberg, D., Donahue, D. W., & Ghanem, A. (2004). Application of chitosan-entrapped β-galactosidase in a packed-bed reactor system. *Journal of Applied Polymer Science*, *91*(2), 1294-1299.

Won, K., Kim, S., Kim, K. J., Park, H. W., & Moon, S. J. (2005). Optimization of lipase entrapment in Ca-alginate gel beads. *Process biochemistry*, *40*(6), 2149-2154.