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PURIFICATION OF B-GALACTOSIDASE ENZYME BY PROBIOTIC *LIMOSILACTOBACILLUS FERMENTUM* LF08

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1. INTRODUCTION AND AIM

Enzymes, also known as biological catalysts or biocatalysts, accelerate biochemical reactions in living organisms. They can be extracted from cells and used to catalyse a wide range of commercially significant reactions. Enzymes play important roles in a variety of industrial applications, such as sweetener production, antibiotic modification, and the manufacture of washing powders and cleaning products. They are also necessary components of analytical devices and assays used in clinical, forensic, and environmental settings (Robinson, 2015).

Enzyme purification is a critical step in enzyme biotechnology, it is the process of removing impurities such as other proteins, nucleic acids, lipids, and small molecules from crude enzyme preparations obtained through microbial fermentation or tissue extraction. Purification increases the enzyme's specific activity, making it more efficient and cost-effective for industrial applications. Furthermore, purification improves enzyme stability, extends shelf life, and ensures consistent performance across batches. Purified enzymes are easier to characterise, which leads to a better understanding of their properties such as enzyme kinetics, substrate specificity, pH, and temperature optimums. This knowledge is critical for optimising process conditions and increasing enzyme performance. Furthermore, purified enzymes are required for biomedical applications such as diagnostic assays, drug development, and gene cloning, where contaminants in crude enzyme preparations can produce incorrect results.

β-Galactosidase, an enzyme with widespread industrial applications, plays a crucial role in the hydrolysis of lactose into its constituent monosaccharides, glucose, and galactose. This enzymatic activity has significant implications across various industries, including food, dairy, pharmaceuticals, and biotechnology. In the food industry, β-galactosidase hydrolyzes lactose in milk and dairy products, making them suitable for lactose intolerant individuals. It is also used in the production of low-lactose or lactose-free dairy products like cheese, yoghurt, and ice cream, which improves digestibility and expands market reach. Furthermore, β-galactosidase has potential applications in biotechnology, particularly in glycobiology. It is used to enzymatically synthesise galactooligosaccharides (GOS), which have prebiotic properties and are used as functional food ingredients and dietary supplements.

Probiotic microorganisms have garnered significant attention as potential sources for industrial enzyme production, including β-galactosidase. These microorganisms have several advantages, including their GRAS (Generally Recognised as Safe) status, robustness, and ability to produce high levels of β-Galactosidase under appropriate fermentation conditions. *Limosilactobacillus fermentum*, formerly known as *Lactobacillus fermentum*, is a probiotic bacterium that can be found in fermented foods and the gastrointestinal tracts of both humans and animals. It produces β-galactosidase during metabolism, making it a promising candidate for industrial production.

In this context, the optimization of fermentation and purification processes for β-galactosidase produced by *Limosilactobacillus fermentum* is of paramount importance. This thesis explores the potential of *Limosilactobacillus fermentum* LF08 as a source for industrial β-galactosidase production and help in developing an efficient fermentation and purification protocol for highly pure and active enzyme preparations. However, depending on the enzyme's properties, unique challenges may arise during the purification process. These challenges may include enzyme insolubility and loss of enzyme activity. While several traditional and modern methods exist to address these issues, enzyme purification remains a significant challenge (Dako et al. 2012). Classic methods include salting out, chromatography, and dialysis, whereas more recent approaches use affinity chromatography, ultrafiltration, and protein engineering techniques. Despite the availability of these methods, determining the best purification strategy and optimising purification conditions for a specific enzyme can be difficult and time-consuming. Furthermore, the purification process must be closely monitored to ensure the preservation of enzyme activity and stability while achieving the desired level of purity.

The objective of this thesis is to assess the purification protocol for β-galactosidase obtained from the probiotic strain *Limosilactobacillus fermentum* LF08. The purification process consists of several steps, including enzyme fermentation, cell disruption, ammonium sulphate saturation, dialysis, and FPLC chromatography. The study aims to evaluate the efficacy of each purification step and its impact on enzyme purity and activity by calculating purification factors. Furthermore, the study seeks to determine the best conditions for key purification steps like ammonium sulphate saturation and enzymatic cell lysis. Finally, the study aims to identify potential modifications to the purification protocol that will improve enzyme stability and overall purification efficiency, aiming to enable more effective enzyme isolation in future applications.

2. LITERATURE REVIEW

2.1 β-galactosidase overview

2.1.1 Definition and specificity

"*A β-galactosidase (EC 3.2.1.23, β-D-galactoside galactohydrolase), commonly known as lactase is known to catalyze, not only hydrolyze β-D-galactoside linkage of lactose or other β-galactosides into monosaccharides, glucose and galactose but also has transgalactosylation activity to synthesize galactooligosaccharides*"(Lee, 2008). It is well-known for its uses in the dairy and food industries, where it can be used to produce a variety of goods, including galactooligosaccharides , allolactose, and hydrolyzed lactose milk (Ruiz-Ramírez & Jiménez-Flores, 2023).

Such an example of β-galactosidase application is found in carbohydrates that contain galactose in which the glycosidic bond is positioned above the galactose molecule itself. Studies conducted by Huber and Gaunt in 1983 also have shown that β-galactosidases exhibit substrate specificity, acting on diverse molecules such as ganglioside GM1, lactosylceramides, lactose, and various glycoproteins. Although the enzyme is very specific for D-galactose (Huber' & Gaunt, 1983). The 2,3 and 4 positions of hydroxyl groups on the galactose molecule are crucial to its function, β-galactosidase also catalyses reactions with β-D-galactopyranosides with an oxygen glycosidic (Lederberg, 1950).

Moreover, for the enzyme to catalyse the reaction the hydroxyls need to be present in the correct orientations in the mentioned position. Under certain circumstances, such as very high sugar concentrations or the absence of particular hydroxyl groups at different positions, β-galactosidase catalyses the conversion of D-glucose into other sugars like D-galactopyranose, L-arabinopyranose, D-fucopyranose, and D-galactal in the reverse reaction (Huber' & Hurlburt, 1986). In addition, p-nitrophenyl-α-L-arabinopyranoside and p-nitrophenyl-β-D-fucopyranoside are also substrates for β-galactosidase; however, their binding efficiency is low, and their pace of reaction is slower than that of other substrates (Loeffler et al., 1979).

Consequently, sugars that have undergone modifications at the D-galactose's C6 hydroxyl position are still substrates, although poor ones, while sugars that have changed anywhere else (aside from D-galactal) are inert. The reason D-galactal interacts is because it produces a relatively stable covalent intermediate when Glu537 combines with the C1 with a concomitant proton addition at the C2 location (Otieno, 2010). As a result, 2-deoxy-galactose is formed and covalently bonded, which is released after hydrolysis. The covalent entity combines with glucose in the reversion reaction at high D-galactal and glucose concentrations (Juers et al., 2000).

2.1.2 Hydrolytic and transgalactosylation activities of β-galactosidase

In the hydrolysis reactions it catalyses, β-galactosidase first binds to the substrate lactose and changes its conformation to fit the substrate into its active site (Figure 1a). Particular amino acid residues in this active site help to break the glycosidic bond in lactose between glucose and galactose. Concurrently, glucose is released from the active site and the galactose moiety is moved to a nucleophilic acceptor molecule to form an enzyme–galactosyl complex.
Lactose is effectively broken down into its component monosaccharides by this process, allowing the cell to use them. The enzyme's adaptability in catalysing trans-galactosylation reactions as well as hydrolysis is demonstrated by the transfer of the galactosyl group to an acceptor molecule, which aids in lactose metabolism and cellular homeostasis (Otieno, 2010).

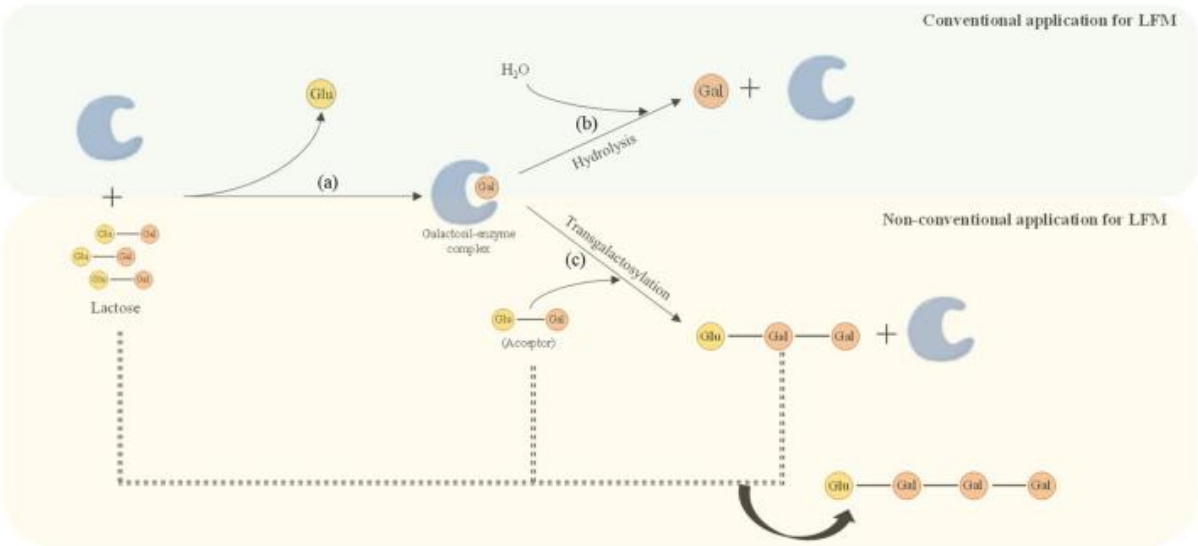

Figure 1. β-galactosidase enzyme's catalytic mechanism using lactose as substrate: The formation of the enzyme–galactosyl complex (a); the hydrolytic reaction (b); and the transgalactosylation mechanism (c). Lactose-free milk (LFM), glucose and galactose are denoted by the letters Glu and Gal respectively *(Liburdi & Esti, 2022).*

If water serves as the acceptor molecule, galactose is obtained as one of the products along with glucose. The enzyme catalyzes the transfer of the galactosyl group from lactose to a water molecule, which results in the formation of galactose and the release of glucose (Figure 1b) (Otieno, 2010).

Apart from water, the medium's lactose can also act as an acceptor molecule in the β-galactosidase-catalyzed transgalactosylation process. In these conditions, the enzyme promotes the synthesis of galactooligosaccharides rather than hydrolyzing lactose into glucose and galactose (Figure 1c). This happens when the enzyme creates GOS products by moving a galactosyl group from one lactose molecule to another. The source of the enzyme and the particulars of the reaction determine which of the two processes is preferred: transgalactosylation or hydrolysis. At the same lactose concentration, different enzymes produce different amounts of GOS due to their varied affinities for water and saccharides (Zarate & Lopez-Leiva, 1990) .

2.1.3 Structure of β-galactosidase

A variety of factors, including variations in quaternary structure, gene regulation, organism-specific modifications, and evolutionary differences, can cause variations in β-galactosidase structures across different sources. The three-dimensional structures and amino acid sequences of these enzymes vary because they have independently evolved throughout the evolutionary history of various animals.

One of the recently studied β-galactosidase enzyme is from *Lacticaseibacillus casei w*hich is one of the most often used probiotic *Lactobacillus* species. It is anticipated that the GH35 protein (GnbG) derived from *L. casei* BL23 is 6-phosphobeta-galactosidase (EC 3.2.1.85).

According to crystal structure study, subsite -1 of LBCZ_0230 is not suited for binding to 6-phospho-β-D-galactopyranoside since its structure is highly similar to that of *Streptococcus pneumoniae* β-galactosidase BgaC (Figure 2). Based on these structural and biochemical tests it can be concluded that LBCZ_0230 is a β-galactosidase (Saburi et al., 2023).

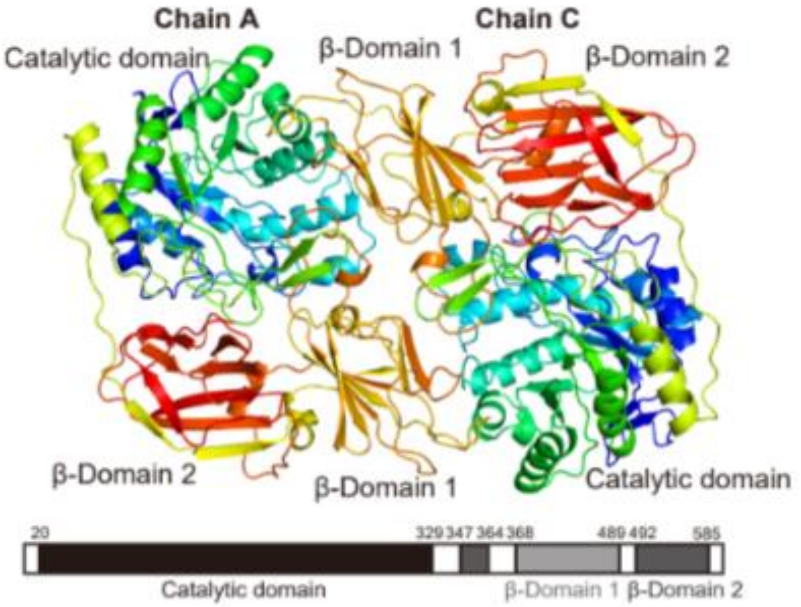

Figure 2. Structural analysis of LBCZ_0230, Similar monomers, four of which make up LBCZ_0230, found in an asymmetric unit. In solution, chains A and C, as well as B and D, form stable dimers. Three domains make up the monomer of LBCZ_0230: two β-domains that come after the catalytic domain and the (β/α)8-barrel catalytic domain. *(Saburi et al., 2023).*

Matthews (2005) conducted studies on *E.coli* β-galactosidase, which is a tetramer of four identical 1023-amino acid chains. Each chain has five domains, the third of which makes up the majority of the active site and is an eight-stranded α/β barrel. Nonetheless, parts of this site come from different subunits and domains. One of the subunit interfaces is formed in part by the polypeptide chains' N-terminal regions.

When combined, these characteristics offer a structural foundation for the widely recognised characteristic of α-complementation. The creation of a covalent galactosyl intermediate with Glu537 is the first step in the catalytic process, which involves both "deep" and "shallow" types of substrate binding. The four monomers of the tetramer, arranged around three two-fold axes of symmetry that are mutually perpendicular. One could think of these axes as forming three unique interactions involving various monomer pairs. Monomer A contributes its Domain 2 loop to complete Monomer D's active site, as shown in Figure 3. Similarly, a loop from Monomer A's active site is completed by Monomer D. Monomer B and C also reciprocally donate and receive each other, resulting in a total of four functioning active sites. Based on their clear separation, these active sites most likely function separately (Matthews, 2005).

Mg^{2+} and Na^{+} are both necessary for β-galactosidase to function at its highest level (Wallenfels & Weil, 1972).

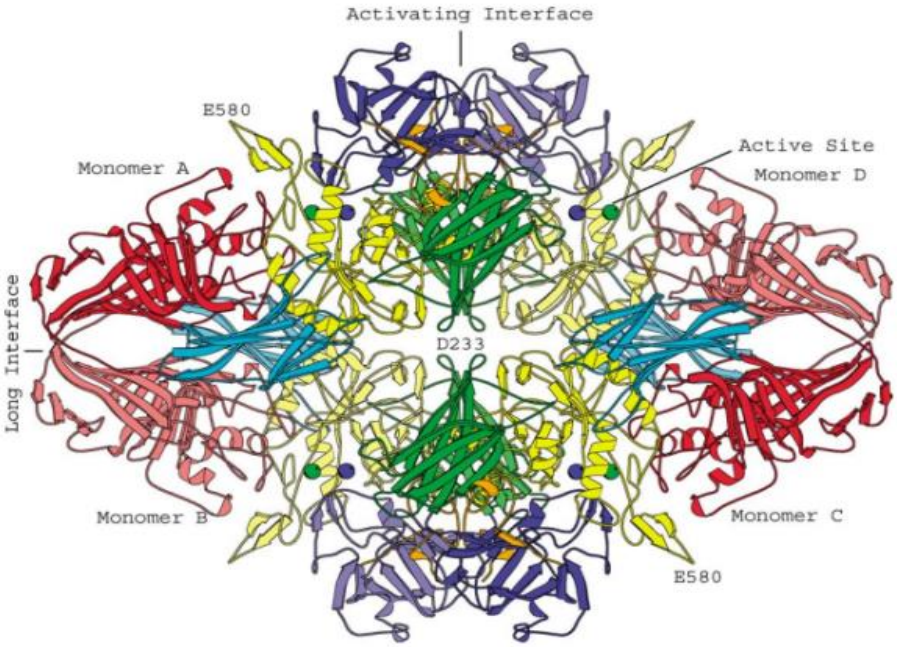

Figure 3. View of the β-galactosidase tetramer *(Juers et al., 2000).*

In the active site of both crystal forms of the native enzyme, a magnesium ion was found. The higher-resolution structure also revealed a number of additional potential Mg^{2+} binding sites, although no functional importance was assigned to them. Five potential locations for binding potassium were found through studies of crystals soaked in potassium and rubidium. The remaining ones are on the protein's surface, with one being in the active site (Juers et al., 2000).

2.1.4 Microbial sources of β-galactosidase

β-Galactosidase is present in fungi, yeasts, and bacteria. However, *Aspergillus* and *Kluyveromyces* are usually the most widely used sources of β-galactosidase for commercial and industrial purposes. These microorganisms have a number of benefits for large-scale production, such as strong growth traits, simplicity in cultivation, and high enzyme yields. Thus, lactose hydrolysis, transgalactosylation, and galactooligosaccharide synthesis are just a few of the processes that use β-galactosidase, which is derived from *Aspergillus* and *Kluyveromyces*, extensively in food processing and other industries (Zhou & Chen, 2001).
Lactic acid-producing bacteria are widely utilized as probiotics due to their numerous beneficial effects.

These microbes are essential for defending the host against dangerous germs, bolstering the immune system, increasing feed digestibility, and easing metabolic disorders (Naghmouchi et al., 2020). Probiotics such as *Lactobacillus* and *Bifidobacterium* species are well known and often used because of their ability to support gut health and general well-being. Additionally, these bacteria are excellent providers of β-galactosidase. From a variety of bacterial strains *Bifidobacterium infantis* CCRC14633, *Bifidobacterium longum* CCRC 15708, and *Bifidobacterium longum* CCRC15708 have shown exceptionally high β-galactosidase enzyme activity (Hsu et al., 2007).

Significantly for their involvement in the production of different fermented milk products are *lactobacilli* that were isolated from the gastrointestinal tract of piglets. Furthermore, β-galactosidase obtained from swine strains of *lactobacilli* exhibits the ability to break down lactose in cow's milk. The ability of the colon's microbiota to ferment intestinal lactose is indicated by the activity of β-galactosidase. Because of the vital role this enzyme plays in lactose digestion and general gut health (He et al., 2008).

Additionally, different yeast species have different potentials when it comes to providing β-galactosidase for different industrial uses. Namely, β-galactosidase has been identified from microbial sources such as *Saccharomyces lactis* (Yashphe & Halvorson, 1976). *Tolulopsis versatilis* M6, *Tolulopsis sphaerica* J28, *Candida pseudotropicalis* B57, and A60 (Itoh et al., 1982) , and fungal strains like *Rhizomucor pusillus* (Panesar et al., 2018).

Furthermore, the capacity to produce β-galactosidase has been characterised in yeast strains linked to domestic alcohol production in Northeast India (Das et al., 2017). The properties of bacterial, yeast and fungal β galactosidase are presented in Table 1.

The superior efficiency and robustness of β-galactosidases derived from fungal and yeast sources make them the preferred option for industrial applications. Due to their high catalytic activity, lactose can be hydrolyzed or transgalactosylated quickly and effectively. They also exhibit exceptional resistance to pH and temperature variations, which makes them ideal for use in a variety of industrial processes that might be harsh. In industrial applications like dairy processing, food production, and bioremediation, their stability under a range of environmental conditions guarantees consistent performance and prolonged enzyme activity, all of which contribute to increased productivity and cost-effectiveness (Movahedpour et al., 2022).

Table 1. Properties of bacterial, yeast and fungal β-galactosidases *(Saqib et al., 2017).*

Microorganism	Location	Temperature	pH
Bacteria			
Escherichia coli	Intracellular	40	7.2
Lactobacillus thermophilus	Intracellular	55	6.2
Bacillus circulans	Intracellular	56	6.5
Leuconostoc citrovorum	Intracellular	65	6.0
Yeast			
Kluyveromyces lactis	Intracellular	30-35	6.5-7.0
Kluyveromyces fragilis	Intracellular	30-35	6.0
Fungi			
Aspergillus niger	Unknown	55	3.5-4.5
Kluyveromyces fragilis	Cell bound	35-45	6.3-6.5
Aspergillus foetidu	Extracelluar	66-67	3.5-4.0
Aspergillus oryzae	Extracellular	55–60	4.5–5

2.2 *Limosilactobacillus fermentum* as a producer of β-galactosidase

Species belonging to the *Lactobacillus* genus rank among the most significant in terms of human nutrition and food microbiology. Certain species are employed as probiotics, vaccine carriers, and food preservatives in addition to being capable of producing fermented foods (Salvetti et al., 2012). Gram-positive bacteria of the species *Limosilactobacillus fermentum* are present in a variety of environment, such as dairy products, fermenting plant materials, and fermented cereals. Usually, it takes the form of rod- or coccoid-shaped cells. Since this bacterium produces a range of metabolites, it is regarded as heterofermentative. It can live in both aerobic and anaerobic conditions because of its tolerance to some amount of oxygen. Furthermore, *L. fermentum* has been found in environments like sewage, manure, and faeces (Dowarah et al., 2018).

Strains of *Limosilactobacillus fermentum* have been demonstrated to benefit the host's antioxidant and anti-inflammatory systems in both experimental and clinical trials. In diabetic conditions, these effects help to improve glucose homeostasis (Lacerda et al., 2022).

Two neighbouring genes, lacL (encoding the large subunit) and lacM (encoding the small subunit), which share 17 overlapping nucleotides, encode the LacLM β-galactosidase present in *Lactobacillus fermentum* K4. This enzyme is closely related to other β-galactosidases found in *Lactobacillus* species, according to phylogenetic analysis (Liu et al., 2011).

The probiotic strain *Lactobacillus fermentum* CM33, which was isolated from healthy infants, was selected because of its capacity to generate β-galactosidase.

This bacterium produces β-galactosidase with transgalactosylation activity, which enables the synthesis of galactooligosaccharides , and it displays other traits that are characteristic of probiotics. It is expected that these GOS will work in concert with the living cells of *L. fermentum* CM33 to create a symbiotic nutraceutical formulation (Sriphannam et al., 2012).

2.3 Lactose operon

Genes in prokaryotic organisms frequently form operons, which contain multiple coding regions controlled by a single promoter. Operons typically contain genes required in the same metabolic pathway. This collective regulation enables the cell to efficiently control entire pathways, activating and deactivating them as needed. This streamlined regulation improves cell efficiency and adaptability by optimizing metabolic processes and coordinating gene expression.

The lac operon is made up of three adjacent coding regions: lacZ, lacY, and lacA (Figure 4). Each gene plays a specific role in lactose metabolism. LacZ encodes β-galactosidase, which breaks down lactose into simpler sugars. LacY encodes lactose permease, which helps lactose molecules move into the bacterial cell. The lac operon tightly regulates the expression of these genes, resulting in efficient lactose utilization. LacA, which encodes lactose acetylase, serves a less obvious function and is generally less important for lactose metabolism (Clark & Pazdernik, 2013). LacZ gene-encoded β-galactosidase, found in bacteria like *E. coli*, carries out three essential enzymatic functions that are vital for lactose metabolism. First, it catalyses the hydrolysis of lactose, converting it into glucose and galactose, which the body can use for glycolysis to produce energy. Second, allolactose, a crucial inducer molecule, is formed when lactose is trans-galactosylated with the help of β-galactosidase. Thirdly, it converts galactose and glucose back into allolactose. The significance of allolactose lies in its ability to bind to the lac repressor protein, releasing it from the lac operator region of the lac operon. The transcription of the lacZYA genes, including lacZ, is made possible by this release. As a result, the production of β-galactosidase is induced by the accumulation of allolactose, thereby facilitating efficient lactose metabolism and creating a positive feedback loop. Thus, through the feedback loop mediated by allolactose, β-galactosidase not only facilitates lactose utilisation but also regulates its own expression (Huber et al., 1976).

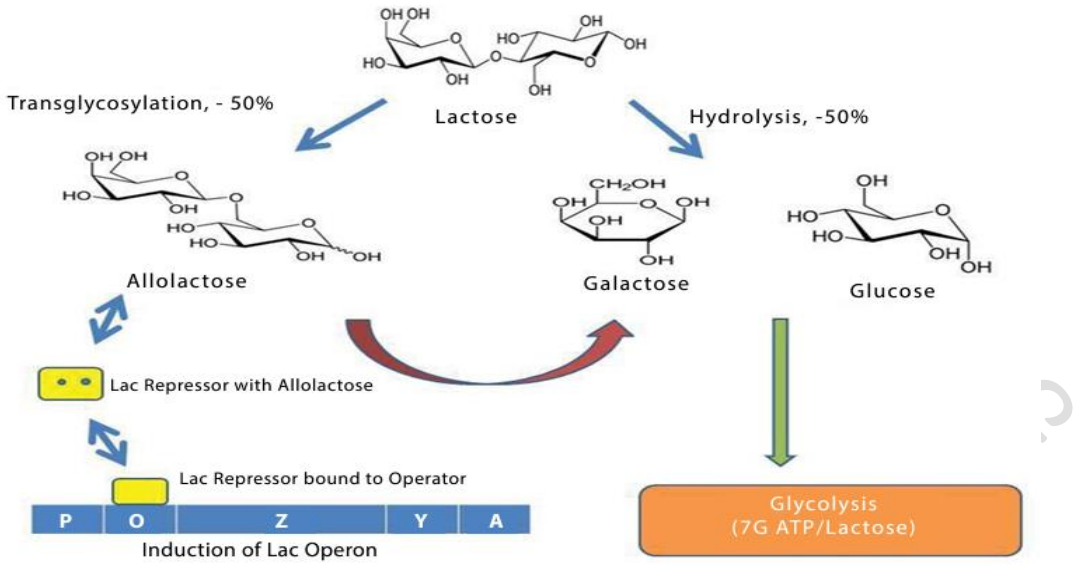

Figure 4. Diagram describing β-galactosidase's roles within the cell *(Huber' & Hurlburt, 1986).*

2.4 Application of β-galactosidase in the Food Industry

β-galactosidase is widely used in food processing, dairy processing, and pharmaceutical applications as a biocatalyst for lactose hydrolysis. For example, whey, a byproduct of making cheese, has a high (4–4.5%) lactose content that makes it difficult to digest and is frequently thrown away, which pollutes the environment. β-galactosidase helps to hydrolyze lactose in whey so that, once the rate reaches 80%, it can be converted into feed or other useful products (Du et al., 2022).

Essential applications of β-galactosidase are transglycosylation activity to synthesise prebiotic galactooligosaccharides, also the production of milk with low concentration of lactose and lactose free milk or dairy products for lactose intolerance affecting 75% of the global population caused by β-galactosidase enzyme deficiency that is crucial for lactose breakdown (Liburdi & Esti, 2022).

2.4.1 Galactooligosaccharides production

Blends of linear and branched oligosaccharides with degrees of polymerization (DP) ranging from 2 to 8 are known as galactooligosaccharides . Lactose is the substrate used by microbial β-galactosidases to synthesise these compounds (Lu & Xiao, 2017).

The transfer of galactosyl groups from lactose to lactose molecules or to hydrolysates like glucose or galactose is catalysed by β-galactosidases. The product of this sequential transfer process is a mixture of galactooligosaccharides (Lu et al., 2020). GOS can be synthesised in free or immobilised forms using β-galactosidases. Enzyme immobilisation is especially appealing for large-scale synthesis due to its ability to be reused, enhance stability, and facilitate product separation (Panesar et al., 2018).

GOS generated by β-galactosidases consist of a blend of transgalactosylated oligosaccharides, along with residual unreacted lactose, glucose, and galactose. Yeast cells have the capacity to ferment these mixtures because they can utilize lactose and monosaccharides separately. High-purity oligosaccharides are produced as a consequence of this fermentation process, which can be used to treat intestinal disorders or by people with diabetes.

An important source of low-glycemic-index carbohydrates for dietary management and medicinal applications is the enrichment of oligosaccharides in the fermentation product, which is made possible by the yeast cells' selective consumption of lactose and monosaccharides (Aburto et al., 2018).

Recently, an advanced system has been created that allows a single organism to be used for the synthesis and purification of high-purity galactooligosaccharides. *Kluyveromyces lactis* cells are treated with ethanol in this system to improve substrate entry and product release (Figure 5). These treated cells are then cultured with lactose to produce GOS. After ethanol treatment, the resultant product mixture is fermented with active *K. lactis* cells to eliminate unwanted lactose, galactose, and glucose. During fermentation, ethanol is simultaneously produced and can be recycled for the first cell treatment. This novel strategy provides an effective and recyclable way to produce GOS with purity levels higher than 95% (Sun et al., 2016).

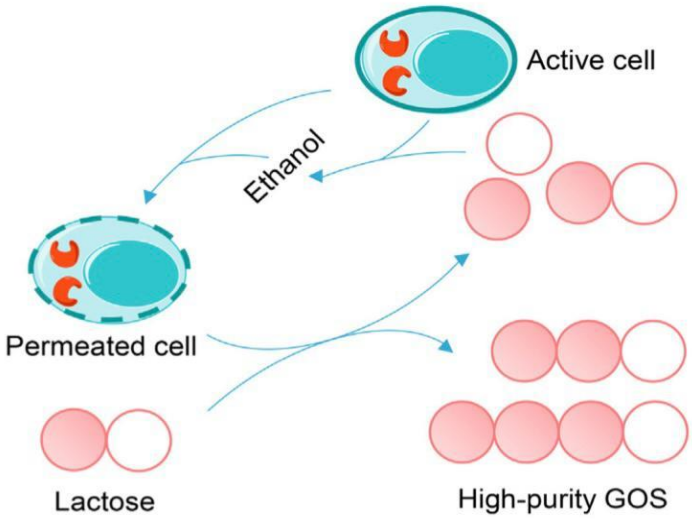

Figure 5. High-purity GOS is synthesised by sequentially using *K. lactis* cells that are permeated and active *(Lu et al., 2020).*

2.4.2 Whey utilisation

Whey is a byproduct of cheese making that includes minerals, proteins, and lactose. Some of it is disposed of, contaminating water, in many developing countries, even though some is used for permeates and protein concentrates. Whey, on the other hand, can be processed to yield useful products like ethanol and β-galactosidase, which is increasingly employed in the production of lactose-free products and has advantages for the environment and the economy (Kokkiligadda et al., 2016). The hydrolysis of lactose in whey permeate into glucose and galactose is one possible application for whey utilisation and this can be accomplished through using immobilized β-galactosidase (Ryan & Walsh, 2016). When compared to lactose alone, hydrolyzed lactose solutions have a greater sweetening power. This is because the relative sweetness of sucrose is roughly 80% for glucose and 60% for galactose and because of their increased sweetness, hydrolyzed lactose solutions are used in a variety of industries, including the manufacturing of soft drinks, ice cream, and confections. They could take the place of corn syrup or sucrose in these goods (Gänzle et al., 2008).

Two important byproducts of milk processing are milk whey (MW) and milk whey permeate (MWP). Making use of β-galactosidases' transgalactosylation activity is a great way to increase the value of these co-products. Using immobilised β-galactosidase, this enzymatic method allows for the synthesis of galactooligosaccharides , providing a viable method to enhance MW and MWP (Hackenhaar et al., 2021).

2.5. Enzyme fermentation

Enzymes for industrial use are produced through the process of fermentation. It makes use of microorganisms like yeast and bacteria. Submerged fermentation and solid-state fermentation are the two primary fermentation techniques used to produce enzymes (Renge et al., 2012).

Enzyme production is affected by a number of fermentation parameters, including β-galactosidase. The ideal temperature for β-galactosidase activity, as well as other factors like incubation time, temperature, inoculum level, moisture content, starting pH, agitation speed, and fermentation medium size, are some of them (Akcan, 2018).

In an investigation to look into the production of galactooligosaccharides from lactose by *Lactobacillus bulgaricus* strain 43's β-galactosidase. At a pH of 6.5 and a temperature of 55°C, the enzyme showed maximum activity. Furthermore, its activity was boosted by the presence of Mg^{2+}, Mn^{2+}, and Ca^{2+} ions (Arsov et al., 2022). Fermentation parameters for β-galactosidase production in *Lactobacillus leichmannii* 313 (ATCC 7830™) were also investigated. The initial pH ranged from 5.5 to 7.5, and the incubation temperature ranged from 30 to 55 °C. Tested carbon sources included lactose, galactose, fructose, sucrose, and glucose. Initial pH of 7.0 and 15.29 g/L of lactose were found to be the optimal conditions for maximum β-galactosidase production (Deng et al., 2020).

2.6. Enzyme purification

Enzyme purification is essential for understanding the structural and functional properties of an enzyme and predicting its potential applications. The intended use of the enzyme determines the necessary level of purity. Achieving the highest yield of the desired enzyme with the best catalytic activity and purity is the aim of purification techniques. Numerous purification techniques that are frequently employed in lab research can be expanded for use in industrial scale.

These techniques include precipitation and different chromatographical steps as gel filtration chromatography and ion-exchange chromatography, as well as centrifugation, ultrafiltration, diafiltration. Every technique has its own advantages and can be customised to meet the needs of the purification process as well as the unique properties of the enzyme (Bajpai, 2014). During the first stages, it is advised to use simple and affordable purification techniques, particularly when working with large volumes. More advanced and expensive methods can be used as the volume drops.

Three essential requirements should be satisfied by an effective purification plan: 1) reaching a high level of purity at the end; 2) preserving a high level of overall enzyme activity recovery; and 3) guaranteeing that the purification procedure is repeatable. These requirements are crucial for getting pure enzymes that are appropriate for a range of uses, all the while maximising yield and preserving consistency between batches (Salem, 2001).

2.6.1. Cell Disruption of Gram-positive bacteria

Gram-positive bacteria have thick mucopeptide layers in their cell walls, which are also referred to as peptidoglycan networks. These strains of lactic acid bacteria include *Lactobacillus, Streptococcus, Lactococcus, Leuconostoc,* and others. This polymeric substance greatly increases the cell wall's rigidity. It is composed of N-acetyl-D-glucosamine and N-acetyl-muramic acid units that alternate and are linked together in polysaccharide (glycan) chains via β-(1-4) linkage (Wicken, 1985). Intracellular products from microbial cells can be released using a variety of techniques (Figure 6), such as mechanical, physical, chemical, enzymatic, and combination approaches.

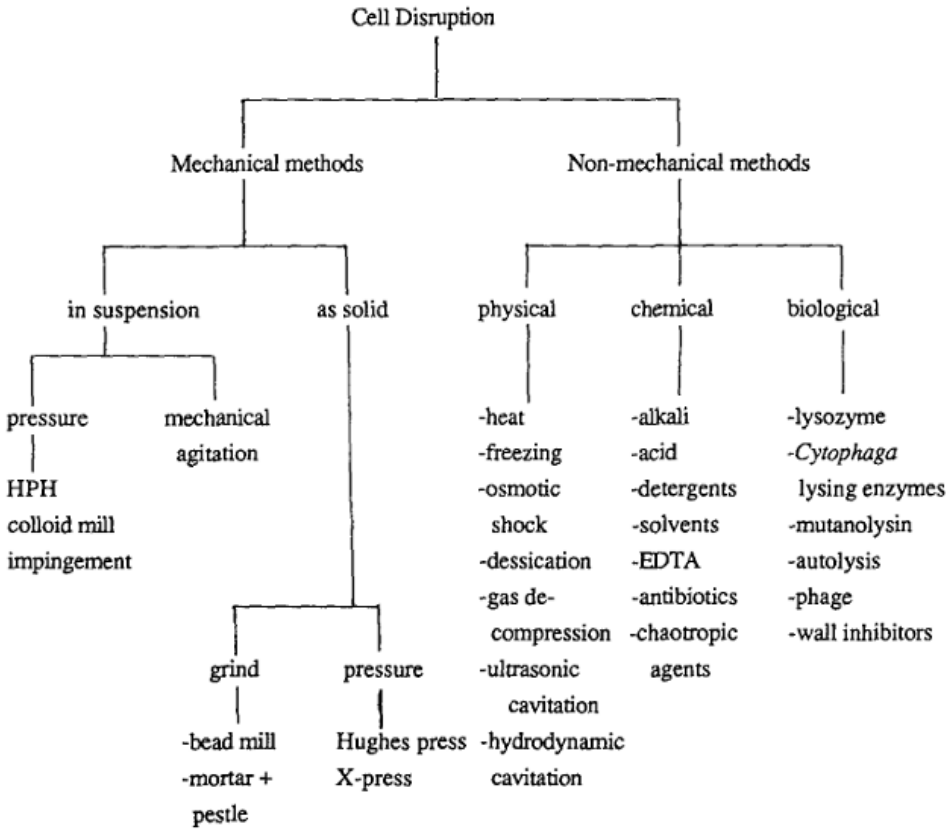

Figure 6. The classification of techniques reported in the literature *(Harrison, 1991).*

The type of intracellular products and the microbial species used determine how effective these techniques are. The selection of a technique is contingent upon various factors, including the microorganism's type, the target product's characteristics, and the desired level of purity (Geciova et al., 2002).

Lysozyme is one example of an enzymatic lysis agent; it works by hydrolyzing the cell walls of certain Gram-positive bacteria, such as lactic acid bacteria (LAB). By cleaving β 1, 2, 3, and 4 linkages between N-acetylglucosamine and N-acetylmuramic acid components of the bacterial cell wall's peptidoglycan structure, lysozyme accomplishes this (Carrillo et al., 2014). On the other hand, it has been shown that treating a suspension of Gram-positive bacteria with two volumes of acetone at 4°C for five minutes, and then treating the suspension with 0.1 volumes of 1% SDS, can release proteins in a manner similar to that which is obtained by sonication or agitation with glass beads (Harrison, 1991).Another study suggests that by using varying concentrations of Tris buffer, EDTA, and lysozyme, researchers can optimize the lysis process to achieve optimal release of intracellular components from different microbial species (Brown et al., 1962).

One technique that is frequently used for protein purification is the French press cell disruptor. This method of mechanical cell disruption can effectively lyse cells and extract proteins with a low levels of oxidation or proteolysis. The French press cell disruptor causes the cells to break and discharge their contents by quickly applying strong pressure to them. The quality and purity of the extracted proteins are increased as a consequence of this method's shown ability to lower extract viscosity brought on by genomic DNA contamination and cell debris (Grabski, 2009). Furthermore, the French press cell disruptor has been employed in conjunction with additional methods, such lysozyme pretreatment and sonication, to enhance the subsequent purification processes of certain proteins, like mecasermin (Haddad et al., 2015).

2.6.2. Precipitation with ammonium sulphate

The process of "salting-in" occurs when globular proteins become more soluble when salt is added at concentrations lower than 0.15 M. However, protein solubility usually decreases at higher salt concentrations, leading to precipitation. Salting-out is the term used to describe this opposite effect (Green & Hughes, 1955).

Ammonium sulphate precipitation is thought to be one of the mildest protein precipitation methods available, with very few exceptions. On a large scale, it usually does not result in significant denaturation or loss of protein activity (Rahman et al., 2014). This method helps to purify the enzyme by enabling its selective precipitation and separation from other substances (Jakoby, 1971).

Ammonium sulphate precipitation is a widely used method for purifying β-galactosidase enzyme. This technique involves adding different concentrations of ammonium sulphate to the enzyme solution, resulting in the precipitation of the protein. For instance, purified the enzyme from *Leuconostoc mesenteroides* using ammonium sulphate concentrations of 40%, 50%, and 60% (Priadi et al., 2018). Also utilized ammonium sulphate precipitation to purify β-galactosidase from *Lactobacillus brevis* PLA28 (Bhalla et al., 2018). Gnanakani isolated and partially purified β-galactosidase from *Streptococcus thermophilus* using ammonium sulphate precipitation at a concentration of 70% (Gnanakani et al., 2013).

2.6.3. Dialysis

Dialysis is an essential procedure for the concentration and purification of enzymes. This technique uses a semipermeable membrane to separate molecules according to size (Figure 7). By allowing only the small molecules to diffuse through selectively permeable membranes, conventional dialysis isolates small molecules from large molecules. Dialysis is typically used to alter a solution containing macromolecules by adding salt (small molecules) (Andrew et al., 2002). After the first concentration step, precipitation with ammonium sulphate, in the purification of β-galactosidase, dialysis is mainly used to remove salt. It is usually carried out in diafiltration mode, makes it easier to remove salts using ion-exchange chromatography elution and precipitation.

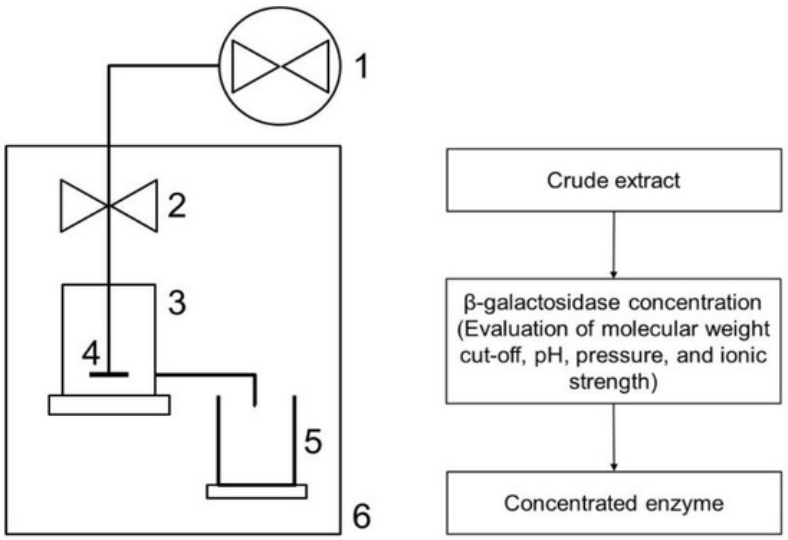

Figure 7. Scheme of the UF system used for β-galactosidase concentration: (1) nitrogen cylinder with valve; (2) pressure control; (3) ultrafiltration cell; (4) stirrer; (5) collector; (6) refrigeration *(Lemes et al., 2023).*

2.6.4 Fast protein liquid chromatography (FPLC)

A chromatographic method called fast protein liquid chromatography (FPLC) was created to purify proteins with excellent repeatability and resolution. It uses glass or plastic columns filled with small-diameter beads to provide a high loading capacity. Aqueous buffers are used as the mobile phase in FPLC to enable a range of chromatography modes, such as gel filtration and ion exchange, etc. The system uses a pump to regulate high flow rates, usually between 1 and 5 mL/min, at low buffer pressure. Through charge interaction in one buffer and elution in another, proteins can bind to the resin. FPLC is adaptable for both analytical and preparative applications, handling samples of purified proteins ranging in size from milligrams to kilogrammes (Pontis, 2017). Using multiple chromatographic steps is often necessary to achieve a high level of purity in protein purification. Numerous methods are available, such as hydrophobic interaction chromatography, gel filtration, affinity chromatography, and anion and cation exchange chromatography, which can be performed at various pH levels. With careful application, each of these methods can improve the purification process and help the target protein reach the appropriate degree of purity (Asenjo & Andrews, 2009).

β-galactosidase has been purified through the use of gel filtration and chromatography in numerous investigations. In the purification procedure of β-galactosidase (Hussien & Doosh, 2022) precipitated the protein by ammonium sulphate, which was then followed by gel filtration chromatography using a Sephadex G-100 column and ion-exchange chromatography.

Using Streamline DEAE resin in conjunction with pH and ionic strength parameter optimisation, ion-exchange chromatography was effective in obtaining stable β-galactosidase from *Kluyveromyces lactis*. With NaCl concentrations ranging from 0.1 to 0.4 M, the β-galactosidase fraction was effectively eluted, yielding 51.65 ± 0.17%. (Carvalho et al., 2022). Percival and co-workers (2019) partially purified β-galactosidase from a novel probiotic *Bacillus subtilis* SK09 using ion exchange chromatography with DEAE cellulose column. The partially purified enzyme's specific activity was 137.02 U/ml, demonstrating how well ion exchange chromatography worked to purify the enzyme.

2.6.5 β-Galactosidase stabilisation

In applied and basic enzymology, "enzyme stabilisation" is an important field. Understanding the fundamentals of enzyme stabilisation in basic enzymology requires examining the reasons and mechanisms behind enzymes' loss of biological activity. Understanding the relationships between structure and stability in enzymatic molecules is necessary for this. The main goal of applied enzymology is to effectively use biocatalysis to produce valuable compounds (Gianfreda & Scarfi, 1991).

Common methods include enzyme immobilisation, which involves attaching enzymes to solid supports, chemical modification with cross-linking agents, pH and temperature optimisation, the addition of stabilising agents such as sugars and salts, water removal via lyophilization, and encapsulation within microspheres or nanoparticles. Immobilisation on supports such as Immobead modified with acid or glutaraldehyde (Eberhardt et al, 2020). When immobilised on glyoxyl-agarose, novel β-galactosidases from *Lactobacillus plantarum* demonstrated great activity and stability (Rosado et al, 2022). When β-galactosidase is immobilised on Amberlite MB-150 beads, the enzyme preparation is significantly stabilised and nearly loses none of its activity over a 12-month period at room temperature (Dwevedi & Kayastha, 2009).

3. MATERIALS AND METHODS

3.1 Materials

3.1.1. Microorganism

The applied probiotic *Limosilactobacillus fermentum* LF08 strain was purchased from Probiotical S.p.A.

3.1.2 MRS for bacteria growth and fermentation

For the purpose of growing the bacteria, the probiotic strain was added to a flask containing a modified MRS medium with the composition shown in Table 2. After the preparation, the flask was autoclaved for 15 minutes at 121℃.

Table 2. Composition of lactose containing MRS medium.

Component	**Amount**
Lactose	20g
Protease- peptone	10g
Beef extract	8g
Yeast extract	4g
Sodium– acetate	5g
Triammonium-citrate	2g
Dipotassium-hydrogen-phosphate	2g
Tween 80	1ml
Magnesium sulphate	0.2g
Manganese sulphate	0.05g
Distilled water	1000 ml

3.1.3 Buffers and solutions

- 4-Nitrophenyl-β-D-galactopyranoside substrate

  50 ml of distilled water was used to dissolve 0.0225 g of 4-nitrophenyl β-D-galactopyranoside to prepare the substrate. The substrate was stored in a refrigerator at 4°C for further use.

- Sorensen buffer (pH 6.5)

  The following ingredients were used to make the Sorensen buffer at pH 6.5: KH_2PO4 at a concentration of 9.0178g/L and Na_2HPO_4*12 H_2O at a concentration of 23.876g/L.

- Cetyl-trimethyl-ammonium bromide solution

  The preparation of the solution for cell lysis the concentration of 0.45 mg/ml cetyl-trimethyl-ammonium bromide (CTAB) was used.

- 0.1 N Na_2CO_3 solution

  0.1 N Na_2CO_3 was used for stopping the enzyme reaction. 5.13g Na_2CO_3 was dissolved in 1000 mL distilled water.

3.2 Methods

3.2.1 Enzyme fermentation

The fermentations are initiated by inoculating Erlenmeyer flasks containing 100-500 mL modified MRS medium - based on the experimental design - with 1 v/v% cell suspensions of one-day inoculum. For enzyme fermentation, we modified the carbohydrate content of MRS medium (Table 2) by supplementing it with a 1:3 glucose: galactose ratio with a final sugar concentration of 0.5%. After that, these flasks were incubated for 16 hours at 37°C. After fermentation, the samples were refrigerated at 4°C until further use.

3.2.2 Enzyme activity assay

Because the β-galactosidase is an intracellular enzyme, the activity was determined after cell disruption. The enzyme activity is determined using the artificial substrate p-nitrophenyl-β-D-galactopyranoside (pNPG).

When the enzyme hydrolyzes the substrate, p-nitrophenol is released, and because of this, the solution takes on a yellow colour. The absorbance of released p-nitrophenol is measured by a spectrophotometer. A pH 6.5 Sorensen buffer was used during the activity assay. The reaction mixtures for determination of the enzyme activity were prepared according to the instructions given in Table 3. The enzyme activity test starts by pre-incubating the tubes at 50 degrees for 5 minutes. The reaction was started by the addition of 0.2 ml of suitable diluted sample. After 5 minutes of reaction time, 5.0 ml of 0.1 N Na_2CO_3 was added to the tubes to stop the reaction. A spectrophotometer was used to measure absorbance at 405 nm.

Table 3. Composition of the reaction mixture for enzyme activity assay.

	Distilled water (ml)	**Substrate (ml)**	**Buffer (ml)**	**Enzyme solution (ml)**
Zero blank	0.7	-	0.3	-
Substrate blank	0.2	0.5	0.3	-
Enzyme blank	0.5	-	0.3	0.2
Sample	-	0.5	0.3	0.2

The following equation was used to measure the enzymatic activity:

$$U = \frac{(A\ sample\ -\ A\ enzyme\ blank\ -A\ substrate)\ *\ dilution\ *\ reaction\ volume}{enzyme\ volume\ *\ reaction\ time\ *\ 2.995}$$

A unit of enzyme activity (U) is defined as the amount of enzyme that, at the specified reaction conditions (50°C, pH 6.5), is capable of releasing one µmol of p-nitrophenol per minute.

3.2.3 Bradford method for protein content determination

The Bradford method is a commonly used assay for determining protein content in samples. It is predicated on the equilibrium between several Coomassie Blue G dye forms (Tantray et al., 2023). This assay involves binding protein molecules to Coomassie dye under acidic conditions, causing a colour change from brown to blue. This method detects the presence of basic amino acid residues (arginine, lysine, and histidine) that contribute to the formation of protein-dye complexes. This method used Bio-Rad's protein dye reagent.

10 µl of the sample was added to 200 µl of the dye. The sample was left with the dye for 2 minutes and after that, the absorbance was measured at 595 nm.

The following equation was used or determine the protein content:

$$Protein\ content\ (mg/mL) = \frac{(A\ sample\ - A\ blank) * dilution\ factor}{0.6246}$$

3.2.4 Cell lysis by chemical disruption

To determine the optimal ammonium sulphate saturation, the cells were resuspended in 20 ml Sorensen buffer, and lysis buffer (CTAB - cetyl-trimetyl-ammonium-bromide) was added in ratio 2:1.

3.2.5 Cell lysis by enzymatic digestion

Lysozyme was added to washed and homogenised cells to release the enzyme from the cell wall. A 40 mg/ml lysozyme solution was prepared and 300 µl of this solution was pipetted into 2 ml samples. The applied temperatures and reaction time were set based on the experimental design.

3.2.6 Purification of β-galactosidase

Several steps and techniques were used to purify the enzyme including cell lysis by enzymatic digestion and mechanical cell disruption, ammonium sulphate precipitation, dialysis and chromatography.

3.2.6.1 Cell lysis by mechanical disruption

Following the primary fermentation of 500mL, cells were harvested by centrifugation at 10.000 rpm for 10 minutes at 4°C, then they were washed three times with 10mL of Sorensen buffer (pH 6.5). The washed cells were dissolved in 20 ml of buffer and then subjected to mechanical disruption using the French press, the enzymatic activity was measured and the sample was refrigerated at 4°C until further purification steps.

3.2.6.2 Protein precipitation with ammonium sulphate

Following cell disruption, the refrigerated sample was supplemented with the appropriate amount of ammonium sulphate (Table 4) under continuous stirring until the desired saturation percentage was reached. The mixture was then refrigerated overnight, allowing the precipitate to develop. The precipitate was then collected and dissolved in a 2 ml Sorensen buffer (pH 6.5), and then kept refrigerated for further purification steps.

Table 4. Ammonium sulphate saturation and respective concentrations.

	Ammonium sulphate saturation (%)	**Ammonium sulphate concentration (g/L)**
1	65%	430g/L
2	70%	472 g/L
3	75%	516 g/L
4	80%	561 g/L
5	90%	662 g/L
6	100%	767 g/L

3.2.6.3 Dialysis

Dialysis with Sorensen buffer (pH 6.5) was used to remove salts after precipitation. An Amicon Ultrafilter with a 10 kDa cutoff value was used. Throughout the procedure, pressure was applied from above, and the entire process was carried out on ice to ensure cooled conditions.

3.2.6.4 Chromatography

To purify the substance, first ion exchange chromatography was performed. A column of Amersham Biosciences Q Sepharose was connected to the Pharmacia Biotech FPLC system, which included a high-precision pump P-500, a Pharmacia Biotech UV-MII detector, and an LCC-501 Plus controller. During ion-exchange chromatography, the mobile phase consisted of Sorensen buffer (pH 6.5) and Sorensen buffer (pH 6.5) with 1 M NaCl. The flow rate was kept at 4 ml/min and the fraction size was 4 ml.

3.2.7 Evaluation of protein purification

Protein purification outcomes are assessed across various parameters, encompassing enzyme activity (U/ml), protein content (mg/ml), specific activity (U/mg), and yield (%). The determination of total enzyme activity and total protein content involves utilising the sample volume and available data for enzyme activity and protein content, respectively.

Specific activity serves as a crucial metric for evaluating enzyme purity. This metric is derived by dividing the total activity of the protein by the total protein content obtained throughout all purification steps. A higher specific activity indicates a more refined enzyme preparation, whereas a lower value may suggest partial enzyme inactivation or the presence of impurities. Essentially, specific activity serves as an indicator of purification success.

In contrast, yield reflects the overall quantity of product obtained. It is anticipated to diminish after each purification step due to inherent losses within the process.

4. RESULTS AND DISCUSSION

4.1 Enzyme fermentation

This phase aims to maximise the synthesis of β-galactosidase by considering the effect of multiple parameters such as the pH, the optimum inoculum size, and fermentation time. A study by Zhang and colleagues (2021) on the production of high-purity galactooligosaccharides suggested that the optimal fermentation parameters for *Lactobacillus*-derived β-galactosidase are 35°C, pH 7.0, and 400 g/L lactose concentration. Furthermore, a study on different *Lactobacillu*s strains by Wang and Sakakibara (1996) suggested the inoculation with 1% of starter cultures for each strain and incubated at 37°C for 16 hours to obtain the highest activity of β-galactosidase crude. Vasudha and Gayathri (2023) found that the β-galactosidase from *Lactiplantibacillus plantarum* activity was high at pH 7.0 and a temperature of 37℃. According to Mahadevaiah et al. (2020) on the optimal production of β-galactosidase from *Lactobacillus fermentum,* among the different carbon sources used, *L. fermentum* was found to produce the highest β-galactosidase on galactose compared to lactose, glucose, sucrose, fructose and maltose. Additionally, Arukha et al. (2014) determined that when glucose is used in combination with galactose it increases enzyme production.

However, Hristovski et al., (2024) determined that the highest β-galactosidase activity from *L. fermentum* LF08 is achieved using glucose and galactose as carbon at a ratio of 1:3 after 1% (v/v) inoculation and fermentation for 16 hours at 37˚C . These parameters were applied during my experiment and 7.229 U/mL cells β-galactosidase activity was achieved after 16 hours fermentation.

4.2. Determination of the optimal saturation of ammonium sulphate

This approach makes protein extraction easier and is regarded as the gold standard in purification operations. Optimal ammonium sulphate saturation can considerably boost enzyme purification, resulting in higher specific enzyme activity and protein content after precipitation (Karaboğa & Loğoğlu, 2019). After fermentation at optimum conditions, the cells were chemically disrupted with CTAB solution. After cell lysis the samples were centrifuged and the supernatant was supplied with different saturations of ammonium sulphate to determine which saturation will result on the highest β-galactosidase activity. Figure 8 presents the effect of the concentration gradient from 65% to 100% of ammonium sulphate on the enzyme activity.

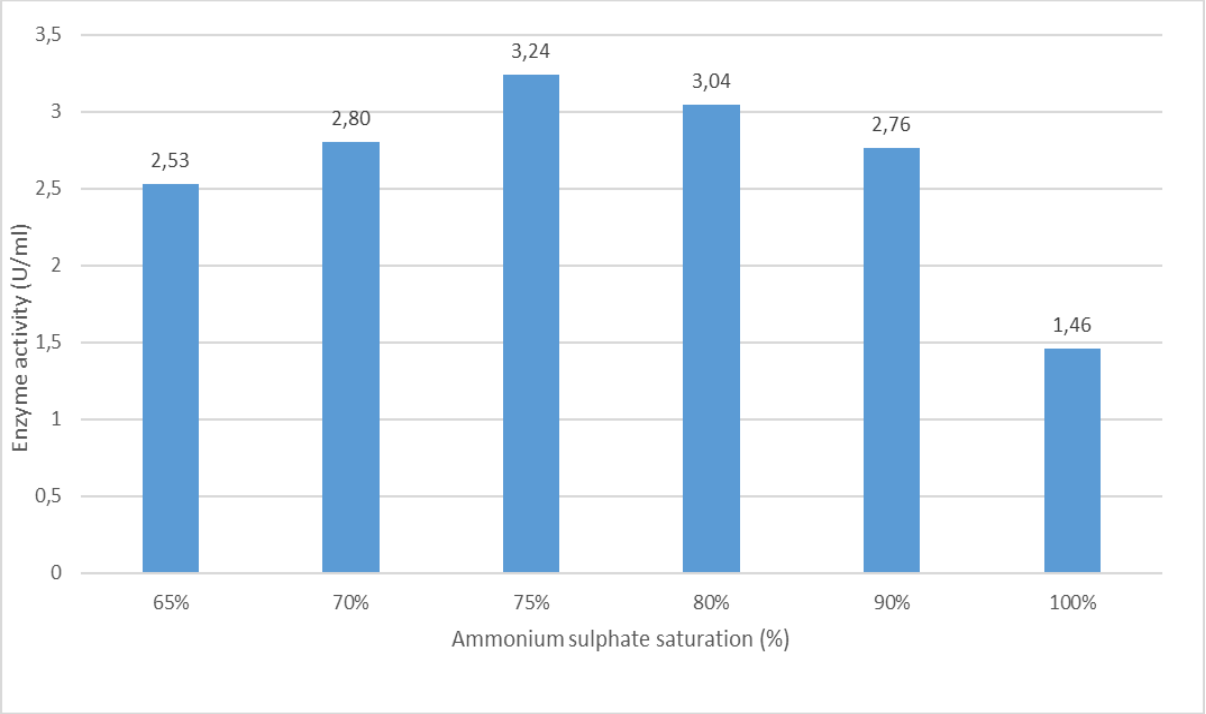

Figure 8. β-Galactosidase activity after precipitation with different saturations of ammonium sulphate

The results reflect that the highest β-galactosidase activity was obtained at 75% saturation with ammonium sulphate. This result correlates to other findings, according to Al-easawi and colleagues (2015) the highest specific activity of β-galactosidase was achieved at 70% saturation, Gnanakani et al. (2013) also used the same 70% saturation to purify β-galactosidase from *streptococcus thermophilus* while 80% saturation was used to purify the enzyme from *Lactobacillus* strains Mozumdel et al. (2011) and *Lactobacillus leichmannii* 313 (Xu et al., 2020). Higher concentrations of ammonium sulphate can cause excessive precipitation of the enzyme and contaminants, resulting in decreased yield. Conversely, lower concentrations may not effectively remove all contaminants or precipitating the entire enzyme, resulting in impure enzyme preparations (Green & Hughes, 1955). Accordingly, 75% ammonium sulphate saturation is used for further purification steps in larger scale.

4.3 Optimization of cell disruption

As chemical disruption by CTAB was used to determine the optimal ammonium sulphate saturation. However, one concern with CTAB lysis buffer is that it is poor at releasing the intracellular enzyme. The enzyme is attached to the cell wall, which causes inefficiency.

CTAB lysis buffer is inefficient at breaking down the cell wall, resulting in low enzyme release. For successful extraction, alternate lysis procedures that efficiently destroy the cell wall, such as lysozyme treatment or sonication, may need to be investigated. We tested the efficiency of lysozyme and the mechanical disruption using the French press for cell disruption. In this part of the results, we will discuss our findings regarding this.

4.3.1 Enzymatic digestion by lysozyme

Lysozymes are hydrolytic enzymes that cleave β-(1,4)-glycosidic bonds in peptidoglycan, a key structural component of bacterial cell walls. This hydrolysis process weakens the cell wall's integrity, resulting in bacterial lysis. Lysozyme works by targeting, hydrolyzing, and breaking down the muco polysaccharide portion of the bacterial cell wall. Similarly, this enzyme can degrade glycosidic linkages in chitin (Nawaz et al., 2022). Lysozyme treatment has been extensively used to release bacterial biomass and β-galactosidase purification by Chanalia et al, (2017) and Sangwan et al, (2014). This test aimed to determine the most effective incubation temperature for lysozyme to achieve the highest enzyme activity, Figure 9 shows the effect of incubation temperature on the enzymatic activity.

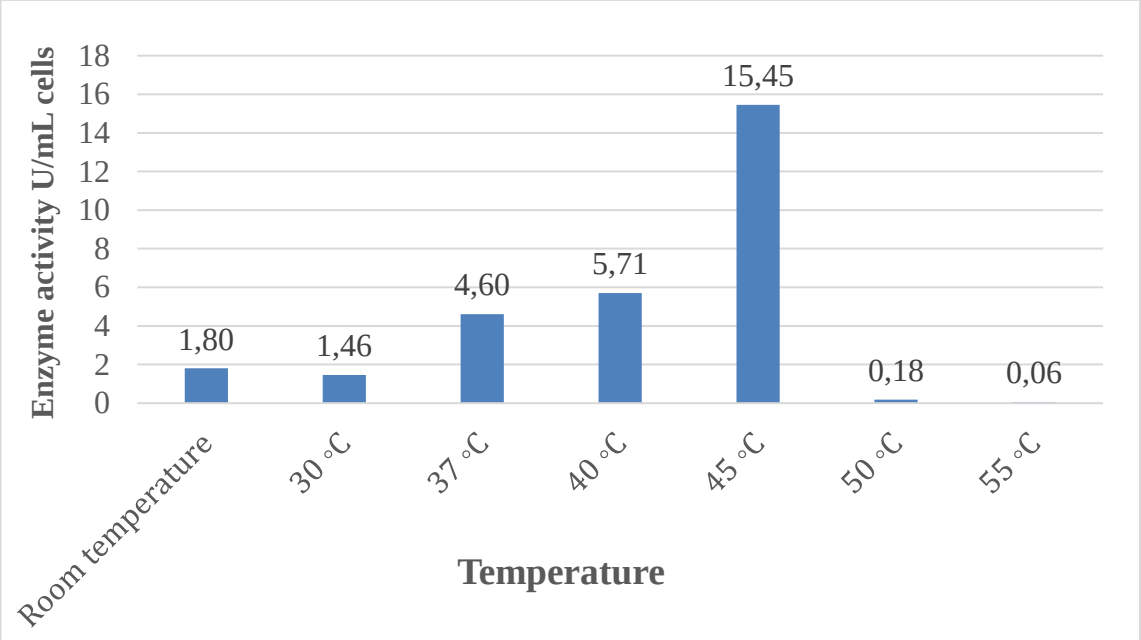

Figure 9. Enzyme activity against the lysozyme incubation temperature

The figure illustrates that the highest β-galactosidase activity was achieved when lysozyme was incubated with the sample at 45°C for 4 hours. The effect of lysozyme on cell lysis is influenced by many factors and parameters, the choice of the used concentration parameter of lysozyme was based on a study of Hristovski, (2020) where he found that 40 mg/ml resulted in the highest enzymatic activity. A study on lysis techniques for Gram-positive bacteria also found a correlation between reaction time and lysozyme concentration with lysis efficacy (Chassy & Giuffrida, 1980). The lysis effect of lysozyme is usually assessed with the supplementation of EDTA which has an accelerated influence on the process (Brown et al., 1961). Dias and co-workers (2015) studied the effectiveness of lysozyme on *Lactobacillus* strains. They used LiCl pre-treatment on the cells to remove the protein from the cells' surface and found it to be effective similar to the addition of 20% (v/v) ethanol. Lysozyme may not always be the most successful approach for protein purification because it is selective to bacterial cell walls, lacks specificity for other organisms, may not fully lyse cells, can contaminate the sample, and may not offer sufficient purity. Other purification methods provide more selectivity and purity for certain proteins.

4.3.2 Mechanical disruption by the French press

Mechanical cell disruption techniques, such as the French press, offer a high potential for extracting intracellular materials from a wide range of microorganisms. These methods allow for the optimization of parameters to increase extraction yield, and they are relatively quick and do not involve the use of toxic chemicals, making them safer and more efficient options for enzyme extraction. During the investigation of the optimum ammonium sulfate saturation, the CTAB method of cell lysis resulted in enzyme activity before precipitation of 1.663 U/mL, whereas the enzyme activity before precipitation was 7.229 U/mL using the French press method.

Factors such as microorganism form, cell wall structure, size, and thermolability all contribute to the efficacy of these approaches (Nemer et al., 2021). Given these advantages and the higher resulting enzyme activity, the French press method was preferred over chemical and enzymatic alternatives.

4.4 β-galactosidase purification by *Limosilactobacillus fermentum* LF08

We used multiple procedures to purify β-galactosidase, including ammonium sulphate precipitation, dialysis, and FPLC chromatography. Each approach had a distinct purpose in the purifying process. Ammonium sulphate precipitation enabled the initial isolation of the enzyme by selectively precipitating proteins depending on their solubility.

Dialysis helped remove contaminants and undesirable chemicals from the enzyme containing ammonium sulphate after the precipitation. Finally, FPLC chromatography allowed for high-resolution separation of the enzyme from remaining impurities, confirming the purity and quality of the finished product. This multi-step process ensured that β-galactosidase was thoroughly purified, satisfying the needs of a variety of research and industrial applications. Figure 10 summarises the purification steps and techniques used.

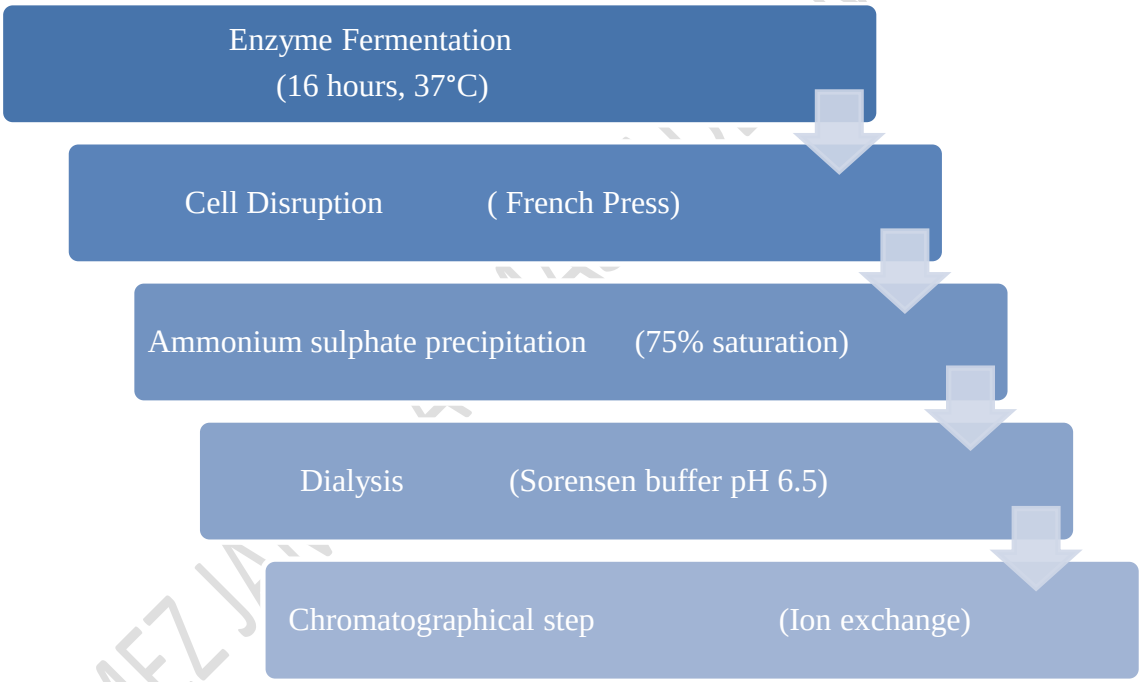

Figure 10. Steps of the purification process of β-galactosidase from *L. fermentum* LF08

4.4.1 Dialysis

Following ammonium sulphate precipitation, dialysis is used to remove salts and small molecules from the sample before concentrating and exchanging it into a buffer suitable for further protein purification and characterization. Following this process, the sample undergoes a 10-fold dilution, and an activity assay reveals enzyme activity of 1.49 (U/ml).

4.4.2 Evaluation of chromatography stage

FPLC is a type of high-performance chromatography that uses small-diameter stationary phases to attain high resolution. Originally intended for proteins, it includes high loading capacity, biocompatible buffers, quick flow rates, and a variety of chromatography modes. Automation, such as autosamplers and gradient control, ensures consistent separations. FPLC has a wide range of applications, including proteins, oligonucleotides, and plasmids. Anion exchange in proteins is a typical FPLC experiment (Madadlou et al., 2016).

Following dialysis, the sample underwent ion exchange chromatography. The resulting fractions were tested for absorption at 280 nm as β-galactosidase contains tryptophan residues, absorption at 280 nm is employed to measure its quantity and purity. Microplates were also employed to test β-galactosidase activity at 405 nm.

Furthermore, evaluating FPLC results entails analysing peak patterns of the mentioned absorption values and grading purity based on peak shape and size, ensuring separation efficiency and calculating yield.

The sample was loaded for the run in two parts from the equal volume under the same exact running conditions and the two resulting chromatograms were analysed. Figure 11 graphically displays the findings of measuring enzyme activity at 405 nm and protein concentration in the samples at 280 nm.

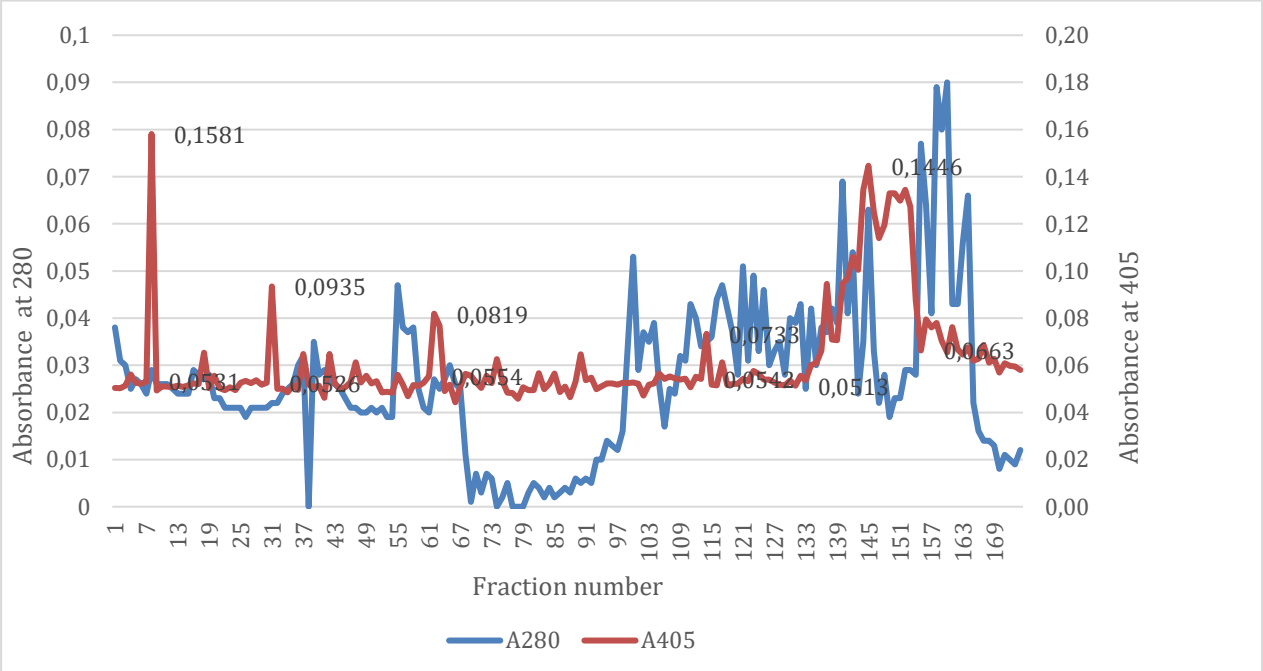

Figure 11. Ion-exchange FPLC chromatogram for β-galactosidase purification (first run)

In the initial fractions of the first run, the chromatogram indicates increased absorption at 405 nm of 0.1581, coinciding with the highest recorded activity. Conversely, absorption at 280 nm indicates lower protein concentrations during this phase, with larger quantities detected in later fractions. This could suggest that the earlier fractions show better purity of β-galactosidase. Additionally, the overlapping peaks in absorption readings (A280 and A405) could indicate that other proteins in the sample could potentially contribute to the observed enzymatic activity peaks. These observations differ slightly during the second chromatography run of the sample illustrated in Figure 12.

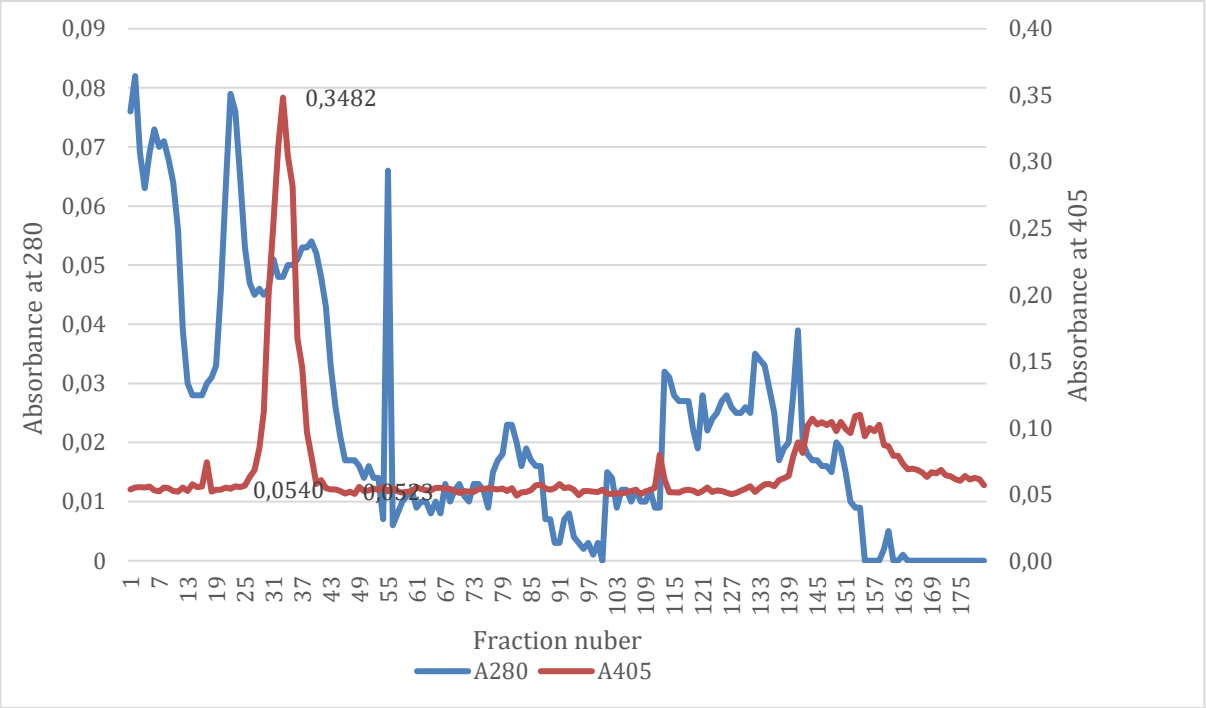

Figure 12. Ion-exchange FPLC chromatogram for β-galactosidase purification (second run).

In the second run, which was under the same conditions, a distinct peak at 405 nm is observed in the initial fractions, similar to the first run, but with a higher absorption value of 0.3482. This could indicate an increased concentration of β-galactosidase. In addition, these early fractions have a larger protein content compared to the first run while showing lower content later in the same run. The well-defined peaks noted in the initial fractions of both runs could suggest that the purification process is reproducible, with β-galactosidase being eluted in the early fractions. The early elution of β-galactosidase may be attributed to its specific characteristics and the conditions of the ion exchange chromatography using Q Sepharose at pH 6.5. This includes factors such as its close proximity to the pH 6.5 isoelectric point, potentially weaker binding

affinity to the Q Sepharose resin at this pH, and the reproducibility of the purification process. These collective factors indicate that the conditions used for ion exchange chromatography effectively isolate β-galactosidase from the sample matrix, leading to its early elution in the initial fractions of both runs.

A number of methodological changes could be taken into consideration in order to enhance the β-galactosidase purification process and further optimise the ion exchange chromatography procedure. The efficiency of protein separation and the target protein's purity can be greatly enhanced by optimising the chromatographic conditions, including pH and salt concentration. By adjusting the pH within a specific range, purification efficiency can be increased by increasing β-galactosidase's binding affinity to the ion exchange elution. Better resolution and purity of β-galactosidase can be achieved by varying the salt concentration in the elution buffer, which can affect the ion exchange interactions between the protein and the resin. Moreover, altering the flow rate during chromatography can change the protein's residence time on the column, enabling more effective elution and separation.

De Medeiros and co-workers (2012) conducted an experimental design and response surface methodology to optimize the elution pH and gradient volume for β-galactosidase purification by ion exchange chromatography using the anion exchange Q Sepharose Fast Flow resin from Amersham Biosciences. A gradient volume of 62.8 mL and an elution pH of 5.5 were found to be the ideal parameters. Under these conditions, β-galactosidase was recovered in a single chromatographic step with an 85.5% purification factor, which is a significant improvement over previous studies that used the same technique for enzyme purification. Ion-exchange chromatography was performed after gel chromatography on Sephadex G-150 in an attempt to purify β-galactosidases from *Bacillus circulans*. Even though β-galactosidase activity was eluted as a single peak in both chromatography steps, specific activity only slightly increased, most likely as a result of ultrafiltration-mediated partial purification. On the other hand, β-galactosidase activity was mostly eluted as a single peak at 0.2 M NaCl in ion exchange chromatography (Mozaffar et al., 2014). Using a gradient buffer in ion exchange chromatography enables selective elution of β-galactosidase based on its net charge, optimisation of separation conditions, and enhanced resolution of closely related proteins. Benavente and co-workers (2015) used different elution buffers for the chromatographic separation: 100 mM NaOH (Eluent A), 100 mM NaOH and 50 mM NaOAc (Eluent B), and

100 mM NaOH and 1 M NaOAc (Eluent C) to determine the pH dependency of enzyme activity and the effect of temperature on enzyme activity.

Chromatographical methods are easily manipulated and adjusted, these methodological refinements can collectively contribute to the optimization of ion exchange chromatography, resulting in higher purity and yield of β-galactosidase. Therefore, by fine-tuning the chromatographic conditions, we aim to enhance the reproducibility and efficiency of the purification process, ultimately improving the quality of the β-galactosidase sample obtained.

4.4.3 Quantitative evaluation of β-galactosidase purification protocol.

The efficiency of the β-galactosidase purification protocol was assessed quantitatively at various stages of the process. Throughout the entire process, several properties were measured and calulated after each purification step to evaluate purification efficiency. These properties included enzyme activity (U/ml), protein content (mg/ml), specific activity (U/mg), and yield (%). Enzymatic activity was measured using absorbance at 405 nm to assess the functional activity of β-galactosidase, and the specific activity of the enzyme was calculated. The Bradford method was utilized to determine the protein concentration, which enabled the monitoring of β-galactosidase yield during the purification procedure.

Furthermore, by comparing the amount of enzyme recovered at each purification stage to the initial amount in the crude extract, the yield of β-galactosidase could be determined. The purification protocol's recovery efficiency is assessed by contrasting the ultimate yield of β-galactosidase with the initial quantity found in the crude sample. While a lower value might suggest partial enzyme inactivation or the presence of contaminants, a higher specific activity indicates a more refined enzyme preparation. Consequently, a particular activity can be utilised to assess the progress of the purification process. Conversely, yield indicates the total amount of the product that was obtained. Table 5 shows the obtained results during different purification steps.

Table 5. Quantification results of the purification protocol

Purification step	Activity (U/ml)	Protein content (mg/ml)	Volume (mL)	Total activity (U)	Total protein (mg)	Specific activity (U/mg)	Yield (%)
After cell disruption	7.229	0.625	8	57.835	4.998	11.572	100
After precipitation	11.963	0.518	2	23.925	1.037	23.075	41,368
After dialysis	1.490	0.449	16	23.843	7.196	3.315	41,225

The enzyme demonstrated a specific activity of 11,572 U/mg after cell disruption, with an activity of 7.229 U/ml and a protein content of 0.625 mg/ml. The increasing relatively specific activity indicates purification of the enzyme preparation. The enzyme activity increased to 11.963 U/ml upon precipitation, while the protein content decreased to 0.518 mg/ml. In comparison to the preceding step, this led to a notable increase in specific activity to 23,075 U/mg, indicating a more refined enzyme preparation.

Nevertheless, the yield dropped to 41.368%, indicating that some enzyme was lost during the precipitation process. Despite a drop in protein content, the decrease in specific activity seen following dialysis to 3.315 U/mg may result from the removal of the enzyme's essential cofactors or coenzymes. Furthermore, the exchange of the buffer surrounding the enzyme during dialysis might have had a dilution effect, which decreased the enzyme activity per unit volume. Despite the decrease in specific activity, the yield remained relatively stable at 41.225%.

Because β-galactosidase is intracellular and contains other biological components, the purification process for it might be complicated. To produce an extremely pure enzyme, a variety of purification methods including dialysis, chromatography, and precipitation are frequently needed. Evaluating aspects like purification yield, specific activity, and fold purification achieved is part of comparing the purification procedure with other research. This comparison aids in assessing the purification protocol's efficacy and efficiency as well as its suitability for use in biotechnological and industrial settings.

Various chromatographic methods were used to purify the intracellular β-galactosidase from thermophilic *Alicyclobacillus acidocaldarius*, the purification procedure included ammonium sulphate precipitation, gel permeation chromatography, ion-exchange chromatography, and affinity chromatography, followed by preparative electrophoresis. After purification, the enzyme had a specific activity of 113 U/mg protein, a purity factor of 163, and an 8% yield. (Gul-Guven et al., 2007). *Lactobacillus acidophilus* produces β-galactosidase, which was extracted and purified using a series of procedures such as ammonium sulphate precipitation, dialysis, and gel filtration using Sephadex G-200 chromatography.

When the ammonium sulphate concentration was optimised at 80%, the precipitate displayed a 73% yield, a purification fold of 4.7, and a specific activity of 0.00000108 U/mg. The specific activity rose to 0.00000154 U/mg after dialysis, yielding a 55.7% yield and a purification fold of 6.7 (Ahmad & et al, 2014). Sephadex G-100 and Q-Sepharose columns were used in gel filtration and anion exchange chromatography, respectively, to purify the enzyme derived from the probiotic *Pediococcus acidilactici*. The specific activity rose during the purification process, going from 0.288 U/ml in the crude extract to 0.883 U/ml in the anion exchange chromatography final phase. The yield attained in the end was 28.26% (Chanalia et al, 2018). A novel intracellular β-galactosidase produced by *Lactobacillus plantarum* HF571129 was isolated from traditional fermented milk product curd was purified and characterised. With an overall recovery of 30.41%, the enzyme was refined 7.23 times via ultrasonication, ultrafiltration, and gel filtration chromatography.

7. CONCLUSION AND SUGGESTION

Enzyme purification involves several techniques such as precipitation, chromatography, centrifugation, ultrafiltration, and diafiltration. These techniques aim to achieve the highest yield of the desired enzyme with optimal catalytic activity and purity. This thesis investigates the purification technique for β-galactosidase extracted from the probiotic strain *Limosilactobacillus fermentum* LF08. The study calculates purification factors to determine the efficacy of each purification process. Furthermore, the study attempts to find the best conditions for essential purification procedures such ammonium sulphate saturation and enzymatic cell lysis.

The choice of purification method depends on the properties of the enzyme and the intended application. Three essential requirements for an effective purification plan are achieving high purity, maintaining overall enzyme activity recovery, and ensuring repeatability. Gram-positive bacteria have thick mucopeptide layers in their cell walls, making it crucial to employ effective cell disruption techniques. Mechanical, physical, chemical, enzymatic, and combination approaches can be used to release intracellular products. Lysozyme is an example of an enzymatic lysis agent that hydrolyzes the cell walls of certain Gram-positive bacteria. Other methods include treating bacterial suspensions with acetone or optimizing lysis using Tris buffer, EDTA, and lysozyme.

Using the mechanical cell disruption method has proven to result in a higher enzymatic activity than using the chemical method utilizing CTAB buffer alone. Furthermore, the French press is a more efficient and gentle way of cell destruction. It enables complete cell lysis, preserves enzyme function, lowers contamination hazards, and is easily scaled for industrial use.

Lysozyme has shown the highest activity when incubated at 45°C, at this temperature, the enzyme's structure is stable enough to efficiently hydrolyze the β-(1,4)-glycosidic linkages in the peptidoglycan layer of bacterial cell walls, resulting in effective bacterial lysis. However, at higher temperatures, lysozyme may denature and lose its action.

Ammonium sulfate precipitation is a widely used method for enzyme purification. It enables selective precipitation and separation of the enzyme from other substances without significant denaturation or loss of protein activity. 75% saturation of ammonium sulfate was found to have optimal effect on the enzymatic activity.

Dialysis is used mainly to remove salt after the initial concentration step. However, Further optimization of the this step is recommended to maintain both purity and activity of β-Galactosidase.

Fast protein liquid chromatography (FPLC) is another effective method for protein purification, providing high repeatability and resolution. It utilizes glass or plastic columns filled with small-diameter beads and is adaptable for both analytical and preparative applications.

This thesis suggests future investigation and optimization of the β-galactosidase purification process using the probiotic *Limosilactobacillus fermentum* LF08:

- β-Galactosidase enzyme fermentation
  Applying glucose and galactose as carbon source at a ratio of 1:3 after 1% (v/v) inoculation and fermentation for 16 hours at 37˚C β-galactosidase by *L. fermentum* LF08 can be produced.
- Optimizing the Dialysis Stage:
  Fine-tune dialysis parameters such as buffer composition, pH, and duration to maintain the purity and activity of β-Galactosidase while reducing enzyme loss and increasing purification efficiency.

Future research could also focus on comparing the effectiveness of different purification techniques to further improve enzyme purity and activity. Investigating the potential impact of different chromatographic conditions on the efficiency of the purification process and exploring alternative enzyme stabilization methods to enhance enzyme stability during the purification process are also recommended.

6. SUMMARY

The purification protocol for β-galactosidase from *Limosilactobacillus fermentum* LF08 involved several crucial steps including enzyme fermentation, determination of optimal ammonium sulphate saturation, and cell lysis. The fermentation process was optimized using a glucose and galactose ratio of 1:3, with 1% (v/v) inoculation, and fermentation for 16 hours at 37°C, achieving the highest enzyme activity.

Optimal saturation of ammonium sulphate was determined to be 75%, resulting in the highest β-galactosidase activity, which correlated with previous findings.

The highest β-galactosidase activity was achieved when lysozyme was incubated with the sample at 45°C for 4 hours, showing the highest activity compared to other incubation temperatures.

The purification process involved precipitation, dialysis, and FPLC chromatography. The enzyme demonstrated specific activity of 11,572 U/mg after cell disruption and 23,075 U/mg after precipitation. However, the specific activity decreased after dialysis to 3,315 U/mg, indicating potential loss of essential cofactors or coenzymes. The overall yield remained stable at around 41%, suggesting the purification protocol's efficiency.

Comparisons with other studies on β-galactosidase purification from different probiotic strains further validated the effectiveness of the purification protocol for Limosilactobacillus fermentum LF08. These results provide valuable insights for industrial applications and future research.

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DECLARATION

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