Chapter

Hydrolysis of Lactose: Conventional Techniques and Enzyme Immobilization Strategies on Polymeric Supports

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Abstract

This chapter explores lactose hydrolysis, emphasizing conventional techniques and the noteworthy immobilization of β-galactosidase on polymeric matrices to enhance the process. Lactose, present in milk and dairy, poses challenges for lactose-intolerant individuals, requiring enzymatic hydrolysis for lactose-free product development. The presence of other milk components, such as proteins and minerals, can indirectly influence the efficiency of lactose hydrolysis by potentially interacting with β-galactosidase enzyme or affecting its stability and activity, making it necessary to control factors such as enzyme concentration, temperature, pH, and reaction time to improve lactose hydrolysis rates. The chapter delves into established methodologies, covering enzymatic kinetics, reaction conditions, and substrate concentrations. It also describes the innovative approach of immobilizing β-galactosidase on polymeric supports to enhance enzyme stability, reusability, and overall efficiency in lactose hydrolysis. Discussions include the design of suitable polymeric matrices, providing insights into mechanisms governing catalytic performance. This comprehensive exploration contributes to understanding lactose hydrolysis, offering valuable insights for developing efficient and sustainable enzymatic processes applicable to the food and pharmaceutical industries.

Keywords: dairy products, lactose hydrolysis, enzyme immobilization, advanced polymers, polymeric supports

1. Introduction

The production and consumption of dairy products worldwide have increased in recent years, driven by factors such as population growth, rising incomes, and dietary preferences. Specifically, milk production is projected to reach 1020 million tons by 2030, making it the most consumed dairy product, followed by cheese [1].

These products are characterized by containing high amounts of lactose, or β-D-galactopyranosyl-(1 → 4)-D-glucose, which is a reducing disaccharide composed of the monosaccharides D-glucose and D-galactose linked by a β-(1 → 4)-glucosidic bond [2]. Lactose is naturally present in the milk produced in the mammary glands of mammals during the lactation period to provide energy to newborns. Lactose represents the predominant carbohydrate in milk, ranging from 4.4% to 5.2% in cow's milk and 6.5–7.5% in human milk [3]. The enzyme β-galactosidase, also known as lactase, found within the brush border microvilli of small intestine enterocytes in newborns [4], plays a crucial role in facilitating the hydrolysis of lactose, breaking it down into D-glucose and D-galactose. These resultant sugars are then absorbed, providing the essential energy required for growth and development (**Figure 1**) [2].

As previously stated, the global consumption of dairy products is substantial, leading to a significant lactose intake. Nonetheless, lactose intolerance issues prevent two-thirds of the population from consuming these products [5].

Lactose intolerance arises from the absence or deficiency of the enzyme β-galactosidase (hypolactasia) in the small intestine, impairing its digestion. Undigested lactose passes into the large intestine, where intestinal bacteria ferment the disaccharide. This fermentation process typically manifests within a short period, often between 30 minutes and 2 hours after consumption, leading to symptoms such as flatulence, bloating, abdominal cramps, or diarrhea [4, 5]. The prevalence and geographical distribution of lactose intolerance vary worldwide. For instance, over 90% of the population in Asia experiences lactose intolerance, while the figure is merely 2% in northern Europe. This disparity is attributed to genetic factors. Populations with historical and continued high consumption of dairy products, notably milk, harbor genetic mutations that sustain the enzymatic activity of intestinal β-galactosidase into adulthood, unlike other populations [4, 6].

β-Galactosidase deficiency can be classified into two main types: Primary, which is genetically determined, and secondary, which occurs because of diseases affecting the small intestine. Primary β-galactosidase deficiency includes congenital cases, present from birth and relatively rare, as well as β-galactosidase non-persistence, characterized by the natural decrease in the enzyme's activity after weaning. Conversely, secondary β-galactosidase deficiency is caused by underlying diseases that affect the small intestine [7]. The prevalence of β-galactosidase intolerance worldwide has spurred the development of industrial processes aimed at producing lactose-free products. These products are designed to be consumed by lactose-intolerant individuals, providing them with essential nutritional compounds found in dairy products without causing discomfort.

To qualify as "lactose-free," a product must contain a glucose-galactose disaccharide concentration below a specified threshold. This threshold is set for milk at 0.1 g/100 mL, as established by the European Food Safety Authority (EFSA) [7].

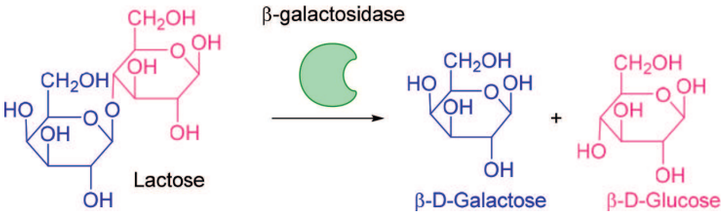


Figure 1.
The hydrolysis of lactose through the action of β-galactosidase.

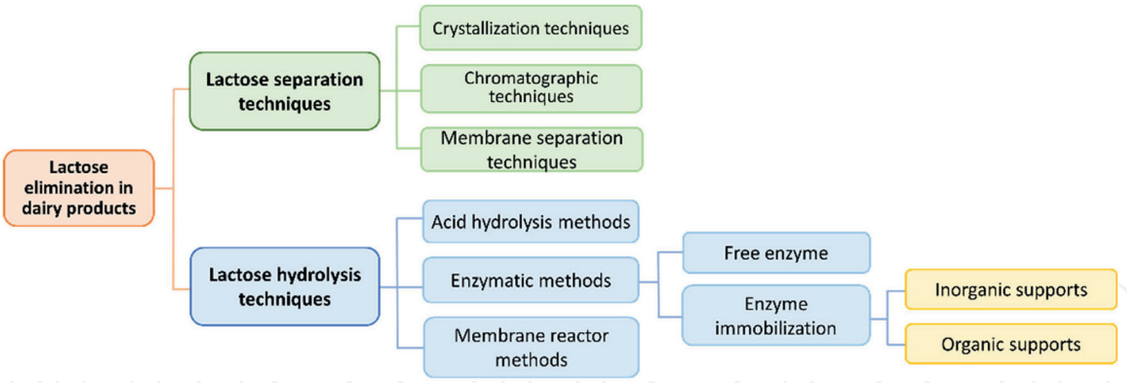


Figure 2.
Diagram of lactose removal techniques.

The removal of lactose from dairy products can be accomplished through separation or hydrolysis techniques. In separation methods, lactose is isolated from other milk components. Conversely, hydrolysis techniques involve the enzymatic or acidic breakdown of the β-(1 → 4)-glucosidic bond (**Figure 2**).

Industrially, most lactose-free products are produced through enzymatic hydrolysis techniques, where β-galactosidases are employed either as additives or coadjuvants. These enzymes are utilized either in free form, which can incur high costs and enzyme wastage, or in immobilized form, aiming to enhance enzyme stability and operational efficiency [8].

This chapter describes diverse techniques (illustrated in **Figure 2**) employed for lactose removal aimed at yielding lactose-free products. It offers a comprehensive exploration of separation and hydrolysis methodologies, elucidating their mechanisms and presenting instances from academic and industrial realms. Moreover, it conducts an in-depth description concerning the latest advancements in the immobilization of β-galactosidase onto polymeric supports for lactose elimination.

2. Lactose separation techniques

Lactose removal from a product can be achieved through separation techniques, where no enzymatic or chemical reactions are involved in breaking down this disaccharide; instead, it is simply isolated and removed. This lactose separation can be conducted through methods like crystallization, chromatography, and membrane separation. The resulting pure lactose obtained through these techniques finds application in the food industry as an ingredient in the production of various food items. Incorporating lactose into foods provides a range of unique functionalities, including low sweetness, reducing sugar properties, color/flavor carrying properties, protein stabilization, selective fermentation, or crystallization control, as well as physiological benefits such as prebiotic functions. It's widely used in various food products such as milk powder, infant formula, confectionery, baked goods, and as a base for dry food mixes [3].

2.1 Crystallization techniques

Crystallization is a physical process characterized by the formation of a crystalline solid resulting from cooling a saturated solution. Lactose exists in two anomeric

forms, α and β due to the existence of a chiral carbon. The most encountered form is a hydrated α-lactose monohydrate crystal that crystallizes at temperatures below 93.5°C [3, 9]. On an industrial scale, lactose crystallization is achieved through several sequential stages, including concentration, crystallization, and purification. Initially, dairy products undergo an evaporation process to eliminate water and concentrate lactose. The concentrated solution is subsequently transferred to a crystallization reactor, where lactose crystals gradually form via controlled cooling. Following crystallization, the crystals are separated from the solution using centrifugation. In the final purification stage, the crystals undergo washing and centrifugation again to remove impurities, yielding the desired final product. To optimize operational efficiency, it is crucial to maintain the temperature within specific ranges: Between 65°C and 70°C after the evaporation stage and between 25°C and 20°C during the crystallization process [9, 10]. It's important to understand that proteins can act as crystallization modifiers, since they have a significant impact on the formation and growth of crystals by affecting factors such as nucleation, crystal growth, and crystal habit. Studies suggest they diminish lactose solubility [11], expedite nucleation [12], and reduce lactose crystal size [13]. Although the precise mechanism of proteins' influence on lactose crystallization remains unclear, it's theorized that their water-binding capacity creates localized areas of lactose supersaturation [12]. Alternatively, some researchers suggest proteins may serve as nucleation centers, facilitating heterogeneous lactose nucleation [14, 15].

In industrial crystallization processes generally fine lactose crystals are obtained, while ideally the production of large and uniform crystals is essential to facilitate subsequent separation and handling. Preferably, lactose crystals should measure between 200 and 300 μm in size and exhibit a tomahawk shape [3, 15]. The size, distribution, and shape of crystals are contingent upon solution type and operational conditions. Additionally, the size and distribution of lactose seeds significantly impact nucleation and growth rates in crystallization. Therefore, in addition to conventional methods, negative temperature procedures to favor crystal growth [10], ultrasound to diminish induction times for nucleation and increase crystallization rate [16–18], or a flow of CO_2 and N_2 gases to increase the nucleation rate and yield [19] can also be employed during the crystallization stage to enhance crystal production. While the primary objective of this technique is not necessarily to produce lactose-free products, its application in removing lactose from dairy products can significantly contribute to the production of lactose-free products obtaining two products with added value in a single process. Understanding how varying percentages of lactose removal influence the production process can shed light on the scale and challenges associated with achieving lactose-free products using crystallization techniques. For instance, exploring how different levels of lactose removal affect the final taste, texture, and overall quality of the product provides valuable insights into the feasibility and optimization of producing lactose-free products [10].

An exemplary large-scale application of this technique is demonstrated by Fonterra, a dairy product company based in New Zealand. In 2016, Fonterra developed and patented an industrial crystallization process, primarily for lactose production, which indirectly facilitates the production of lactose-free dairy products. In this patented process, dairy products are initially heated to temperatures ranging between 50°C and 90°C in an evaporator to concentrate the lactose. Subsequently, the temperature is reduced to 8–25°C, promoting the formation of lactose crystals. These crystals are then separated from the rest of the solution through settling, followed by centrifugation and washing to eliminate impurities [20].

2.2 Chromatographic techniques

Chromatography is a versatile technique used to separate the various components of a mixture based on their differential retention while traversing through a supporting medium. This technique encompasses different variants depending on the characteristics of the stationary and mobile phases, as well as the interactions between the mixture components and these phases. Examples of chromatography types include ion-exchange chromatography (IEC), hydrophobic interaction chromatography (HIC), and affinity chromatography (AC), each one leveraging distinct interaction between the stationary phase and the mixture compounds for separation. Additionally, size-exclusion chromatography (SEC) represents another variant, wherein the separation is dictated by the size of the compounds [21].

Chromatographic techniques are not widely employed for lactose separation to produce lactose-free products on an industrial scale, primarily due to economic considerations. However, they find utility in determining lactose content and other compounds in dairy products. A notable exception is the application of chromatography to obtain lactose-free milk using a chromatographic column composed of poly(aniline) (PANI) functionalized with glutaraldehyde (GA) and lectin. This technique facilitated the removal of 47% of lactose from milk, leveraging lectin's high affinity for this disaccharide [22].

2.3 Membrane separation techniques

Membrane technologies rely on the application of pressure or electrostatic gradients to drive the passage of components in a solution through a semipermeable porous membrane. Components that are of a size compatible with the membrane pores and pass through are termed permeate, while those that are unable to permeate the membrane and are retained are referred to as retentate [21, 23].

In the dairy industry, membrane filtration techniques such as ultrafiltration (UF) and nanofiltration (NF) play a pivotal role in the recovery of various compounds from dairy products, including proteins, amino acids, and lactose. These methods operate on the principle of segregating particles based on their size using specialized membranes. UF membranes, with pore sizes ranging from 1 to 100 nm, facilitate the passage of small molecules like lactose, water, and minerals while retaining larger molecules such as proteins and fats. In contrast, NF membranes feature smaller pores (1–10 nm), enabling the retention of lactose and molecules of similar size while permitting smaller molecules like water and minerals to traverse through. Additionally, electro-dialysis (ED) techniques utilize electrically charged membranes to achieve separation via an electrostatic gradient, providing an alternative approach [23, 24]. Consequently, these techniques offer the potential to derive products with low lactose content or lactose-free products as byproducts of the filtration process.

The proper selection of membrane materials (ceramic or polymeric) is essential to ensure optimal separation efficiency. Polymeric membranes are more widely used for their affordability and lower energy demands compared to ceramic alternatives. Polymeric membrane materials used in production can be categorized into modified natural polymers, such as cellulose acetate, synthetic polymers including polysulfones, polytetrafluoroethylene, polyamides, polysulfides, and polypropylenes. Cellulose acetate membranes generally have lower thermal, chemical, and biological resistance compared to synthetic polymers, which offer

greater resilience to varying operational conditions such as temperature and pH. Conversely, ceramic membranes boast greater resilience owing to their physical, hydrothermal, and chemical stability properties. Depending on specific operational needs or applications, various materials such as Si, Zr, Ti, Al oxides, and silicon carbide (SiC) are utilized due to their distinct surface charge characteristics in solution. Ceramic membranes find common use in microfiltration (MF), UF, and NF applications. Additionally, they can be effectively cleansed using standard sanitizing products commonly employed in the food industry, thus enhancing their reusability (**Table 1**) [23, 25].

Considering the potential for obtaining lactose-free products through filtration and ED techniques, a study demonstrated the successful production of lactose-free powdered milk by integrating UF and NF techniques with ED [24]. Initially, the proteins and fats present in the dairy product are retained using a poly(ether sulfone) (PES) UF membrane. Subsequently, mineral salts are captured by the ED membrane to prevent fouling of the NF membrane and enhance the efficiency of lactose removal. It is noteworthy that the retained salts are reintroduced into the final lactose-free product at the conclusion of the process. Finally, lactose is retained in the NF membrane. This process achieved lactose-free powdered milk with a lactose content of less than 0.2%, along with lactose with a purity of 95.7%.

In addition to UF and NF membranes, MF membranes are utilized for their high permeation flow and efficiency in separating lactose and proteins in dairy products. A recent study [26] showcased the effectiveness of ceramic ZrO_2 and Al_2O_3 MF membranes in removing lactose from skim milk while simultaneously concentrating the protein in a single step. This resulted in 87.73% lactose removal and a product containing less than 5 g/L of lactose.

On an industrial scale, a Finnish dairy company (Valio) acquired a patent in 2010 for developing a process to produce milk with less than 0.01% lactose using membrane separation techniques. In an example of this process, 30 L of pasteurized milk with a 1.5% fat content undergo filtration at 50°C through a UF GR61PP membrane having a cut-off value of 20,000 Da, allowing lactose, mineral salts, and water to pass through while retaining proteins and fats. Subsequently, lactose is separated from the mineral salts and water using Millipore Nanomax-50 NF membrane, with lactose retained in the membrane and mineral salts and water forming the permeate. The mineral salts and water, comprising the NF permeate, are concentrated using a reverse osmosis membrane (Nanomax-95), where mineral salts are retained (NaCl retention > 94%) in the membrane while water passes through. Finally, the mineral salts recovered in the reverse osmosis stage (10.5 g) are mixed with the retained in the UF stage (69.2 g) and water (20.3 g). Then, 0.35 g of HA lactase is added and allowed to hydrolyze for 24 hours at 10°C resulting in lactose-free milk with identical organoleptic properties [27].

Membrane material	Optimum working pH	Optimum working temperature (°C)
Cellulose polyacetate	3–8	<50
Synthetic polymer	2–12	<80
Ceramic	0–14	>300

Table 1.
Optimal operational conditions according to the material of the membrane.

3. Lactose hydrolysis techniques

As highlighted earlier in this book chapter, various pathways have been devised for hydrolysis of lactose's β-(1 → 4)-glucosidic bond. These pathways encompass the chemical route, employing acid hydrolysis, and the biological route, employing enzymatic hydrolysis. Acid hydrolysis is facilitated by the presence of inorganic and organic acid compounds, while enzymatic hydrolysis utilizes biological catalysts such as enzymes. In addition to these two approaches, a third method known as membrane reactor method has emerged, which integrates enzymatic hydrolysis with separation stages. Below, we delve into the methodologies associated with acid hydrolysis, enzymatic hydrolysis, and membrane reactor techniques.

3.1 Acid hydrolysis methods

Acid hydrolysis of lactose involves the cleavage of the β-(1 → 4)-glucosidic bond through the action of acidic compounds like HCl, H_2SO_4, citric acid, and H_3PO_4 [28, 29]. In this process, the pH of the medium typically drops to around 1–2, and high-temperature conditions (100–150°C) are maintained [30]. While this method is commonly employed in whey or products obtained after milk UF, its aggressive conditions can result in protein degradation and the formation of undesired byproducts. Consequently, acid hydrolysis is not typically utilized for producing lactose-free products. However, it has found application in obtaining alternative products, such as lactose-free whey syrup with high glucose and galactose content, where hydrochloric acid combined with ultrasound reduces hydrolysis time to just 1 hour [31].

3.2 Enzymatic hydrolysis methods

The methods of enzymatic hydrolysis primarily involve the enzyme β-galactosidase, which, as mentioned, catalyzes the hydrolysis of the β-(1 → 4)-glucosidic bond present in galactosides. This tetrameric protein has an approximate molecular weight of 464 kDa, which may vary depending on its source. β-Galactosidase can be extracted from various biological sources, including bacteria, fungi, yeast, and plants. However, enzymes sourced from fungi such as *Aspergillus oryzae* and *Aspergillus niger*, as well as yeasts like *Kluyveromyces lactis* and *Kluyveromyces fragilis*, are the most commonly utilized in industrial applications [32]. The utilization of enzymes to produce lactose-free products can be accomplished by employing the enzyme in its free form or by immobilizing it on a support. Enzymatic lactose hydrolysis methods involve introducing the enzyme into a reactor containing a dairy product, facilitating the production of a lactose-free product through its catalytic action.

The optimal pH and temperature conditions for β-galactosidase, which exhibits maximal catalytic activity, are contingent upon its biological origin. Therefore, selecting the appropriate source of β-galactosidase is crucial to match the operational conditions required for effective hydrolysis. For instance, when hydrolyzing lactose in acid whey, β-galactosidases isolated from bacteria are preferred due to their optimal pH range between 2.5 and 5.4. Conversely, β-galactosidases sourced from yeasts are typically employed for lactose hydrolysis in milk and whey, as they demonstrate optimal pH activity within the range of 6.0–7.0 [32]. **Table 2** summarizes the key characteristics of β-galactosidases commonly utilized on an industrial scale.

Despite the availability of various enzymes sourced from different biological origins, efforts have been made to develop more efficient enzymes capable of

Biological origin of β-galactosidase	Microorganism type	Optimal temperature (°C)	Optimal pH
Aspergillus oryzae	Fungi	55–60	4.5–5.0
Aspergillus niger	Fungi	55	3.5–4.5
Kluyveromyces lactis	Yeast	30–35	6.5–7.0
Kluyveromyces fragilis	Yeast	30–35	6.0

Table 2.
Characteristics of the most common β-galactosidases employed in the dairy industry.

functioning across a broader pH range and at elevated temperatures (thermostable). Genetic engineering techniques have played a pivotal role in attaining this objective, enabling the genetic modification of organisms to facilitate the expression of proteins, such as tailored modified β-galactosidase, designed to fulfill precise specifications. These techniques involve the manipulation of genetic material to introduce, remove, or modify genes, thereby altering the genetic makeup of organisms to elicit desired traits or functions. In this context, the targeted modification of organisms to express a modified form of β-galactosidase underscores the versatility and precision afforded by genetic engineering in tailoring proteins to meet specific industrial, scientific, or therapeutic needs. β-Galactosidase has been subject to modification to enhance its stability, activity under particular conditions, or to confer specialized properties conducive to specific applications [33, 34].

3.2.1 Methods of enzymatic hydrolysis with free enzyme

In a study investigating the enzymatic hydrolysis of lactose in milk, cheese whey, and whey permeate ultrafiltrate using free β-galactosidases sourced from *A. oryzae* and *K. lactis*, researchers explored the effects of enzyme concentration, temperature, and reaction time depending on the biological origin of the enzyme [35]. At a temperature of 10°C, which is of particular interest for preserving nutritional and sensory composition while preventing microbial growth, regardless of concentration and reaction time, β-galactosidase from *K. lactis* demonstrated more efficient lactose hydrolysis in the mentioned dairy products compared to that from *A. oryzae*. However, complete hydrolysis of lactose, reaching 100%, was only achieved using *A. oryzae* at its optimal temperature of 55°C and after a reaction time of 12 hours.

On an industrial scale, lactose hydrolysis utilizing β-galactosidase has been widely employed to produce lactose-free dairy products such as milk, yogurt, cheese, and ice cream, among others, in two different processes, batch and aseptic. In the batch process, the enzyme is introduced into a reactor containing raw or thermized (15 seconds at 65°C) milk and allowed to incubate for 24 hours at temperatures between 4°C and 8°C to inhibit microbial growth. Then, the milk is pasteurized (this process also inactivates the enzyme), homogenized, and packaged. In the aseptic process, the first step in the milk sterilization following the ultra heat treatment (UHT) procedure (2 seconds at 142°C) and the sterile enzyme is then injected just before packaging. Because UHT milk is often stored in quarantine for around 3 days at room temperature, there is sufficient time for complete hydrolysis before the milk is sent to the retailer. Since pasteurized milk does not undergo a quarantine period, the aseptic process is not utilized for this type of lactose-free milk [36].

In most enzymatic lactose hydrolysis methods, β-galactosidase is typically the enzyme selected. However, as an alternative, β-glucosidase sourced from the archaea *Sulfolobus solfataricus* can be utilized. This enzyme exhibited broad substrate specificity across various oligosaccharides, encompassing lactose and glucose oligomers, alongside high thermostability and thermophilicity, withstanding temperatures of up to 80°C. Additionally, it demonstrated resilience against proteases, organic solvents, and detergents. Therefore, this other group of enzymes presents significant potential for industrial utilization due to their remarkable versatility and resilience [37].

3.2.2 Methods of enzyme immobilization

To mitigate the high operational costs associated with free enzymes, enzyme immobilization methods have emerged, involving the attachment of enzymes onto solid supports via covalent bonds or weak interactions. These methods address limitations such as the inability to reuse enzymes in multiple cycles and the susceptibility of enzymes to denaturation under working conditions, resulting in a rapid loss of catalytic activity. Immobilization reduces operational costs by facilitating enzyme reuse, enabling continuous operation, and enhancing enzymatic stability while also allowing for the separation of the enzyme from the product [38]. Achieving optimal stability and efficacy in the support-enzyme system requires careful selection of immobilization strategies to prevent conformational changes in the enzyme structure that could otherwise diminish its enzymatic activity and alter its kinetic properties [8].

Immobilization methods encompass both physical and chemical techniques. Physical methods include adsorption and entrapment, while chemical methods involve covalent binding and crosslinking (**Figure 3**). Adsorption immobilization entails attaching enzymes to support via non-covalent interactions like dipole-dipole interactions, ionic interactions, hydrophobic forces, van der Waals forces, and hydrogen bonds. This method is favored for its simplicity, requiring mild operational conditions of pH and temperature, supporting reuse potential due to the reversible bond between enzyme and support, and, crucially, preservation of enzyme structure, thereby maintaining catalytic activity. However, the weak interaction inherent in this immobilization type may lead to enzyme desorption from the support under varying operational conditions. Notably, ionic adsorption of enzymes on ion exchange resins is a notable subtype within adsorption immobilization [8, 39].

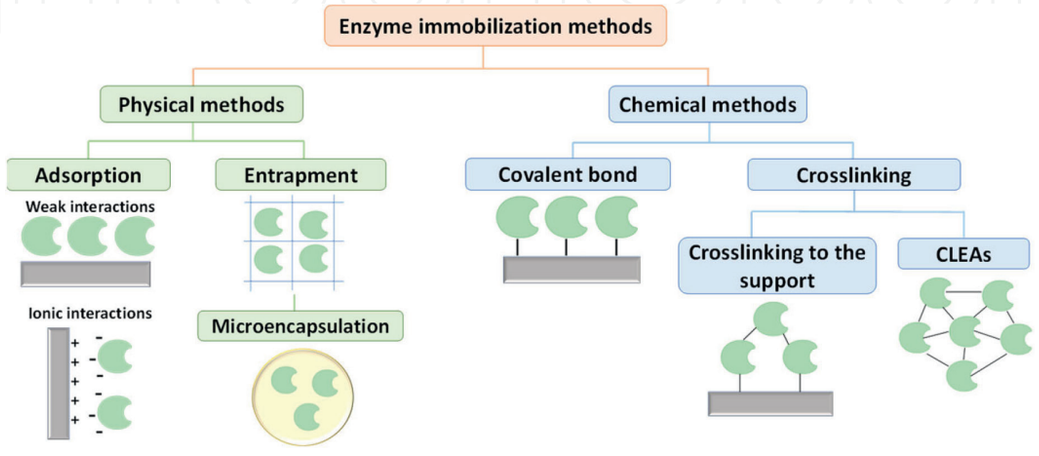


Figure 3.
Classification of different enzyme immobilization methods.

On the contrary, entrapment immobilization involves encapsulating the enzyme within a matrix. This physical method is known for its simplicity and ability to preserve the enzyme's structure, thus retaining enzymatic activity. However, the lack of strong interaction between the matrix and the enzyme may result in enzyme release under certain operational conditions. Moreover, diffusional limitations can impede substrate-enzyme active site contact or final product release [8, 40]. This method encompasses techniques such as microencapsulation, where the enzyme is enclosed within a semipermeable spherical membrane with controlled porosity [39].

In chemical methods, covalent binding immobilization involves the creation of covalent bonds between functional groups on the support's surface and amino acid residues on the enzyme's surface. This process can occur directly between the enzyme and the support or with the assistance of a bridging compound. Notably, this immobilization is typically irreversible. While the formation of strong bonds enhances the stability of the immobilized enzyme under varying operational conditions, it's important to acknowledge that this bond's nature may also induce structural changes in the enzyme, potentially impacting its catalytic activity and stability [41].

On the contrary, crosslinking immobilization utilizes a di- or multifunctional reagent such as GA, isocyanate, or *N*,*N*′-ethylenebismaleimide, with GA being the most prevalent, to form covalent bonds between enzyme molecules. Often, this method is coupled with other immobilization techniques to enhance system stability during operational conditions. Notably, crosslinking enables enzyme immobilization without any supports, known as crosslinked enzyme aggregates (CLEAs), by creating covalent bonds between enzymes [8, 40].

In addition to the various enzyme immobilization methods, it's crucial to underscore the significance of the different supports utilized for this purpose. A suitable support must fulfill several criteria, including stability under operational conditions, high surface area, biological inertness, resistance to biological degradation, cost-effectiveness, chemical and mechanical stability, and the presence of reactive groups on its surface [38]. Supports can be categorized as either inorganic or organic based on their nature. Inorganic supports are renowned for their exceptional mechanical, chemical, and electrical properties, along with their high surface area, porous structures, and cost-effectiveness due to their abundance. Among the frequently employed inorganic materials for immobilization are silica (SiO_2), metal oxides like ZnO, Al_2O_3, FeO, and Fe_2O_3, as well as minerals such as clay and bentonite [42]. Additionally, the growing utilization of nanomaterials, such as ZnO nanoparticles, is noteworthy due to their nanoscale dimensions, extensive contact surface area, resistance to mass transfer, and ability to enhance enzyme stability under varying operational conditions of temperature and pH [5].

Polymers are frequently utilized for enzyme immobilization when it comes to organic supports. Polymers are large molecules composed of monomers, which are repeated units along the chain. These supports are classified based on the origin of the polymer into natural, synthetic, and modified natural polymers. Natural polymers, or biopolymers, such as chitosan, agarose, and cellulose, are derived from living organisms and are recognized for their biocompatibility, biodegradability, low toxicity, and strong enzyme affinity [42]. In contrast, synthetic polymers such as polyethylene glycol (PEG) and poly(vinyl alcohol) (PVA) are synthetic and valued for their high versatility, ease of functionalization for specific applications, structural stability, and ability to shield biomolecules from degradation, thereby enhancing their stability [42, 43]. Hybrid or composite supports, consisting of a blend of inorganic and organic materials, offer a unique combination of properties derived from both components.

Commonly, these supports integrate nanomaterials with polymeric materials to leverage the advantages of both constituents [42, 44].

There is a growing interest in the development of effective supports for immobilizing β-galactosidase, given its significance in various industries. While carbon nanotubes and inorganic supports, particularly nanomaterials like nanodiamonds, ZnO nanoparticles, SiO_2 nanoparticles, and silver nanoparticles, have been utilized for this purpose due to their remarkable mechanical properties [45–51], the recent trend favors the use of polymeric supports. This preference is attributed to the abundance of polymeric materials, their versatility, and biocompatibility, offering promising prospects for β-galactosidase immobilization.

On an industrial scale, the Milan-based company Centrale de Latte (1975) pioneered the development of an industrial entrapment immobilization process for β-galactosidase, facilitating lactose-free milk production [52]. This process entails introducing milk into a reactor containing the enzyme immobilized in cellulose acetate fibers at a temperature of 5°C for 20 hours, resulting in a 75% lactose hydrolysis rate and enabling the production of 10,000 L of lactose-free milk per day. After each cycle, the fiber requires cleaning with a sterile buffer solution containing Mg^{2+} and treatment with a quaternary ammonium compound to prevent psychrophilic bacteria growth on the fiber's surface. Despite achieving significant operational stability, with only a 10% loss after the 50th cycle, this method does not operate continuously. The implementation of enzymatic immobilization on an industrial scale has paved the way for designing other immobilization systems for lactose-free product production. For instance, Corning company produced lactose-free whey by immobilizing β-galactosidase in porous glass beads. Valio, a Finnish company, also obtained lactose-free milk with the enzyme immobilized in a phenol-formaldehyde resin through adsorption and crosslinking with GA [52, 53].

3.3 Membrane reactor methods

In membrane reactors, a combination of UF membranes and enzymatic hydrolysis using β-galactosidase enables separation stages. The process hinges on the distinct interactions of lactose, the hydrolyzed product, and the enzyme with the UF membrane. In the general process, hydrolysis occurs in a protein-free milk or serum stream, purified by UF, where lactose is enriched in the permeate while the retentate has lower lactose content. The permeate then undergoes lactose hydrolysis in a reactor with β-galactosidase. The hydrolyzed product, passing through a second UF membrane, is mixed with the solution retained in the first UF stage to yield a lactose-free product. Meanwhile, the enzyme retained on the second UF membrane is recovered for another operational cycle [21, 54]. Despite the potential for continuous operation, microbial growth induced by non-sterile feedstock and operational complexities hinders widespread industrial adoption of this method, dampening commercial interest in membrane reactors [21].

In membrane reactor methods, the enzyme can either be immobilized on the UF membrane or in free form in the reactor. As an illustration of this, a work described a process where the lactose underwent hydrolysis using two hyperthermophilic β-galactosidases derived from archaea *S. solfataricus* and *Pyrococcus furiosus* [55]. This process occurred in a continuously stirred reactor integrated with a crossflow 10-kDa UF membrane, facilitating enzyme recovery. Throughout the operational period, which spanned over 2 weeks, the reactor maintained a consistent conversion rate of 80% while utilizing lactose as the substrate. In a separate instance, 43% of the lactose

present in skim milk underwent hydrolysis within a reactor featuring a PES UF membrane. In this setup, the enzyme was immobilized and crosslinked with 4% (v/v) GA [56]. Remarkably, the enzyme retained over 90% of its initial activity after 6 operational cycles. Likewise, lactose contained in cheese serum has been successfully hydrolyzed within a reactor housing β-galactosidase from *K. fragilis*. This enzyme was immobilized on a 10 kDa PES UF membrane activated with GA at a temperature of 55°C and a pH of 6.9 [57].

4. Enzyme immobilization on polymeric supports

The selection of the appropriate support for enzyme immobilization is paramount to ensure system efficiency. The material properties of the support significantly influence the enzyme's performance. Ideally, the support should exhibit high affinity with proteins, possess functional groups on its surface for enzyme interaction, demonstrate high mechanical stability, and be non-toxic, biocompatible, and biodegradable [58]. In addition, the presence of proteins or other components in milk can have complex effects on β-galactosidase activity in immobilized enzymes, which may require careful consideration and optimization in lactose hydrolysis processes.

Polymeric supports offer notable advantages over inorganic ones, particularly due to their versatility and exceptional mechanical properties, making them the preferred choice for enzyme and protein immobilization applications. As discussed previously, polymeric supports are categorized into two groups based on the polymer's origin: Natural and synthetic. Natural polymers, or biopolymers, are derived from living organisms and are specifically sourced from biologically derived polysaccharides or proteins. These polymers are esteemed for their biocompatibility, biodegradability, and non-toxicity. The natural origin of these polymers is responsible for their high biocompatibility with enzymes, which helps to minimize changes in the enzyme's structure and properties and achieve greater retention of enzymatic activity. Moreover, these materials are cost-effective in industrial settings as they originate from natural sources and can be readily obtained from nature or through industrial processes that generate them as byproducts. For all these reasons, polymeric supports based on biopolymers are widely used in enzyme immobilization. In these supports, the presence of various functional groups, such as -OH, -NH_2, -COOH, -CHO, and epoxy, on the surface allows enzyme immobilization through adsorption and covalent bonding. Additionally, the tendency of these polymers to form gels, along with their different configurations and geometries, makes them highly suitable for entrapment immobilization [50, 51].

Specifically, hydrogels, characterized by their crosslinked polymer network, are renowned for their high water adsorption capacity and the preservation of their three-dimensional structure during swelling, thus effectively entrapping enzymes within [59]. Among the biopolymers frequently utilized in immobilization, chitosan, cellulose, alginate, κ-carrageenan, pectin, and agarose stand out as the most prevalent options (**Figure 4**) [42, 43].

In contrast to natural counterparts, synthetic polymers are artificially synthesized through step-growth or chain-growth polymerization of monomers. Their synthetic origin enables tailored design, modification, and functionalization of monomers to suit specific enzyme immobilization processes and applications. It is noteworthy that monomers dictate the polymer's chemical structure, thereby influencing properties like solubility, porosity, stability, and mechanical attributes. The diverse array of polymers that can be synthesized facilitates the selection of a polymer containing

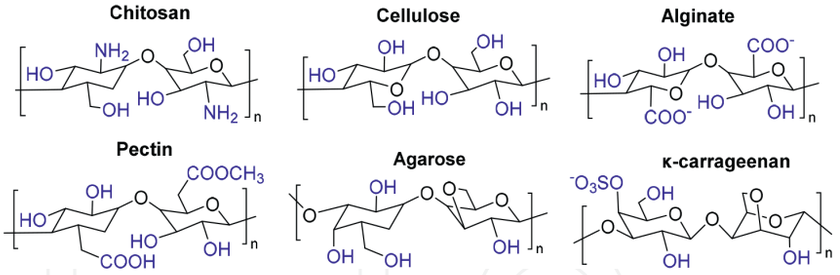


Figure 4.
Structure of the most commonly used biopolymers as polymeric supports.

the most suitable functional groups for anchoring enzymes to the support, aligning with the chosen immobilization strategy. Common functional groups found in synthetic polymers encompass carbonyl (-CO-), carboxylic acid (-COOH), hydroxyl (-OH), epoxy, amine ($-NH_2$), diols, hydrophobic alkyls, and alkylamines ($-NR_2$). Furthermore, surface modification of these supports enhances enzyme affinity while minimizing nonspecific interactions. An additional advantage lies in the ability to regulate spacer length between the polymer matrix and enzymes [60].

Polymeric supports derived from synthetic polymers are extensively employed in immobilization techniques, particularly those involving physical adsorption and covalent bonding. Among the most prevalent are poly(4-vinyl pyridine) (P4VP), polystyrene (PS), polyurethane (PUR), polylactic acid (PLA), polyethylene glycol (PEG), and poly(vinyl alcohol) (PVA) (**Figure 5**) [43, 59].

It's important to note that enzyme immobilization involves establishing weak or covalent interactions between the functional groups on the surface of the polymeric support and those on the amino acids present on the enzyme's surface [49]. Examples of these immobilization methods are depicted in **Table 3**, delineating the functional groups of the polymeric support, the reactive groups on the enzyme, and the nature of the interactions, which are elaborated upon below.

Hydrophobic adsorption immobilization (**Table 3**, entry 1) occurs through interactions between the alkyl groups on the support's surface and the enzyme's hydrophobic regions. Conversely, electrostatic interactions are established between the ionic groups on the support and the enzyme in ionic adsorption immobilization by ion exchange. Within this category, cationic exchange ionic adsorption (**Table 3**, entry 2) involves interactions between protonated amino groups (NH_3^+) on the support and deprotonated carboxylic groups (COO^-) on the side chains of aspartic and glutamic acid residues on the enzyme's surface. Conversely, in anionic exchange adsorption (**Table 3**, entry 3), the anionic groups on the support interact with the cationic groups on the enzyme's lysine and arginine amino acid side chains [46].

Moreover, the existence of electrophilic groups like aldehyde (-CHO) and epoxy groups on the polymeric support's surface facilitates the formation of covalent bonds with nucleophilic groups found on the enzyme's surface (-OH, $-NH_2$, -SH),

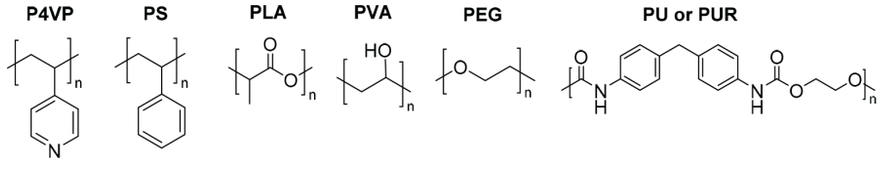


Figure 5.
Structure of the most commonly used synthetic polymers as polymeric supports.

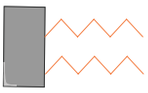
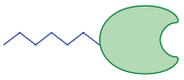
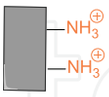
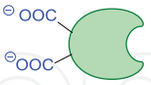
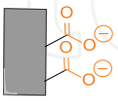
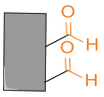
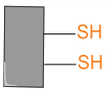
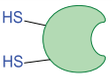
Entry	Functional group of the polymeric support	Surface reactive group of the enzyme	Type of interaction	Immobilization method
1			Hydrophobic interaction	Hydrophobic adsorption
2			Ionic interaction	Ionic adsorption by cation exchange
3			Ionic interaction	Ionic adsorption by anion exchange
4			Covalent bond through epoxy group chemistry	Covalent bond
5			Covalent bonds through aldehyde group chemistry	Covalent bond
6			Disulfide covalent bond	Covalent bond

Table 3.
Some functional groups are present on the surface of the polymeric support and the enzyme, as well as the interaction between these groups.

predominantly engaged in covalent immobilization [58]. In covalent immobilization, the aldehyde groups on the polymeric supports interact with the amino groups (-NH_2) on the enzyme's surface, forming secondary amines through Schiff base formation (**Table 3**, entry 5). In contrast, epoxy groups can interact with various nucleophilic groups on the enzyme, primarily hydroxyl (-OH), primary amine (-NH_2), and thiol (-SH) groups (**Table 3**, entry 4). When an epoxy group reacts with a primary amine, it forms a secondary amine; with a thiol, it forms a thioether; and with an alcohol, it results in an ether. Heterofunctional supports utilize epoxy groups for immobilization through a two-step process involving ion exchange or hydrophobic adsorption, followed by covalent immobilization based on epoxy group chemistry [58, 61, 62]. The primary amine group (-NH_2) is the most significant nucleophilic group found on the enzyme's surface. In fact, proteins typically react through terminal amino groups, specifically the terminal primary amines of the amino acid lysine.

Furthermore, the presence of cysteine in the enzyme provides thiol (-SH) groups capable of forming disulfide bonds with supports modified with -SH groups (**Table 3**, entry 6). Similarly, the presence of tyrosine contributes to an increase in the number of hydroxyl (-OH) groups, which interact with the electrophilic groups on the support [58] (**Table 3**, entry 4).

The modification of polymeric supports is relatively straightforward and allows for the addition of spacer molecules, which act as a bridge between the support's surface and the enzyme's surface, enhancing enzyme mobility. The length of these molecules impacts the enzyme's properties: Longer spacers increase the enzyme's conformational flexibility, thereby enhancing mobility, while shorter ones confer thermal stability and reduce the likelihood of enzyme detachment from the support [58].

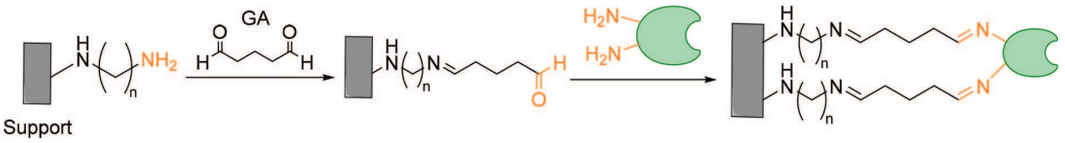


Figure 6.
Activation of the amino groups of the polymeric support with GA and covalent immobilization of the enzyme.

On the other hand, polymeric supports may feature nucleophilic groups on their surface, predominantly amino (-NH_2) and hydroxyl (-OH) groups, facilitating support functionalization with electrophilic groups for enzyme anchoring via covalent bonds. Supports with amino groups are activated using difunctional reagents like glutaraldehyde (GA), enabling the incorporation of aldehyde groups on the surface capable of reacting with amino groups on the enzyme, forming Schiff bases (depicted in **Figure 6**). The resulting imine can then be stabilized by reduction with $NaBH_4$, minimizing enzyme leakage but potentially compromising enzyme activity. Conversely, supports with hydroxyl groups can be activated with cyanogen bromide (BrCN), which reacts with these groups to form imidocarbonates, subsequently engaging with amino groups on enzymes (particularly lysine) to forge covalent bonds [61].

4.1 Immobilization of β-galactosidase on polymeric supports

As previously mentioned, β-galactosidase functions as a hydrolase, an enzyme that catalyzes the breakdown of chemical bonds by adding a water molecule. In the case of lactose, β-galactosidase breaks the β-(1 → 4)-glucosidic bond present in this disaccharide. Notably, this enzyme also exhibits transglycosidic activity, enabling the transfer of galactose from a galactosyl donor compound to an acceptor compound, leading to the formation of oligosaccharides or glucosides [63]. Industrially, β-galactosidase holds significant importance, particularly in the production of lactose-free products within the food industry and the synthesis of galacto-oligosaccharides (GOS). These GOS serve as non-digestible prebiotics, fostering the growth of beneficial bacteria in the intestine and contributing to human health by modulating immune system activity and potentially preventing conditions like cancer, among other positive effects [63]. Given its pivotal role in industrial processes, considerable research and development efforts have been dedicated to creating polymeric supports for its immobilization.

The structural features, composition, and arrangement of amino acids on the surface of β-galactosidase facilitate its immobilization through various methods such as adsorption, entrapment, covalent bonding, and crosslinking. Notably, the presence of specific amino acid residues like aspartic acid, glutamic acid, lysine, and arginine on the enzyme's surface plays a crucial role in its immobilization onto ion-exchange resins through ionic adsorption. For instance, the carboxyl (-COOH) groups located on the side chains of aspartic and glutamic acids can undergo deprotonation by adjusting the pH, allowing them to electrostatically interact with cationic groups present on the surface of the polymeric support. Conversely, the amino groups (-NH_2) found on the side chains of lysine and arginine can be protonated and interact with anionic groups on the support. On the other hand, covalent bonding to the support is achieved through various functional groups, including the amino groups on lysine side chains, the terminal amino groups of the enzyme's subunits, the hydroxyl groups on tyrosine residues, the thiol groups of cysteine, and the imidazole groups of histidine (**Figure 7**) [58].

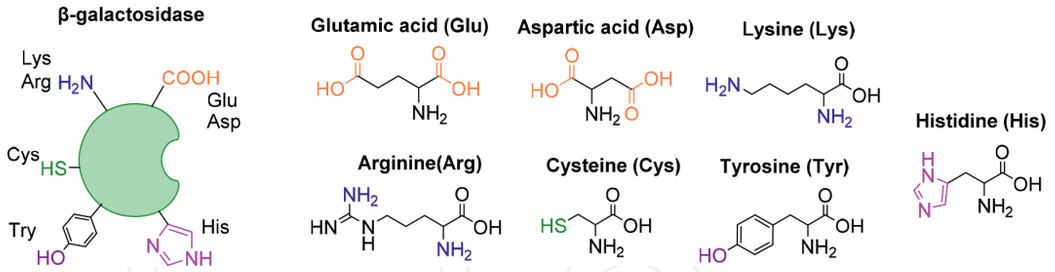


Figure 7.
Schematic representation of β-galactosidase and the amino acids involved in its immobilization.

4.1.1 Immobilization of β-galactosidase by adsorption

The even distribution of anionic amino acids, such as glutamic acid and aspartic acid, across the surface of β-galactosidase facilitates its immobilization through ionic adsorption onto ion-exchange resins [64, 65]. This method was successfully applied in the immobilization of β-galactosidase from *A. oryzae* on an anion-exchange resin functionalized with quaternary ammonium groups (**Figure 8A**) [65]. The process resulted in a 76% yield in enzyme immobilization under conditions of 30°C temperature and a pH of 7. The objective of this immobilization was to synthesize lactulose, a bioactive disaccharide widely utilized in pharmaceuticals and as a prebiotic ingredient in food products (**Figure 8B**).

In another study, β-galactosidase from *A. oryzae* was immobilized on a commercial anion-exchange resin, Duolite™ A568 (phenol-formaldehyde resin) [66]. This method involved activating tertiary amine groups present on the support's surface in an acidic medium. Optimization was achieved by combining ionic adsorption with enzyme crosslinking using GA. Notably, the adsorption-based immobilization retained 51% of the initial enzymatic activity after 30 operational cycles, while the adsorption-crosslinking immobilization retained 90%.

Anion-exchange resins stand out as the preferred polymeric support for the ionic adsorption immobilization of β-galactosidase due to their ability to yield high rates of

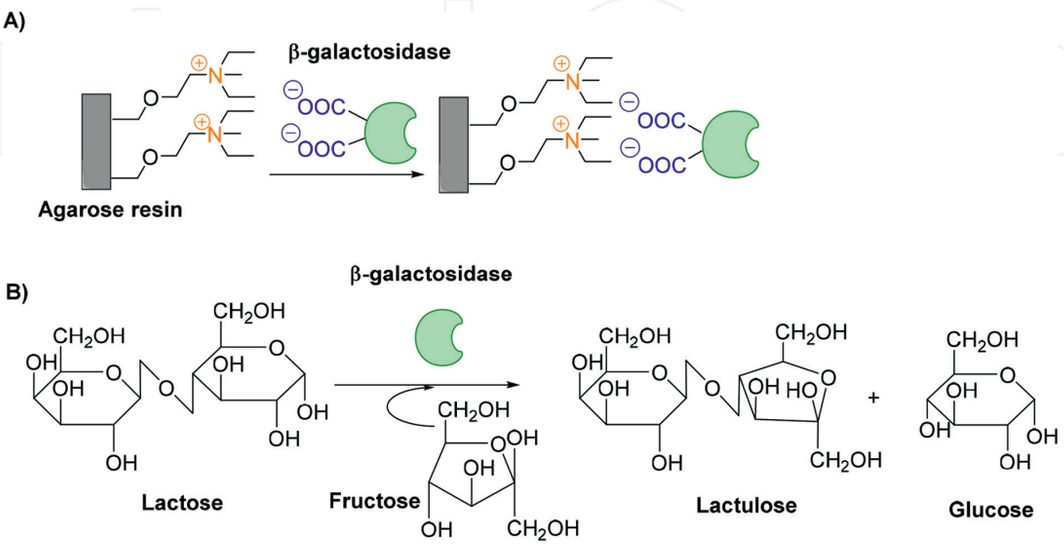


Figure 8.
Ionic adsorption immobilization of β-galactosidase: (A) immobilization system and (B) lactulose synthesis reaction carried out by the enzyme-support system.

enzyme immobilization. Nevertheless, variations in the pH of the medium can trigger enzyme desorption from the support, underscoring the criticality of pH control during both the immobilization process and lactose hydrolysis [67].

4.1.2 Immobilization of β-galactosidase by entrapment

The entrapment immobilization of β-galactosidase commonly utilizes hydrogels based on natural polymers, prized for their biocompatibility, biodegradability, and lack of toxicity. For instance, a crosslinked chitosan hydrogel incorporating acrylic acid and *N*′,*N*′-methylenebisacrylamide has been employed to hydrolyze lactose in both commercial lactose solution and UHT milk [68]. Over 10 lactose hydrolysis operational cycles in the commercial lactose solution, the activity of the immobilized enzyme decreased from 98% to 56%, while in UHT milk, it decreased from 98% to 72%. Similarly, Arabic gum hydrogels crosslinked with acrylamide have shown promise in lactose hydrolysis since, after three operational cycles, enzymatic activity was recorded at 53% and 94% in standard lactose and UHT milk, respectively [68, 69].

It's also commonplace to utilize hydrogels formed by the combination of two biopolymers. For instance, β-galactosidase has been immobilized in a pectin hydrogel incorporating varying percentages of pine residue (5% and 10%) [70]. Pine residue, comprising cellulose, hemicellulose, and lignin, bolsters the mechanical resilience of polysaccharide-based hydrogels owing to its chemical composition. Furthermore, its affordability, biocompatibility, and eco-friendly attributes make it particularly intriguing to develop solid supports. Immobilization studies with this composite revealed that at pH 4.0 and after 600 minutes, the enzyme's immobilization capacity was 181 mg of enzyme per gram of dry hydrogel for the 5% pine residue and 183 mg enzyme per gram of dry hydrogel for the 10% pine residue. However, at pH 5.6, the immobilization capacity increased to 220 and 219 mg of enzyme per gram of dry hydrogel for the 5% and 10% pine residue, respectively. An enzyme immobilization within a pectin hydrogel was also conducted to contrast the outcomes with those obtained from the prior support. The immobilization capacity within this pectin hydrogel was observed to be 242 and 183 mg of enzyme per gram of dry hydrogel at pH 4.0 and 5.6, respectively. It's essential to consider that the carboxyl groups of the pectin hydrogel deprotonate at pH values surpassing the pK_a of pectin, which stands at 3.5. Conversely, the cationic groups on the surface of β-galactosidase prevail at pH values below its isoelectric point, which is 4.8. Consequently, the higher immobilization capacity of the pectin hydrogel at pH 4.0 compared to pH 5.6 is attributed to the robust electrostatic attractions established between the deprotonated carboxyl groups of the hydrogel and the protonated groups of the enzyme, thereby enhancing the stability of the enzyme-support bond. In contrast, at pH 5.6, intense electrostatic repulsion ensues between the negatively charged groups of the hydrogel and the enzyme, resulting in destabilized immobilization. However, a contrasting pattern emerges with the pectin/pine residue hydrogel, as immobilization demonstrates greater stability at pH 5.6 compared to pH 4.0. This shift arises from the pine residue, which mitigates repulsion between the negatively charged enzyme groups and the pectin by positioning itself within the hydrogel pores, thus increasing the separation between these groups. Similarly, in another study [71], lactose hydrolysis was conducted utilizing β-galactosidase immobilized within a hydrogel composed of alginate and gelatin crosslinked with genipin, a natural crosslinking agent derived from gardenia fruit. The immobilized enzyme exhibited remarkable longevity, retaining 90% of its initial activity after 11 operational cycles in a batch reactor and maintaining 80% of its initial activity even after 175 days of storage in a freezer.

Mostly, hydrogels made from natural polymers are chosen for the immobilization of β-galactosidase, although hydrogels made from synthetic polymers can also be employed. One such example is the use of LentiKats®, a PVA hydrogel, for the immobilization of β-galactosidase from *K. lactis* and *A. oryzae* [72]. The study delved into the potential of this enzyme-support system in lactose hydrolysis to yield the monosaccharide D-galactose. Notably, the immobilized enzymes were employed in tandem with yeasts: Initially, β-galactosidase catalyzed lactose hydrolysis, followed by yeast-mediated fermentation of glucose to yield galactose. Results revealed that β-galactosidase from *A. oryzae* retained 80% of its initial activity after 466 operational hours in a batch reactor, generating D-galactose at a rate of 1.9 g/L h. Conversely, under identical conditions, β-galactosidase from *K. lactis* exhibited a lower retention of initial activity at 50%, yet yielded a higher D-galactose production rate of 3 g/L h. Another noteworthy example involves the immobilization of β-galactosidase from *A. oryzae* within cryogels, which in this case, are gels formed at sub-zero temperatures, composed of polyacrylamide (PAM) and poly(2-hydroxyethyl methacrylate) (HEMA) chelated with Fe^{3+} [73]. This study evaluated the enzyme-support system's efficacy in lactose hydrolysis, revealing that the immobilized enzyme exhibited optimal conditions at pH 3.0 and preferred temperatures between 60°C and 65°C. Impressively, it retained 65% of its initial activity after 25 operational cycles and experienced a modest decline of 29% in activity following 70 days of storage.

These findings underscore the prominence of supports based on polysaccharide hydrogels in β-galactosidase immobilization for diverse applications. Their appeal lies in their biocompatibility, substantial immobilization capacity, facilitation of environmentally friendly support utilization, straightforward preparation, and ability to maintain enzymatic activity across numerous operational cycles.

4.1.3 Immobilization of β-galactosidase by covalent bonding

As discussed earlier, covalent immobilization of β-galactosidase typically involves the interaction between nucleophilic groups on the enzyme and electrophilic groups on the polymeric support. Polymeric supports featuring epoxy groups on their surface react with amine, hydroxyl, and thiol groups on the enzyme's surface, forming covalent bonds of secondary amine, ether, and thioether types. For instance, for lactose hydrolysis in milk, a recent study describes the immobilization of *Escherichia coli* β-galactosidase using an acrylic UV-cured polymeric film functionalized with epoxy groups [74]. The enzyme was anchored to the polymeric film through the reaction of the epoxy groups on the support with the amine groups present on the enzyme's surface. The immobilized β-galactosidase exhibited an optimal pH of 6.5 and temperature of 60°C, retaining 51% of its initial activity after 12 operational cycles of lactose hydrolysis. Regarding the support composition, the polymeric film comprises glycidyl methacrylate monomers (20% by weight), trimethylolpropane triacrylate (15% by weight), and poly(ethylene glycol) methyl ether acrylate polymer (65% by weight) (**Figure 9A**). This support is characterized by its excellent resistance to chemicals and abrasion, as well as thermal insulation properties, high thermal and mechanical stability, and strong adhesion to substrates of diverse natures.

Another example of β-galactosidase immobilized in a film-shaped polymeric support was described in the work by Vallejo-García et al. [75]. In this study, β-galactosidase from *A. oryzae* was covalently immobilized via azo linkages in highly manageable polyacrylic films and subsequently employed to hydrolyze lactose from commercial milk (**Figure 9B**). Results demonstrated that these films were capable of

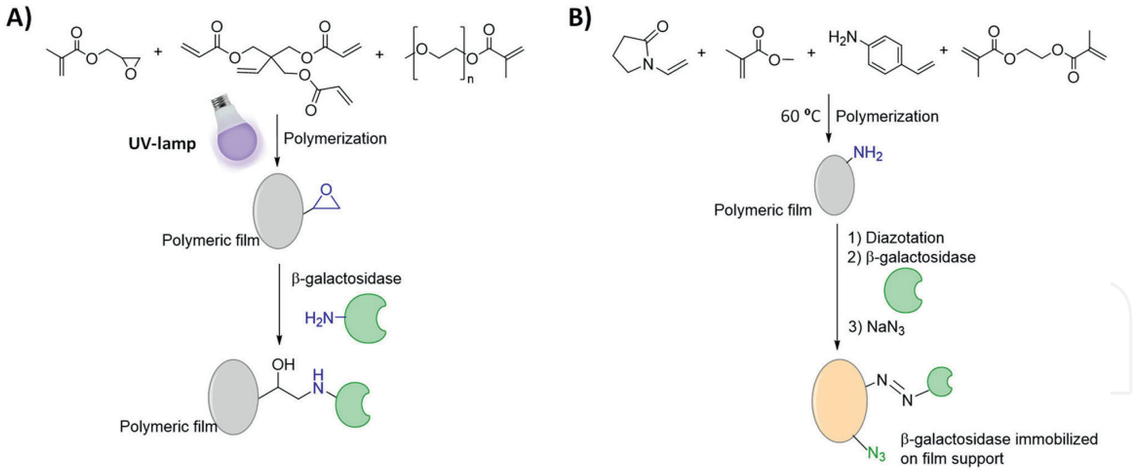


Figure 9.
Immobilization of β-galactosidase on a polymeric film with epoxy groups.

producing lactose-free milk (with concentrations below 0.1 g/100 mL) after 20 hours of incubation at 25°C or 6 hours at 55°C. However, at 4°C, the final lactose concentration after 30 hours was not sufficiently low to be considered lactose-free but rather low-lactose milk. Additionally, the material's operational and storage stability is noteworthy, as it retained its ability to hydrolyze lactose in milk even after 10 cycles and 1 month of storage without any loss of activity, which was attributed to the covalent immobilization method.

When considering commercially available supports, an illustration can be found in the immobilization of β-galactosidase from *A. oryzae* on a commercial polymeric support named epoxy Immobead 150® (Immobead-Gal, **Figure 10A**) [76]. Additionally, the enzyme was immobilized on the same support after modifying it with aldehyde groups, achieved through the reaction of the epoxy groups on the surface with GA, resulting in Immobead-Glu-Gal (**Figure 10B**). The immobilization process on the unmodified support entailed the formation of covalent bonds between the epoxy groups on the support and the amino groups of β-galactosidase (**Figure 10A**). Conversely, on the modified Immobead-Glu support, covalent bonds were established between the aldehyde groups on the support and the amino groups of the enzyme (**Figure 10B**).

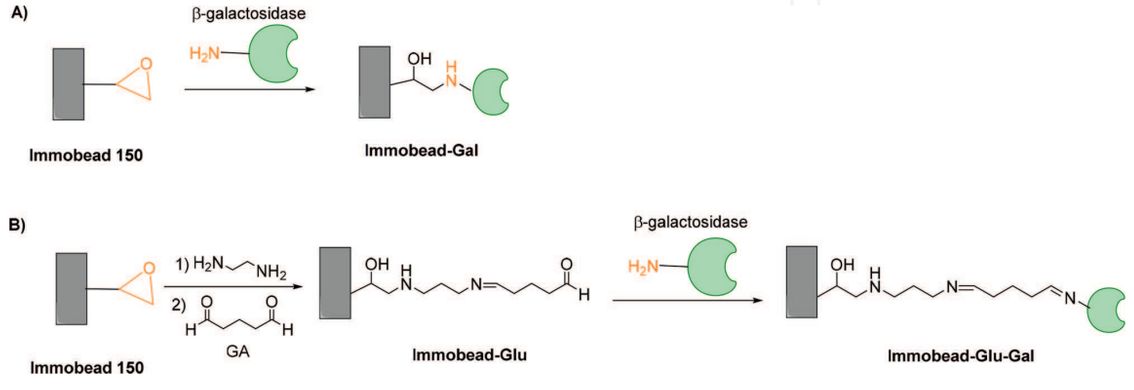


Figure 10.
Immobilization of β-galactosidase by covalent bonding: (A) Immobead 150® support and (B) Immobead-Glu support.

The effectiveness of immobilization was assessed through lactose hydrolysis in various substrates, including an artificial lactose solution, whey filtrate, cheese whey, and skim milk. Both supports exhibited a yield of over 75% in enzyme immobilization, with the enzyme retaining 50% of its initial activity after 20 operational cycles using the lactose solution. Notably, the optimal pH shifted from 5.0 for the free enzyme to 6.0 for the immobilized enzyme. Furthermore, the immobilized enzyme displayed significantly higher activity at 70°C compared to its free counterpart.

In addition to these supports, there is a growing interest in enzymatic immobilization on hybrid supports formed by combining polymeric materials with nanomaterials, leveraging both materials' superior chemical, physical, and mechanical properties. These supports have been used for the covalent immobilization of β-galactosidase in applications requiring lactose hydrolysis. One notable example is the hybrid support Fe_3O_4@PDA@DAPEG-GA, developed for the immobilization of β-galactosidase from *A. oryzae* (**Figure 11**) [77]. Comprising Fe_3O_4 nanoparticles along with the polymers polydopamine (PDA) and polyethylene glycol diamine (DAPEG), this support's surface is functionalized with GA. GA reacts with the amino groups of lysine and arginine residues on the enzyme's surface to achieve immobilization. The enzyme immobilized on this support displayed an optimum pH of 6.0 and an optimum temperature of 45°C, which is 15°C higher than that of the free enzyme. Moreover, the enzyme retained 94% of its initial activity in a lactose hydrolysis process after 96 hours in a continuous reactor.

Up to this point, all the supports mentioned for the covalent immobilization of β-galactosidase are composed of synthetic polymers, but biopolymers can also be used. β-Galactosidase from *A. oryzae* was immobilized on chitosan support, where the support was either fully or partially functionalized with aldehyde groups [78]. In the fully modified support, aldehyde groups are introduced by reacting the surface groups of the support with GA or epichlorohydrin (ECH). The enzyme is then immobilized through the formation of covalent bonds between the amino groups on the enzyme's surface and the aldehyde groups on the support's surface at pH 10, to deprotonate ε-amino groups of the lysine (**Figure 12A**). In contrast, the partially functionalized surface support distinguishes aldehyde groups introduced by reaction with ECH and amine groups previously activated in an acidic medium (pH 5.5) (**Figure 12B**). In this support, immobilization occurs in two stages. Initially, at pH 5.5, the enzyme is immobilized by ionic adsorption between the protonated amino groups on the support's surface and the carboxylate groups on the enzyme's surface.

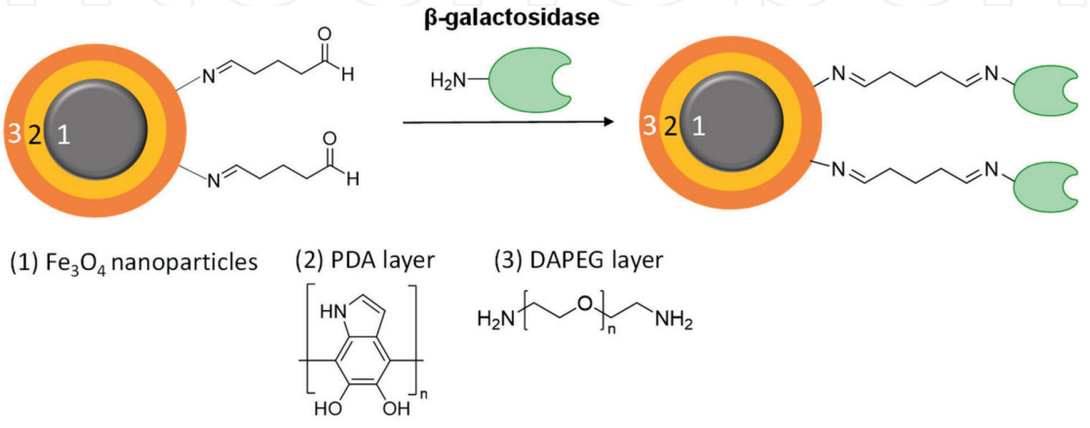


Figure 11.
β-Galactosidase immobilization on the Fe_3O_4@PDA@DAPEG-GA hybrid support.

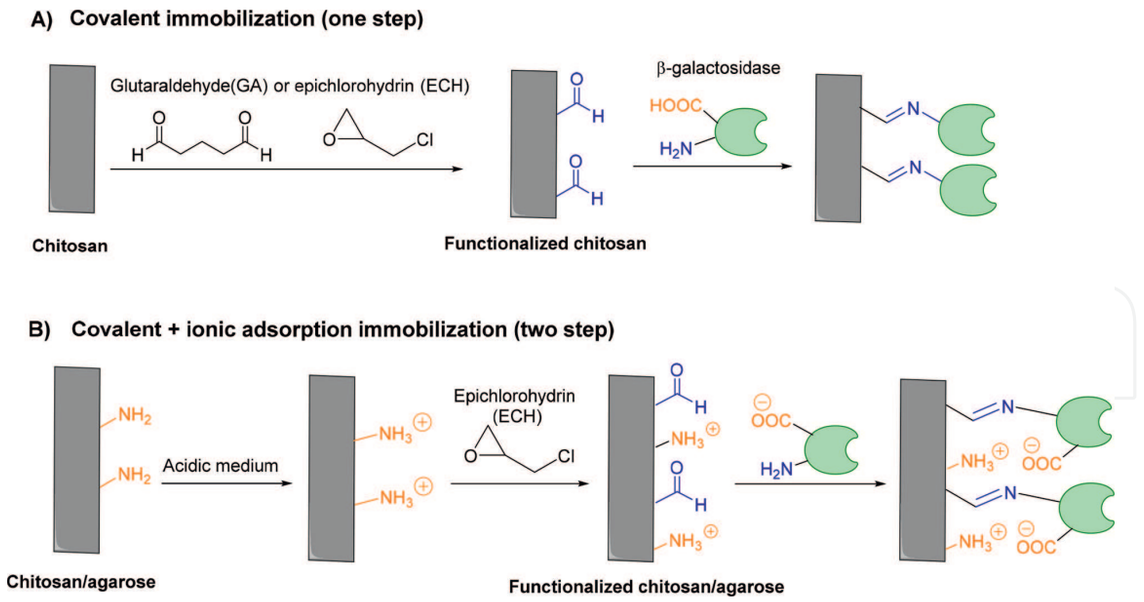


Figure 12.
Immobilization of β-galactosidase on biopolymers: (A) support fully modified with aldehyde groups and (B) support partially modified with aldehyde groups.

Subsequently, at pH 10, covalent immobilization occurs through the formation of covalent bonds between the aldehyde groups on the support's surface and the amino groups on the surface of β-galactosidase (**Figure 12B**).

The percentages of β-galactosidase immobilization on supports fully functionalized with GA or ECH, and on the partially ECH-modified support were 29%, 6%, and 42%, respectively. When the support is fully functionalized with GA, a higher immobilization efficiency is achieved compared to when ECH is used. This is attributed to the versatile chemistry of GA and its various degrees of polymerization, enabling multiple mechanisms for enzyme immobilization, besides Schiff base formation. However, a higher yield is obtained in the support partially functionalized with ECH compared to the previous supports. Here, preliminary immobilization by ionic adsorption occurs, facilitating interaction between the amino groups on the enzyme's surface and the aldehyde groups on the support's surface, leading to intramolecular covalent bond formation and a higher immobilization capacity. Additionally, the partially functionalized support exhibited greater thermal stability, likely due to multipoint attachment with the support and/or a different enzyme orientation.

Given the robust stability of β-galactosidase during operational conditions, covalent immobilization onto polymeric supports is extensively employed in lactose-free product production, ensuring sustained enzymatic activity across numerous operational cycles. Nevertheless, the fabrication processes of these supports can sometimes be intricate. Furthermore, a full comprehension of the covalent bond formation mechanism between the support surface and the enzyme surface is imperative to forestall any alterations to the enzyme's active center, which could lead to a decline in catalytic activity.

5. Conclusions and future perspectives

There is a growing interest in producing lactose-free products via enzymatic hydrolysis using β-galactosidase immobilization methods on supports. This approach

allows lactose-intolerant individuals to incorporate these essential products into their diets without compromising their health. Enzymatic immobilization stands out among other lactose separation or hydrolysis methods due to its ability to reuse the enzyme across multiple operational cycles, resulting in cost reduction in industrial production, ensuring enzyme stability under operational conditions, and preserving the enzyme's conformation and catalytic activity. Additionally, the wide range of available supports offers the flexibility to select the most suitable support for the immobilization process, the enzyme, and the specific application.

In this context, it's crucial to emphasize the pivotal role of polymeric supports, both natural and synthetic, in enzymatic immobilization. Their utilization presents numerous advantages, including biocompatibility, contribution to the development of environmentally friendly supports, versatility, availability of various functional groups for enzyme anchoring through different immobilization methods, and exceptional chemical, mechanical, and biological properties.

Industrially, transitioning from batch processing to continuous processing holds potential economic and environmental advantages, primarily due to the recovery of enzymes, which would reduce the overall cost of enzymatic hydrolysis. However, the commercial development of continuous enzymatic hydrolysis faces obstacles related to scaling up batch reactors and the recovery and cleaning of immobilized enzymes. Additionally, the recent surge in patents related to enzyme immobilization, particularly β-galactosidase, presents a promising advancement. Further exploration into the feasibility and development of large-scale bioreactors utilizing immobilized β-galactosidase that can be efficiently reused is imperative. Thus, there is a crucial need for research focused on theoretically scaling up enzymatic bioreactors to advance the commercialization of this process. Continuous efforts in developing β-galactosidase immobilization processes on polymeric supports are essential for designing efficient and economically viable industrial processes for lactose-free product production.

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Conflict of interest

The authors declare no conflict of interest.

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