Chapter

Optimizing Enzymes for Dairy Processing: A Protein Engineering Perspective

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Abstract

The dairy industry relies heavily on enzymes for various processes, from milk coagulation to flavor development. Enzyme engineering has revolutionized these applications by creating more efficient, stable, and specific biocatalysts. Key advancements include recombinant production of chymosin replacing traditional calf rennet, development of cold-active and thermostable lactases for lactose-free products, creation of regioselective lipases for targeted flavor development, and modification of proteases for controlled cheese ripening. Modern techniques encompass rational protein design, directed evolution, computational modeling, and innovative immobilization technologies such as cross-linked enzyme aggregates and magnetic nanoparticle carriers. These engineered enzymes deliver significant industrial benefits, including higher yields, consistent product quality, reduced energy consumption, and novel dairy product development. Enzyme engineering also addresses growing consumer demands for clean-label products through improved specificity and reduced side reactions. The continuing evolution of these techniques promises further innovations in sustainability, functionality, and nutritional enhancement in dairy processing across global markets.

Keywords: dairy enzymes, directed evolution, rational enzyme design, enzyme engineering, dairy processing, biocatalyst optimization

1. Introduction

It is a vital component of the global economy, contributing significantly to the agricultural and food sectors. The industry in question encompasses a variety of activities, including, but not limited to, dairy farming, the production of milk, and its subsequent processing into various products, such as cheese, yogurt, and butter. The distribution of these products to consumers on a global scale is also an integral aspect of the industry [1]. Dairy products are classified as functional foods due to their inherent or possible enrichment with bioactive components, which confer health benefits that extend beyond basic nutritional requirements. The aforementioned benefits encompass the following: the prevention of disease, the enhancement of metabolism, and the optimization of physiological functions [2].

Technological advancements are significantly transforming the dairy sector. Furthermore, innovations in biotechnology and functional dairy product development are expanding the industry's product range. Enzymes, for instance, play a crucial and transformative role in the dairy industry, significantly enhancing production efficiency, product quality, and environmental sustainability. The utilization of these applications has been demonstrated to enhance the sensory quality and safety of products, while concurrently contributing to the industry's sustainability by minimizing waste and reducing energy consumption [3, 4].

Enzymes are proteins that act as pivotal biological catalysts, thereby accelerating essential chemical reactions in biological systems. It is evident that these substances fulfill a pivotal function in a multitude of physiological processes by accelerating reactions without being consumed in the process. This enables biological processes to proceed under milder conditions, such as lower temperatures and a neutral pH, than would otherwise be feasible [5, 6]. Beyond their role within biological systems, enzymes boast a plethora of industrial applications [7, 8]. Their utilization is pervasive within the food industry, encompassing processes such as starch processing, meat processing, dairy industry operations, wine industry practices, and the manufacture of predigested foods [9, 10]. For instance, they are employed in the degradation of oil spills and waste materials, the removal of fat stains, and the fermentation process for the production of alcoholic beverages.

Enzyme engineering customizes enzymes by altering their amino acid sequences to achieve improved properties. Enzyme engineering optimizes β-galactosidase activity to maximize lactose conversion while maintaining product quality and sensory characteristics, allowing the production of lactose-free or reduced-lactose dairy beverages. Additionally, enzymatic synthesis of prebiotic galactooligosaccharides (GOS) from lactose during fermentation creates functional symbiotic products that combine probiotic and prebiotic effects, further enhancing health benefits for consumers, including those with lactose intolerance.

2. Enzymes in the dairy industry

2.1 Historical overview of enzyme utilization in the dairy sector

In the dairy industry, enzymes are considered to be essential tools for the transformation of milk into a wide range of products, with specific characteristics that are in accordance with the desired product specifications. These enzymes are also known to extend the shelf life of the products and enhance their nutritional profiles [11–14]. The utilization of enzymes in the production of foodstuffs, including dairy products, constitutes an ancient practice with origins extending thousands of years. The earliest documented applications of biological agents in food processing date back to 6000 BCE, with practices such as bread baking, brewing beer, and the production of cheese and wine [7, 9, 15–17]. One of the most well-known and historically significant applications of enzymes in dairy is the use of rennet for milk coagulation in cheese production [13, 18, 19]. It is speculated that cheesemaking may have been discovered serendipitously 4000–5000 years ago when milk was stored in bags made from calf stomachs, where remnants of aspartic proteases from the stomach, along with natural milk flora, led to coagulation [19–21]. The first commercial standardized rennet preparation was produced and sold by Chr. Hansen A/S, Denmark, in 1874, making it arguably the first commercial enzyme of any type [22].

2.2 Applications of enzymes in the dairy industry

The global market for food enzymes, valued at USD 2058 million in 2020, is projected to reach USD 4194 million by 2032, with the dairy sector being a major demand area for enzymes [10, 12]. The dairy industry represents a significant domain for the implementation of enzymatic processes, with a diverse range of food products, including cheese, butter, and yogurt, being produced. The primary exogenous enzymes employed in dairy products are proteases (such as rennet and chymosin), lipases, and lactases, which are classified as hydrolases within the EC 3.x classification system [12].

Enzymes continue to play a pivotal and multifaceted role in modern dairy processing, offering innovative solutions for enhancing product quality, efficiency, and sustainability (curdling) of milk, a critical step in cheese production. The primary function of these enzymes is to hydrolyze κ-casein, a milk protein that stabilizes casein micelles, thereby allowing the micelles to aggregate and form a gel-like curd [16]. Enzymes are vital in the production of cheese, chiefly due to their ability to induce milk coagulation. Rennet, an enzyme complex that is traditionally derived from animal stomachs, is responsible for the breakdown of milk proteins, resulting in the formation of curds and whey. The utilization of sustainable sources of these enzymes, encompassing microbial and genetically engineered variants, has witnessed a marked increase in their application to meet the vegetarian and kosher demands within the industry [23].

Lactose intolerance, a condition characterized by the inability to digest lactose due to a lack of the necessary enzymes, affects a significant portion of the global population. This has resulted in a growing demand for lactose-free products, which are products that do not contain any lactose. Enzymes such as beta-galactosidase are pivotal in the hydrolysis of lactose into glucose and galactose, thus rendering dairy products more digestible for individuals who are lactose-intolerant. This process, catalyzed by enzymes, not only addresses consumers' health requirements but also enhances the sweetness of dairy products without the need for additional sugars [24].

Lactic acid bacteria, which are renowned for their ability to produce compounds that enhance flavor, possess enzymes capable of breaking down milk components into flavor-contributing peptides and amino acids. The fermentation process employs these enzymes to generate complex flavor profiles that are characteristic of products such as yogurt and cheese. Furthermore, proteolytic enzymes derived from these bacteria have been shown to enhance the maturation and flavor profiles of cheese [23].

Proteolytic enzymes have been identified as playing a pivotal role in the modification of milk proteins, thereby enhancing their nutritional and functional properties. Of particular interest are whey protein hydrolysates, which are obtained through enzymatic processes, yielding bioactive peptides that exhibit beneficial health effects such as antihypertensive and antioxidant properties. These modifications can be controlled to produce specific peptides for use in functional foods and nutraceuticals. Whey, a byproduct of cheese making, contains valuable proteins and lactose that can be processed using various enzymes to create high-value ingredients and products. Additionally, enzymes are used to modify whey proteins for improved functionality. Controlled proteolysis can enhance the solubility and emulsifying properties of whey proteins, making them more suitable for use in sports nutrition products and food applications [24, 25].

Enzymes also offer a sustainable alternative for cleaning and sanitation in dairy processing. Enzyme-based cleaning-in-place (CIP) systems are increasingly being

adopted as sustainable alternatives to traditional chemical detergents, offering significant reductions in water and energy consumption as well as environmental impact. Enzymes effectively break down milk residues on processing equipment, reducing biofilm formation and ensuring hygiene without the drawbacks of harsh chemicals [4].

Furthermore, enzymes are pivotal in the transformation of milk into functional foods through the production of bioactive components such as peptides, oligosaccharides, and other health-promoting molecules by means of targeted biochemical reactions.

2.3 Key enzymes in dairy processing

The dairy industry depends on a range of enzymes for the enhancement of product quality, the optimization of processing efficiency, and the development of specialized dairy products (**Table 1**). The following section provides a comprehensive overview of the key enzymes commonly employed in the field of dairy processing. These enzymes are accompanied by a detailed description of their sources, functions, and applications.

Enzymes in milk can be categorized into two main groups: first, indigenous enzymes are naturally present in milk and originate from the cow itself (e.g., blood plasma, mammary epithelial cells, milk fat globule membrane, somatic cells during infection) [14, 26, 27]. While the physiological role of certain indigenous enzymes in milk is not immediately apparent, others have been shown to have a significant impact on the quality and shelf life [26, 28]. Exogenous enzymes are added externally to milk or milk products during processing or are produced by bacteria, molds, or yeasts (microbial enzymes). It is imperative to acknowledge the significance of these components in the manufacturing process of numerous dairy products, which, in the absence of these elements, would remain unattainable [12, 26, 29, 30].

Application	Enzyme	Function/Benefit	Example Products
Cheese manufacturing	Chymosin (Rennet)	Coagulates milk by cleaving κ-casein, forming curds	Cheddar, Parmesan, soft cheeses
Lactose-free dairy products	Lactase (β-Galactosidase)	Hydrolyzes lactose into glucose and galactose, increases sweetness, enables lactose-free products	Lactose-free milk, yogurt, ice cream
Cheese ripening, flavor enhancement	Lipase	Hydrolyzes milk fats, develops characteristic flavors	Blue cheese, Parmesan, butter
Cheese maturation, protein hydrolysates	Protease	Breaks down milk proteins, enhances texture, reduces allergenicity	Yogurt, infant formula, protein supplements
Texture improvement, water retention	Transglutaminase	Cross-links proteins, improves texture and yield	Yogurt, processed cheese
Milk sterilization, shelf-life extension	Catalase	Decomposes hydrogen peroxide, prevents oxidation, preserves quality	Sterilized milk, cheese
Flavor development	Esterase	Hydrolyzes esters, contributes to flavor compound formation	Specialty cheeses

Table 1.
Applications of enzymes in the dairy industry.

2.3.1 Milk coagulants (rennets, chymosin, pepsin)

Due to their critical function in milk clotting, milk coagulants represent some of the most commercially important enzymes in cheese production. This enzymatic coagulation is a two-phase process [31]. In the primary, enzymatic phase, the coagulant, most notably chymosin, specifically cleaves the Phe105-Met106 peptide bond of kappa-casein (κ-casein) [3, 12, 19, 31]. Kappa-casein is located on the surface of casein micelles and typically stabilizes them through steric repulsion and a negative electrical charge [19, 32]. The cleavage of κ-casein yields a hydrophobic para-κ-casein, which remains in the curd, and a hydrophilic glycomacropeptide (also known as caseinomacropeptide), which is liberated into the milk serum (whey) [15, 31]. The secondary, nonenzymatic phase involves the aggregation of these destabilized casein micelles, which then precipitate in the presence of calcium ions (Ca^{2+}) to form a gel or curd [32, 33].

Traditionally, milk coagulants were sourced from animal rennet, primarily derived from the abomasa of young suckling calves, which are rich in chymosin [12, 31, 34, 35]. This type of rennet contains chymosin and pepsin, with their ratio influencing proteolysis during cheese ripening [35]. Calf chymosin is particularly valued for its specific proteolytic activity that coagulates milk without extensively damaging immunoglobulins, a characteristic favored in the evolution of young ruminants [36]. Rennet paste, derived from kid goats and lambs, contains lipase from the animals' glands, which is essential for producing piquant cheeses [32]. Besides calf rennet, other animal sources include lamb chymosin, which has similar coagulation properties but a slightly higher temperature dependency, and less-studied kid and buffalo rennet [35]. Recombinant camel chymosin is also gaining market share, known for its superior coagulation properties for both bovine and camel milk [20, 36].

However, due to limitations in the supply of animal rennet and other factors like increasing cheese production and dietary preferences, there has been a significant shift toward alternative milk coagulants [22, 31]. These alternatives include (I) microbial coagulants: These are produced from various microorganisms, including fungi (e.g., *Rhizomucor miehei*, *Mucor pusillus*, *Endothia parasitica*, *Aspergillus oryzae*, *Irpex lactis*, *Thermomucor indicae-seudaticae*) and certain bacteria (Bacillus subtilis) [3, 12, 15, 31, 32, 37]. While easily produced and inexhaustible, some early microbial coagulants had drawbacks such as heat stability, which could lead to clotting in rehydrated infant formulas, and high levels of nonspecific proteases, causing bitterness and poor yields in long-ripened cheeses [15, 32, 38]. Fermentation-produced chymosin (FPC), which involves cloning the calf chymosin gene into genetically modified food yeasts or molds, has largely overcome these issues and constitutes a significant portion of the market [22, 31, 36]. (II) Plant-derived coagulants: These include extracts from plants such as *Onopordum macracanthum*, kiwi fruit (*Actinidia deliciosa*), *Calotropis gigantea*, *Solanum incanum*, and lemon juice [3, 33, 39, 40]. Plant proteases show variability in their specificity, offering opportunities for diverse cheese characteristics. *Onopordum macracanthum* proteases, for example, have demonstrated promising technological properties [33].

The choice of coagulant significantly impacts cheese quality, affecting aspects such as texture, flavor, and whey drainage properties [14, 20, 28, 34]. Factors influencing milk-clotting activity (MCA) include temperature, pH, and calcium chloride concentration [33, 37]. For instance, increasing calcium chloride concentration can improve MCA by neutralizing negative charges on casein micelles and facilitating aggregation, although excessively high concentrations can reduce activity.

2.3.2 Lipases

Lipases (triacylglycerol acylhydrolases; EC 3.1.1.3) are a crucial class of hydrolase enzymes widely utilized in the dairy industry [12, 28, 41, 42]. These enzymes are primarily responsible for catalyzing the hydrolysis of ester bonds in triglycerides, breaking them down into free fatty acids (FFAs), partial glycerides (mono- and diglycerides), and glycerol, predominantly at the water/oil interface [15, 28, 42–44]. Beyond hydrolysis, lipases are versatile biocatalysts capable of facilitating various other reactions, including esterification, interesterification, transesterification, acidolysis, aminolysis, and alcoholysis, which are vital for modifying lipid properties and creating value-added products [14, 18, 41, 45]. Their importance in the dairy sector stems from their ability to influence flavor, texture, ripening processes, and overall product quality [7, 11, 18, 21, 22, 46]. In addition to their role in flavor development, lipases contribute to the structural stability of dairy products.

Lipases found in dairy applications originate from both indigenous milk components and exogenous sources, namely animal, microbial, and, to a lesser extent, plant-derived materials [12, 20].

Indigenous Milk Lipases: The primary indigenous lipase in bovine milk is lipoprotein lipase (LPL; EC 3.1.1.34) [29, 42, 47]. LPL is a glycoprotein that exists as a homodimer with a molecular mass of approximately 90 kDa [41–43, 48]. It is thought to spill over from the mammary gland into milk, where it is primarily associated with casein micelles [48, 49]. While LPL plays a crucial role in lipid synthesis within the mammary gland, its presence in milk itself does not have an apparent biological purpose [29, 48]. LPL is unstable and can be significantly inactivated by heat treatments, such as High-Temperature Short-Time (HTST) pasteurization (72°C for 15 seconds) [48, 50]. Its optimal pH is around 8–9, and its optimal temperature is around 37°C [41]. Other less-studied indigenous esterases and colostral lipase also exist in milk [12].

Exogenous Lipases (Commercial Sources): Commercial lipases can be obtained from animals, plants, and microorganisms. *(I)* Historically, lipases were sourced from animal rennet, primarily from the abomasa of young suckling calves, kid goats, or lambs [7, 35, 51]. Pregastric esterase (PGE), secreted from the oral tissues of these animals, is particularly notable for its high specificity for short-chain fatty acids at the sn-3 position, contributing to a characteristic 'piccante' (pungent or peppery) flavor in cheeses like Provolone and Romano [12, 19]. *(II)* Due to advantages such as lower production costs, greater stability, and inexhaustible supply, microbial lipases have become the most commercially significant source [7, 15, 16, 46, 52]. These are produced by various microorganisms, including fungi (e.g., *Rhizomucor miehei*, *Aspergillus niger*, *Aspergillus oryzae, Candida* species, *Penicillium*, *Mucor pusillus*, *Mucor javanicus*, *Thermomyces*), yeasts (*Candida, Yarrowia*), and bacteria (*Bacillus subtilis*, *Pseudomonas* species, *Serratia marcescens*, *Staphylococcus aureus*) [3, 7, 15, 38]. Microbial lipases are increasingly important for dairy products, especially low-fat cheeses, where they enhance fatty acid flavor release [53]. While mentioned as a source, microbial lipases are favored for industrial-scale production.

2.3.2.1 Applications and functions of lipases in the dairy industry

Lipases are broadly applied across the dairy industry for a range of purposes, from enhancing flavor to improving product yield and even aiding in equipment cleaning.

Cheese production and ripening: This is arguably the most significant application of lipases in the dairy industry [9, 18, 35]. They contribute to the development of cheese

flavor, texture, and body characteristics by hydrolyzing milk fats [12, 14, 54]. This hydrolysis releases FFAs, which are key flavor components. Short-chain FFAs, such as butyric and caproic acids, impart pungent, tangy, peppery, or cheesy notes [18, 28, 47, 51, 55], while long-chain FFAs tend to develop a softer taste and odor [12, 55]. FFAs also serve as precursors for other volatile flavor compounds like esters, methyl ketones, and lactones, which contribute sweet, fruity, floral, buttery, or musty notes to cheese [38, 45]. Lipases also accelerate the cheese ripening process [9, 12, 15, 56]. They are specifically used in the production of enzyme-modified cheeses (EMCs) to achieve intense flavors [9, 29, 35].

Milk Fat modification and valorization: Lipases are used to enhance the flavor and structure of butter and dairy cream [28, 35]. They are also employed to produce modified milk fat products for other food applications in the confectionery, candy, chocolate, sauce, and snack food industries [22, 30]. Enzymatic interesterification of milk lipids can modify their rheological properties [30, 47], and lipases can be used to produce diglycerides (DGs) and monoglycerides (MGs) that function as emulsifiers in the food industry [47]. Furthermore, they enable the enrichment of milk fat with omega-3 fatty acids and the production of human milk fat substitutes [28, 38, 45].

Yield improvement: Fungal phospholipase A1, a type of lipase, can increase cheese yield, such as in mozzarella production, by improving the retention of fat and protein [12, 29, 35]. This occurs by enhancing the emulsifying property of milk, reducing fat losses in whey and cooking water [35, 38].

Addressing off-flavors (hydrolytic rancidity): While beneficial for specific cheese flavors, uncontrolled lipase activity, particularly from indigenous LPL and bacterial lipases, can lead to undesirable off-flavors in liquid milk and other dairy products, described as rancid, butyric, astringent, or soapy [26, 28, 41, 42, 57]. This "spontaneous lipolysis" can be triggered by factors related to the cow (e.g., stage of lactation, nutritional status) or induced by mechanical handling of raw milk (e.g., agitation, foaming, freezing, homogenization, pumping) that disrupts the milk fat globule membrane (MFGM) [42, 46, 47]. Lipolysis can also reduce the foaming capacity of milk due to the liberation of FFAs [26, 42, 47].

Cleaning-in-place (CIP) in the dairy industry: Lipases, often in combination with proteases and amylases, are employed in cleaning processes to hydrolyze lipid-based fouling deposits and biofilms that accumulate on dairy processing equipment. They represent the third most used enzyme class in detergents, highlighting their role in maintaining hygiene and operational efficiency [44].

2.3.3 Proteases

Proteases, or proteolytic enzymes, are crucial in the dairy industry, catalyzing the breakdown of proteins into smaller peptides or amino acids, thereby influencing the texture, taste, and aroma of dairy products and may also generate bioactive peptides with antihypertensive, antimicrobial, or immunomodulatory effects [3, 58, 59]. They are classified into serine, cysteine, aspartic, and metalloproteases based on their active site and catalytic mechanisms [60]. Proteolytic activity in milk comes from both indigenous enzymes, inherent to the cow, and exogenous enzymes, primarily from microbial contamination [59].

Indigenous milk proteases: Milk contains over 60 indigenous enzymes, many of which can survive heat treatments like High-Temperature Short-Time (HTST) pasteurization [12]. The most prominent indigenous proteinase is plasmin (EC 3.4.21.7), which exhibits trypsin-like activity [61]. Other indigenous proteases like cathepsin

D are also present in milk, originating from somatic cells, and can survive some heat treatments, contributing to post-processing proteolysis [41, 62].

Microbial proteases: Microbial enzymes are widely used in the dairy industry due to their efficiency and cost-effectiveness [58]. However, psychrotrophic bacteria, especially *Pseudomonas* species, are a major source of spoilage proteases in raw milk [41, 63, 64]. These bacteria produce significant amounts of heat-stable extracellular proteases, such as AprX (Protease) from *Pseudomonas fluorescens*, which are difficult to inactivate even with UHT treatment [59, 63, 64]. These heat-resistant proteases can cause undesirable changes like age gelation, off-flavor, and textural defects in processed dairy products, particularly ultra-high temperature processing (UHT) milk, thereby reducing shelf life and nutritional content [41, 63]. *Pseudomonas* proteases primarily degrade casein, with β-casein and κ-casein being particularly susceptible [63]. The thermal stability of AprX is mainly attributed to its flexible tertiary structure, which can refold quickly upon cooling [63]. Reducing the growth of psychrotrophic bacteria in raw milk by rapid processing and maintaining low temperatures (2–4°C) can suppress the production of these heat-stable enzymes [60].

2.3.3.1 Applications of proteases in dairy

Proteases are indispensable in various dairy applications, impacting product characteristics from coagulation to flavor development and shelf-life stability [8, 11, 15, 65].

Cheese production: Proteases are vital in cheese production, contributing to milk coagulation (see 2.3.1), accelerating ripening, and developing unique flavors and textures [9, 22, 50, 65, 66]. Proteases significantly enhance the aroma and flavor of cheese by breaking down casein proteins into various peptides and amino acids during the ripening process [9, 29, 49, 65]. The specific type of flavor generated depends on the amino acids released and the ability of cheese cultures to utilize them [20]. Proteases are also used to make enzyme-modified cheeses (EMCs), where cheese or curd is incubated with enzymes to achieve desired flavor intensity [29].

Shelf-life extension and spoilage prevention: Proteases are among the primary enzymes responsible for the deterioration of dairy products, particularly those with a long shelf life [14, 26, 41, 67]. Uncontrolled protease activity can lead to various defects such as casein coagulation, off-flavor development, textural changes, sedimentation, and creaming [26, 60]. For example, in UHT milk, proteolysis can lead to age gelation, a significant factor limiting shelf life [30, 41, 60]. By controlling these spoilage enzymes and their activities, raw milk quality can be maintained for extended shelf-life products [3, 7, 54].

Hypoallergenic products and protein hydrolysis: Proteases are crucial for the production of dairy protein hydrolysates used in infant, clinical, or sports nutrition, as they help reduce possible allergenic reactions and improve the digestibility of dairy proteins [12, 20, 29, 65, 66, 68]. They break down milk proteins (casein and whey proteins) into smaller peptides or amino acids, enhancing their nutritional value and digestibility [54, 62, 65, 69]. This enzymatic action also improves the viscosity, texture, and taste of fermented products like yogurt, contributing to a smooth and creamy consistency [54].

Cleaning-in-place (CIP) in the dairy industry: Proteases, along with lipases and amylases, are studied for their potential use in cleaning-in-place (CIP) systems in the dairy industry to remove fouling deposits, especially those that accumulate on heated surfaces during thermal processing [3, 44, 65]. These deposits, composed of protein, mineral, and fat, can obstruct flow, reduce heat transfer efficiency, and harbor microbial growth, including biofilms [44]. Proteases, particularly alkaline serine

proteases from *Bacillus* species, are effective in hydrolyzing proteinaceous substrates and disrupting biofilms [44].

2.3.4 Lactases (β-galactosidase)

Lactases, also known as β-galactosidases (EC 3.2.1.23), are a crucial class of glycoside hydrolase enzymes in the dairy industry, primarily catalyzing the breakdown of lactose. This enzymatic action plays a significant role in influencing the taste, texture, and aroma of dairy products and is central to various modern dairy applications [11, 51, 52, 70, 71]. Lactases perform two main types of reactions critical for dairy product development and consumer health.

2.3.4.1 Hydrolysis of lactose

This is the primary function: breaking down lactose into its constituent monosaccharides, d-glucose and d-galactose. This process is vital for producing lactose-free products [12, 32, 55, 70]. Lactose intolerance affects a significant portion of the global adult population, with rates as high as 70–90% in some Asian and American regions [52, 55, 72–74]. This condition stems from a deficiency or inefficiency of lactase in the human digestive system, leading to symptoms like diarrhea, abdominal cramping, and flatulence upon consuming lactose-containing dairy products [12, 20, 52]. Lactase enzymes are used to predigest lactose in milk, enabling the production of "low-lactose" or "lactose-free" dairy options such as milk, yogurt, ice cream, cheese, and dulce de leche [7, 11, 15, 18, 51, 75–77]. This not only facilitates lactose-free product development but also significantly enhances digestive health and comfort for lactose-intolerant individuals. The lactose content in these products can be reduced significantly, with thresholds varying by region (e.g., less than 10 mg/100 g for "lactose-free" in Denmark, 100 mg/100 g in Germany, and no FDA definition in the US beyond "truthful and not-misleading" labeling) [12, 20]. The hydrolysis products, glucose and galactose, are sweeter than lactose itself (approximately 70% of sucrose's sweetness compared to lactose's 20%) [7, 14, 18, 20, 29]. This allows for reduced sugar addition in dairy products like yogurts and desserts [11, 15], while also preventing lactose crystallization (often described as "sandiness") in products like ice cream and sweetened concentrated milk [15, 78]. Beyond lactose-free products, enzymatic hydrolysis improves the overall digestibility of dairy products and enhances fermentation efficiency and yield in products like yogurt and cottage cheese [14, 38].

2.3.4.2 Transgalactosylation for galactooligosaccharide (GOS) synthesis

Lactases can also catalyze transgalactosylation, synthesizing galactooligosaccharides (GOS) or lactulose from lactose [18, 70, 75, 79, 80]. GOS are nondigestible oligosaccharides that act as prebiotics, stimulating the growth and activity of beneficial microorganisms in the colon, such as *Lactobacillus* and *Bifidobacterium* species [12, 75, 80]. They contribute to various health benefits, including immune system support, protection against cancer, and prevention of cardiovascular and metabolic issues [80]. GOS are increasingly used as food ingredients in infant formulas, infant foods, livestock feed, and pet food industries [74, 80]. The successful synthesis of GOS from concentrated whey permeate utilizing a commercial β-galactosidase is reported. The yield of GOS was found to be up to 74% (g GOS/g lactose), with 63% lactose utilization after a mere 0.5 hours of reaction [81]. In a separate study, β-galactosidase from probiotic bacteria,

such as Lactobacillus delbrueckii subsp. bulgaricus CRL450, was employed to efficiently synthesize galactooligosaccharides (GOS) and lactulose oligosaccharides (OsLu) from lactose and lactulose *via* transgalactosylation. The resulting GOS was found to significantly enhance the growth of probiotic strains [82]. In another study, GOS were synthesized from whey permeate using commercial β-galactosidases from *Kluyveromyces lactis* and *Escherichia coli*, achieving yields up to 24%, which demonstrates a viable industrial process to turn a dairy byproduct into a value-added prebiotic [83]. A third approach combines specific enzymes like β-galactosidase with dietary fibers to generate tailored short-chain oligosaccharides that act as highly preferential food sources for probiotics, with the enzymatic process minimizing unwanted glucose production [84]. All these cases highlight that the enzymatic source and conditions can be controlled to create specific prebiotic structures that selectively stimulate beneficial gut bacteria.

2.3.4.3 Whey utilization

Lactase enzymes are used to hydrolyze lactose in whey, a byproduct of cheese production, converting it into more useful food ingredients and syrups for the bakery and confectionery industries [7, 29, 74, 78, 85]. This also provides better biodegradability to whey, making it suitable for ethanol production and addressing environmental concerns related to its high biological and chemical oxygen demand [7, 78].

2.3.4.4 Sources and types of lactases

Lactases can be derived from various sources, predominantly microbial, but also from plants and animals, and confer the desired properties [86–89].

β-galactosidases from yeasts are active at a rather neutral pH (between 6.0 and 7.0), making them highly suitable for producing lactose-free milk due to milk's pH of approximately 6.6 [22, 35, 73, 74]. *Kluyveromyces lactis* is the most commonly used yeast for industrial lactase production [74]. Other yeast sources include *Saccharomyces fragilis* and *Kluyveromyces fragilis* [22, 77]. These enzymes generally have low heat stability, becoming rapidly inactivated above 55°C [77].

Fungal β-galactosidases commonly work in an acidic pH range (from 2.5 to 5.0), making them most effective for lactose hydrolysis in acidic products like acid whey [20, 32, 65, 77]. They typically have a high optimum temperature (55–60°C) [77]. Notable fungal sources include *Aspergillus niger* and *Aspergillus oryzae* [22]. Fungal enzymes may contain other enzymes like amylase, lipase, and protease, limiting their application to high-acid products and pharmaceutical preparations [77].

Bacterial β-galactosidases generally exhibit high enzyme activity, good stability, and high production yield [70]. They are often suitable for milk and sweet whey hydrolysis, operating at a neutral pH (6.5–7.5) [15, 65]. Key bacterial sources include *Bacillus circulans* and *Bifidobacterium bifidum* [70, 75, 77]. Some *Bacillus* species lactases are noted for their thermostability and low product inhibition by galactose [15].

β-galactosidases are also found in plants (e.g., almonds, peaches, and apricots) and the intestinal tract of mammals, but these sources generally have less commercial value compared to microbial enzymes [74, 77, 90].

2.3.5 Transglutaminase (TGase)

Transglutaminase (EC 2.3.2.13) is an enzyme that catalyzes the cross-linking of proteins by forming covalent bonds between amino acid residues, particularly

between lysine and glutamine [8, 12, 15, 18]. TGases have been used to modify the rheological (flow and deformation) properties and improve the texture and stability of dairy products [16, 54, 69]. For instance, it can improve viscosity and texture in yogurt and contribute to firmness in cheese [54]. Furthermore, they have been shown to improve the yield of cheese [14]. Additionally, they are important for innovative dairy products and protein supplements [54].

2.3.6 Other specialized enzymes

Several other enzymes are utilized for specific functions within the dairy industry:

Catalase (EC 1.11.1.6): Breaks down peroxide compounds, preventing oxidation and prolonging the shelf life of dairy products [9, 51, 65, 67]. It acts as a natural preservative and helps control oxygen levels in milk solutions, which is particularly important in cheesemaking and packaging [16].

Lysozyme (EC 3.2.1.17): A hydrolase with antimicrobial properties that breaks down bacterial cell walls (peptidoglycan layer), extending shelf life and preventing spoilage [43, 65, 91]. It is used as a protective agent in cheese production, especially soft cheeses, and as an alternative to potassium nitrate for controlling "late blowing" caused by *Clostridium tyrobutyricum* in hard and semi-hard cheeses [16, 65]. It can also make cow's milk suitable as infant milk [65].

Lactoperoxidase (LPO; EC 1.11.1.7): An antimicrobial enzyme that catalyzes the oxidation of various substrates by hydrogen peroxide, playing a role in the lactoperoxidase-thiocyanate system for cold pasteurization of milk [30, 43, 91]. It is also used as an indicator of effective pasteurization [29, 30].

Xanthine oxidoreductase (XOR) / xanthine oxidase (XO): Can act as a pro-oxidant, is involved in oxidative flavor formation, and plays a role in cheese ripening [12, 30].

Sulfhydryl oxidase: Contributes to the amelioration of cooked flavor and milk preservation [30, 42].

Superoxide dismutase: An antioxidant enzyme involved in milk preservation [30, 42].

Alkaline phosphatase (ALP; EC 3.1.3.1): A phosphomonoesterase whose inactivation is a key indicator for ensuring adequate pasteurization of milk, thereby enhancing microbiological safety [12, 28, 29, 57].

Acid phosphatase (ACP): Involved in cheese ripening and can reduce the heat stability of milk. Its concentration increases during mastitis infection [12, 30].

N-acetyl-β-D-glucosaminidase (NAGase; EC 3.2.1.30): An enzyme whose elevated concentration indicates mastitis infection [29, 42, 73].

Glucose oxidase (EC 1.1.3.4): Used in conjunction with catalase for food preservation, removing harmful oxygen and stabilizing probiotic bacteria in yogurt [9, 65, 67].

The dairy industry's reliance on enzymes is continuously growing. Microbial enzymes offer significant advantages, including large-scale production, cost-effectiveness, environmental friendliness, and the ability to be genetically manipulated for superior properties like higher solubility, increased specificity, and improved product yield.

Advances in DNA technology and protein engineering allow for the production of tailored enzymes that optimize activity for specific applications. Furthermore, enzyme immobilization techniques (e.g., adsorption, covalent binding, entrapment, and encapsulation) are transforming milk processing by improving enzyme stability, activity, and reusability, leading to more efficient and sustainable production processes, reduced costs, and a lower environmental impact.

2.3.7 Enzymatic production of health-promoting molecules from milk

Milk proteins, predominantly casein and whey, comprise encrypted sequences that, upon cleavage by designated proteolytic enzymes (e.g., pepsin, trypsin, chymotrypsin, alcalase, thermolysin), yield bioactive peptides with diverse physiological effects [92–94]. The resultant peptides have been shown to display antihypertensive, antioxidant, immunomodulatory, antithrombotic, anticancer, and antimicrobial activities [92, 94]. The nature of the peptides formed is contingent on both the source substrate and the protease employed. For instance, chymotrypsin can target aromatic amino acids, while trypsin is able to target basic amino acids. This has a consequential effect on the final peptide's structure and function [93].

It is evident that human milk is a natural source of complex oligosaccharides (HMOs), which have been shown to possess significant prebiotic, immune-modulating, and anti-infective properties. However, cow's milk contains them at much lower levels. The employment of engineered enzymes (e.g., transglycosylases, fucosyltransferases, and sialidases) enables scientists to catalyze the formation of human-identical oligosaccharides such as 2'-fucosyllactose (2'-FL), lacto-N-tetraose, and sialyllactoses, either directly in bovine milk or within *in vitro* systems [95–97]. These oligosaccharides function as prebiotics, thereby promoting the proliferation of beneficial gut microbiota and contributing to immune system development and the inhibition of pathogens [96].

Lipolytic enzymes may release bioactive fatty acids and glycerol from milk triglycerides, further enhancing the nutritional profile and health benefits of fermented or ripened dairy products [98].

Enzymes enhance the bioavailability of nutrients in dairy products through various mechanisms. Phytases release minerals like phosphorus by breaking down phytate, making them more absorbable. Proteolytic enzymes hydrolyze proteins into easily digestible peptides and amino acids, improving the nutritional quality of milk proteins [45]. Enzymatic hydrolysis enhances mineral bioavailability by producing Casein Phosphopeptides (CPPs), which are bioactive peptides derived from caseins [99]. CPPs chelate essential minerals like calcium and iron, keeping them soluble in the gastrointestinal tract and preventing precipitation. This process increases the absorption of these minerals, leading to improved bone density and calcification from calcium and better iron uptake by vital organs [99]. In animal feed, fibrolytic enzymes boost nutrient absorption in dairy cows, indirectly increasing the nutrient density of milk [100]. Additionally, lactase breaks down lactose, improving carbohydrate digestibility and energy bioavailability for lactose-intolerant individuals.

3. Protein engineering for enzyme optimization

Enzyme engineering is an emerging field of research that focuses on the modification of enzymes to achieve specific properties for a range of applications [8, 101]. The process of altering the amino acid sequences of proteins through the genetic manipulation of their genes is a fundamental aspect of protein engineering [8, 102].

Natural enzymes often have properties that are not optimal for industrial or therapeutic applications [103–105]. Enzyme engineering aims to improve these limitations. Natural enzymes may not be efficient enough for large-scale industrial production or may be rate-limiting bottlenecks in metabolic pathways [89, 106, 107]. Enzyme engineering can significantly enhance the catalytic activity of the target

enzyme [108–110]. Enzymes obtained from natural sources often exhibit instability under nonphysiological conditions like elevated temperatures, extreme pH, or the presence of organic solvents [110–112]. Enzymes are highly specific to their natural substrates, limiting their application to a broader range of chemicals [113–115]. Many natural enzymes lack the desired stereoselectivity for producing optically pure compounds [8, 104, 116, 117]. Enzyme engineering can go beyond optimizing existing functions to design enzymes with entirely new capabilities not found in nature [118–121]. Enzyme engineering employs several key approaches to modify proteins for improved or novel functions, often used independently or in combination.

3.1 Directed evolution

Directed Evolution (DE) is a revolutionary protein engineering strategy that systematically mimics the process of natural evolution in a laboratory setting [122–126]. Its primary goal is to tailor the properties of proteins, particularly enzymes, to meet specific industrial demands or to design entirely new functions not found in nature [105, 120, 124]. This approach has become a cornerstone in various fields, including biocatalysis, metabolic engineering, and synthetic biology [127].

At its core, directed evolution operates through iterative cycles of introducing genetic diversity (mutagenesis) and then screening or selecting for improved enzyme variants [86, 122, 123, 128, 129]. This process is akin to guiding a "random walk" through the vast protein sequence space, aiming to climb "fitness peaks" on the fitness landscape (a plot of fitness versus sequence) [122, 126]. Unlike rational design, DE does not require detailed prior knowledge of the protein's structure, function, or the specific amino acid substitutions that would confer the desired properties [86–89]. Instead, it relies on the principle that evolution has already solved the complex mapping from amino acid sequence to function [130]. The method involves repeated local searches, taking advantage of the functional promiscuity and clustering of functional sequences in sequence space [129].

The general procedure of directed evolution encompasses two main steps: generating a mutant library to achieve genetic diversification and then screening or selecting beneficial mutants from this library [86, 125, 128].

3.1.1 Genetic diversification (mutagenesis)

This initial step is crucial for creating the raw material for evolution: a diverse library of genetic variants from a parental gene [86, 131]. The rate of natural mutation is typically too low for laboratory purposes, necessitating artificial enhancement of genetic diversification [86].

Random mutagenesis: This approach introduces mutations broadly across the gene sequence, without specific targeting [86, 132]. Error-prone PCR (epPCR) is a widely used technique that employs low-fidelity DNA polymerases to introduce random point mutations during DNA replication [126, 133–135]. EpPCR can be modified by adjusting experimental conditions (e.g., elevating magnesium levels, adding manganese, or using imbalanced dNTP concentrations) to significantly enhance mutation frequency [134]. It is particularly advantageous for changing global characteristics of enzymes, such as thermal and pH stability, and has been used to enhance enzyme activity [136].

Gene recombination/DNA shuffling: This technique reassembles gene fragments from different proteins, organisms, or variants of the same protein to create chimeric

proteins [87, 126, 137]. This can combine functions from parental enzymes or yield entirely new properties [137].

3.1.2 Screening and selection

Identifying beneficial mutants from the massive libraries generated is a critical and often rate-limiting step [111, 124, 138, 139]. Screening involves performing individual assays on each variant, often facilitated by robotics and high-throughput platforms [111, 134, 140]. Examples include colorimetric assays, fluorescent signals, or High-Performance Liquid Chromatography (HPLC) to detect the desired function [134, 140]. High-throughput screening methods, such as droplet-based microfluidics and Fluorescence-Activated Cell Sorting (FACS), enable the isolation and identification of enzymes with improved features [121, 134, 141, 142]. Selection identifies individual variants with a given property by separating them from those without the property, often by directly linking a fitness advantage to the desired activity [126, 140]. Techniques like Phage-Assisted Continuous Evolution (PACE), OrthoRep, and MutaT7 enable continuous mutation and selection *in vivo* [121, 138, 140, 143]. These methods significantly accelerate evolution cycles and can enhance gene mutation rates, overcoming the labor-intensive iterative steps of traditional DE [87, 138].

Directed evolution offers several compelling advantages that make it a powerful tool in protein engineering. A significant benefit is that it does not necessitate a detailed structural or mechanistic understanding of the protein, bypassing the challenges encountered when such knowledge is incomplete or unavailable [8, 86–88, 144]. This makes it particularly useful for enzymes that are not well-characterized [145]. DE can explore a much broader sequence space than rational design, allowing for the evolution of complex functionalities such as enhanced catalytic activity, improved stability (thermal, pH, solvent), altered substrate specificity, and tuned selectivity (enantio-, regio-, chemoselectivity) [86, 120, 125, 129, 133, 139, 146]. It also has the capability to generate novel functionalities or "new-to-nature" activities not found in nature, expanding the enzyme universe for nonnatural reactions [8, 87, 105, 124, 125]. Directed evolution effectively bypasses the limitations of purely rational design, especially when the sequence-structure-function relationship is difficult to predict or when structural information is lacking [126, 131, 147–149]. Additionally, it can identify beneficial mutations at sites distant from the active center, which are often challenging to predict computationally or with rational design approaches. These distal mutations can significantly influence enzyme function, including catalytic activity and allostery [87, 143, 148, 150–152].

Despite its power, directed evolution is not without its drawbacks. The process often requires significant resources, including time, labor, and financial investment, particularly for generating large libraries and implementing efficient screening methods [88, 124, 138, 144, 153–155]. Random mutagenesis techniques often produce libraries where a high percentage (60–70%) of the mutations are deleterious or inactive, while only a small fraction (less than 5%) provides functional gains [87, 88]. This necessitates robust screening methods to sift through many nonfunctional variants [88]. High-throughput screening remains a major challenge, especially for complex desired properties or when sensitive and high-throughput assays are not readily available [86, 138, 142, 144, 154]. Some random mutagenesis methods, such as epPCR using Taq polymerase, exhibit an inherent bias toward certain types of mutations, which can limit the diversity of the sequence space explored [132, 134].

3.2 Rational design

Rational design, also known as computational enzyme design, is a protein engineering strategy that focuses on creating or improving enzymes with desired functionalities by making precise, targeted changes to their amino acid sequences [8, 102, 110, 118, 126]. This approach is fundamentally based on a deep understanding of the relationship between an enzyme's structure and its function [106, 115, 117, 142, 156–158]. Unlike directed evolution, which relies on iterative cycles of random mutagenesis and high-throughput screening, rational design aims to significantly reduce the experimental workload by predicting beneficial mutations *in silico* [88, 89, 126, 159, 160].

The core principle is that an enzyme's three-dimensional (3D) structure dictates its function [160, 161]. Therefore, by rationally altering the protein structure, its properties can be modified [8]. This requires precise knowledge of the enzyme's structure, including substrate-binding pockets, catalytic sites, and sometimes even dynamic conformational changes [89, 110, 162, 163]. A detailed understanding of the enzyme's catalytic mechanism is crucial. This involves comprehending how the enzyme binds to substrates, stabilizes transition states (TS), and releases products, as well as the roles of specific amino acid residues in these processes [120, 157, 160, 163–165]. Rational design identifies "hotspots," or specific amino acid residues, for mutagenesis [162, 166–168]. These sites can be in the active site pocket, substrate-binding tunnels/channels [110, 148, 166, 169], surface regions [110, 115, 167], or even distal sites that influence enzyme function through allostery or dynamics [143, 148, 151, 165]. Highly conserved residues are generally important for protein function and are often non-variable. Highly variable regions, on the other hand, can be targeted for mutagenesis to alter surface properties [122] .

3.2.1 Computational tools and approaches

Rational design heavily relies on computational approaches to predict the impact of mutations and guide the design process, making it a "computer-aided" or "in silico" methodology (**Table 2**) [117, 126, 156, 160, 170].

When crystal structures are unavailable, homology modeling, often using tools like AlphaFold2 (AF2) or RosettaFold, can be used to predict the 3D structure of an enzyme based on known structures of related proteins [156, 158, 171–173].

Molecular docking predicts how a substrate or ligand binds to the enzyme's active site, revealing optimal binding modes and crucial interactions [174, 175]. Tools like AutoDock, Glide, and GOLD are frequently used [164, 167, 170, 175].

Molecular Dynamics (MD) simulations provide insights into the dynamic behavior of enzymes, including conformational changes during catalysis, substrate/product channels, and the flexibility of residues [110, 126, 160]. They can be used to screen for beneficial substitutions that repel/attract surface solvation or to understand mechanisms through which mutations enhance activity [176].

Quantum Mechanics (QM)/Molecular Mechanics (MM) calculations are advanced methods and are used to determine the catalytic mechanism of an enzyme and analyze the potential energy surface of reaction mechanisms, providing in-depth knowledge on the reaction [117, 157, 164, 177]. They are crucial for understanding high-energy transition states [157]. Monte Carlo (MC) simulations explore the conformational space of active sites and ligands to identify optimal positions for catalytic residues or to evaluate system energy [110, 178].

Software/Tool	The primary purpose in rational design	Key features/applications
AlphaFold2/AlphaFold3	Protein structure prediction	Predicts highly accurate 3D protein structures, including complexes with small molecules. Essential for design when experimental structures are unavailable.
RoseTTAFold/ESM-Fold	Protein structure prediction	Rapidly predicts single-sequence protein structures, comparable in accuracy to AlphaFold2. Used for de novo structure prediction.
YASARA	Homology modeling, molecular graphics	Simulates molecular mechanics, builds homology models, visualizes structures, and predicts molecular interactions (hydrogen bonds, salt bridges).
Swiss model	Homology modeling	Automates 3D protein structure homology modeling and assesses model quality.
Phyre2	Protein fold recognition/ homology modeling	An online tool for protein fold recognition, homology modeling, and active site studies.
MOE	Homology modeling and structure analysis	Used for all steps of homology modeling and calculating structural differences (RMSD).
MODELER	Comparative protein structure modeling	Performs comparative protein structure modeling. Performs comparative protein structure modeling.
AutoDock/AutoDock Vina	Molecular docking	Simulates how a ligand binds to an enzyme's active site to understand binding modes.
Discovery Studio (DS)	Homology modeling, docking, structure superimposition	Generates homology models, superimposes structures, and performs docking analysis.
PLIP	Protein-ligand interaction analysis	An automated tool for profiling protein-ligand interactions.
LigPlot+	Intermolecular interaction analysis	Analyzes interactions, hydrophobicity, and surface charge of enzymes and their complexes.
DOCK/GOLD/GLIDE	Molecular docking	Identifies binding sites, calculates distances between residues, and studies stereoselectivity.
PointSite	Binding pocket prediction	Predicts the binding pockets of an enzyme.
Rosetta (Suite)	De novo enzyme design, protein stabilization, activity/specificity enhancement	A versatile computational engine for de novo protein design, enzyme active site design, docking, and structure prediction.
FuncLib	Automated active site design and functional diversification	Designs multi-point mutations at active sites to enhance activity without sacrificing stability.

Software/Tool	The primary purpose in rational design	Key features/applications
SABER	Active site identification and redesign	Identifies active sites suitable for computational redesign and can be used for de novo design.
AsiteDesign	Semi-rational active site design	A Monte Carlo-based method for engineering active sites by introducing new catalytic residues.
PRODA	Computational enzyme design protocol (active site redesign)	Redesigns active sites and generates/ranks mutant sequences based on energy calculations.
OptZyme	Computational enzyme redesign (transition state analogues)	A tool for enzyme redesign based on transition state analogues.
CADEE	High-throughput screening of enzyme variants	A computational framework to screen thousands of enzyme variants for improved properties.
FoldX	Protein stability prediction	Calculates free energy changes upon mutations to predict protein stability.
FireProt/FireProtDB	Automated protein stabilization	An automated platform for designing thermostable proteins using energy- and evolution-based methods.
PROSS	Protein stabilization	Automates protein design for high bacterial expression and stability.
PoPMuSiC	Protein stability prediction	Predicts the effect of mutations on protein stability to aid in improving thermostability.
Disulfide by Design v2.0 (DbD2)	Disulfide bond prediction and optimization	Predicts the formation of disulfide bonds to improve protein stability.
LibGENiE	Mutant library design and deleterious mutation exclusion	Filters sequences to exclude deleterious mutations, creating more effective mutant libraries.
DynaMut / DynaMut2	Mutation impact prediction	Predicts how mutations impact a protein's conformation, flexibility, and stability.
HotSpot Wizard	Identification of hotspot residues and smart library design	Automates the design of targeted mutagenesis libraries by identifying "hotspot" residues critical for function.
IREDFisher	Structure-based computational screening workflow	Prioritizes protein sequences based on predicted activity, reducing lab screening efforts.
TurNup	Turnover number (kcat) prediction	Predicts the turnover number of enzyme variants to avoid those with poor activity.
COMPASS/CompassR	Organic-solvent tolerance / Activity enhancement	Yields enzymes with high tolerance to organic solvents by recombining compatible substitutions.
Pypef	Data-driven protein engineering framework	An integrated framework for data-driven protein engineering, used for screening and exploring combinatorial effects.

Software/Tool	The primary purpose in rational design	Key features/applications
EnzymeMiner	Soluble enzyme mining and alignment filtering	Generates function-specific alignments and filters them based on essential residues, predicting soluble expression.
A2CA	Sequence analysis and enzyme selection	A user-friendly tool to visualize sequence alignments and establish robust sequence-function relationships.
ConSurf	Evolutionary conservation analysis	Visualizes the evolutionary conservation of residues in proteins.
CoeViz	Coevolution analysis	A web tool for analyzing the coevolution of protein residues.
PELE	Ligand binding/unbinding simulations, substrate-induced fit	Predicts dynamic interactions and migration paths of ligands and proteins.
CAVER/CAVER Web	Tunnel and channel analysis	Identifies and characterizes transport pathways (tunnels and channels) within protein structures.
MOLEonline	Access tunnel detection and characterization	Detects and characterizes access tunnels in protein structures to guide substrate preference engineering.
EzMechanism	Automated catalytic mechanism proposal	Infers mechanistic paths for a given active site and reaction based on a database of catalytic rules.
GENZYME	Reaction-conditioned De novo enzyme design	A model that designs enzymes from a catalytic reaction as the input, generating the catalytic pocket and full enzyme structure.
Protein Language Models (e.g., ESM-2, RFdiffusionAA, ProteinMPNN, LigandMPNN, RIFGen, RIFDock)	De novo sequence generation, structure prediction, functional prediction	Models that efficiently generate new protein sequences and structures with desired functionalities.

Table 2.
Software used for rational enzyme design.

Rational design reduces the need for extensive experimental screening by using computational predictions to create "small but smart" mutant libraries. This approach allows for the precise customization of enzyme properties, such as enhancing catalytic activity, improving stability in harsh conditions, and fine-tuning selectivity. It can also create enzymes with entirely new functions, expanding the scope of biocatalysis. By relying on computational simulations, rational design significantly shortens the enzyme development cycle and deepens our understanding of protein function [8, 118, 126, 142, 156, 160, 165, 179].

Rational design has its challenges. It requires a deep understanding of the protein's structure and function, which can be difficult to obtain, especially for new or complex enzymes. Accurately predicting the effect of mutations is also a major challenge. Computational tools can be limited by biased training data and may not always accurately account for complex factors. Furthermore, some advanced methods are computationally expensive, requiring significant resources and expertise to use effectively [8, 88, 110, 117, 145, 180].

Despite these limitations, rational design, especially when combined with advanced computational tools and machine learning, is rapidly evolving and is considered a transformative approach in enzyme engineering, paving the way for more efficient and precise enzyme design [156, 165].

3.3 Semi-rational design

Semi-rational enzyme engineering is a sophisticated approach that strategically combines principles from both rational design and directed evolution to modify and enhance enzyme properties [88, 125, 160, 165, 181]. This hybrid methodology aims to generate "small but smart" mutant libraries, significantly reducing the extensive screening efforts typically required by purely random directed evolution methods, while offering more flexibility than solely rational approaches [88, 125, 173, 182].

Semi-rational design integrates the knowledge-based predictions of rational design with the diversity-generating power of directed evolution [126, 136, 160, 183]. This approach directly addresses the screening bottleneck of classical directed evolution and circumvents the need for an absolute understanding of the complex sequence-function relationship in enzymes, which can be computationally intensive [88]. It allows for a more efficient sampling of the vast sequence space compared to completely random methods, leading to a lower screening burden [88].

A core aspect of semi-rational design is the use of existing biochemical or structural information to guide mutagenesis [88, 117, 144, 148]. This often involves analyzing the enzyme's three-dimensional (3D) structure, particularly focusing on the catalytic pocket, substrate-binding sites, or access tunnels [110, 117, 184].

3.4 Artificial intelligence (AI) in enzyme engineering

Artificial Intelligence (AI), encompassing Machine Learning (ML) and Deep Learning (DL), plays a transformative role in enzyme engineering by enhancing the prediction of mutations, streamlining library design, and optimizing screening processes [147, 154, 185, 186].

In rational design, artificial intelligence (AI) is applied in two main ways. First, machine learning (ML) and deep learning (DL) models are used for predicting mutations. These models analyze huge datasets of protein sequences and structures to learn complex patterns and predict the effects of mutations on an enzyme's properties, like its stability, solubility, activity, and specificity [127, 144, 165, 177, 187]. They are especially useful for handling epistatic effects—nonadditive interactions between mutations that are very hard for traditional methods to predict accurately [106, 126, 188]. Specific tools and algorithms, such as protein language models (PLMs) and structure-based deep learning models, help identify beneficial mutations and predict stability changes [105, 106, 127, 189, 190].

Second, AI is used for designing libraries. By identifying promising "hot spots" for mutagenesis, ML/DL helps create "smart libraries" that have a much higher chance of producing a beneficial mutation. This significantly reduces the size and cost of screening compared to random mutagenesis [124, 142, 191]. ML also aids in combinatorial mutation design, allowing researchers to explore massive sequence spaces and find superior mutants with enhanced properties [103]. Generative models, such as Variational Autoencoders (VAEs) and Diffusion Models, can even create entirely novel protein sequences with functions not found in nature. By predicting variant

performance in a computer, AI greatly cuts down on the experimental work needed in traditional directed evolution [127, 147, 154].

ML approaches accelerate the screening and selection phase by predicting mutants with enhanced characteristics, allowing for multi-parameter optimization (e.g., catalytic activity, enantioselectivity, pH stability, thermostability, and binding affinity) [104, 131, 141, 147].

MLDE (Machine Learning-Guided Directed Evolution) reduces the burden of laboratory experiments by performing *in silico* population selection through model-based fitness prediction [128, 192–195].

These advancements enable rapid identification and engineering of enzymes, significantly reducing the complexity and cost of traditional screening processes.

3.5 Engineering of enzymes used in the dairy industry

Enzyme engineering in the dairy industry focuses on enhancing enzyme performance to improve product quality, process efficiency, and sustainability.

Improving the properties of lactase, particularly its thermostability and activity at low pH, is crucial for its widespread and efficient use in the dairy industry, enabling the production of lactose-free products, enhancing sweetness, and preventing lactose crystallization [12]. Enhancing lactase thermostability is a significant goal for industrial applications because higher temperatures can accelerate reaction rates, increase substrate solubility (especially for high lactose concentrations), and minimize microbial contamination risks [20, 79]. Several lactase enzymes have been successfully engineered. For instance, triple mutations (K166P, G307P, A833P) were introduced into a *Bacillus circulans* β-galactosidase, resulting in enhanced thermal stability at 60°C [7]. The half-life and catalytic efficiency of *Kluyveromyces lactis* lactase have been improved through rational mutation involving the addition of cysteine residues at subunit interfaces [7]. A thermoresistant variant of *Thermotoga maritima* beta-galactosidase was developed through rational design, specifically to improve its specificity for the biosynthesis of galactooligosaccharides (GOS). This engineered enzyme demonstrated optimal activity at 75°C, making it highly suitable for processes requiring elevated temperatures [79]. Rational design has also been successfully employed to overcome product inhibition in β-galactosidase by modifying the galactose-binding motif of *Aspergillus niger* β-galactosidase, thereby reducing the enzyme's affinity for galactose [7]. An evolved variant of β-galactosidase, achieved through directed evolution, showed high activity under industrial conditions relevant to milk processing (pH 6.75, 8°C) [7].

A protease (PB92) from *Bacillus alcalophilus* was engineered using site-directed mutagenesis, increasing its thermostability by up to 31 times at 65°C by identifying key residues like N18L-R143L-S97A and N18L-R143L-G100A [196]. Similarly, engineering the lactate dehydrogenase enzyme from *Bacillus stearothermophilus* altered its specificity from lactate to maleate by replacing glutamine at position 102 with arginine [8]. The thermostability of *Kluyveromyces lactis* β-galactosidase was improved by adding cysteine residues at subunit interfaces through rational mutation. The galactose-binding motif of *Aspergillus niger* β-galactosidase was modified to reduce product inhibition [7]. *Bacillus megaterium* M32 carboxypeptidase was modified through semi-rational design to enhance catalytic efficiency and thermal stability, improving food flavor and reducing bitterness in protein ingredients [17, 68].

Advanced enzyme engineering enables the scalable, controllable production of asymmetric and complex biantennary oligosaccharides that closely mimic those in

human milk, allowing for the enrichment of infant formula and functional foods with tailored health benefits.

4. Conclusions

The dairy industry, a vital component of the global economy, is being transformed by the strategic use of enzymes. Enzymes act as powerful biological catalysts, enabling crucial processes from milk coagulation to flavor development, all while enhancing product quality and sustainability. The global market for food enzymes is projected to grow significantly, with the dairy sector representing a major area of demand.

Enzyme optimization techniques are crucial in advancing the dairy industry, leading to the development of "next-generation" functional dairy products by enhancing their nutritional value, improving their sensory characteristics, extending their shelf life, and addressing specific dietary needs.

Enzyme engineering has emerged as a transformative field, allowing for the modification of enzymes to meet specific industrial needs. Through approaches like rational design, directed evolution, and semi-rational design, scientists can overcome the limitations of natural enzymes, such as poor stability or low catalytic efficiency. Rational design uses a deep understanding of protein structure to make precise, targeted changes, while directed evolution mimics natural selection to screen for improved variants without needing extensive prior knowledge. Semi-rational design combines the strengths of both, creating "small but smart" mutant libraries that reduce the experimental workload.

Modern computational tools and artificial intelligence (AI), including machine learning and deep learning, are increasingly integral to these processes. These technologies help predict beneficial mutations, handle complex interactions between mutations, and design efficient mutant libraries, significantly reducing the time and cost associated with enzyme development.

The continuous evolution of enzyme engineering techniques promises further innovations, offering solutions for higher yields, consistent product quality, and enhanced functionality. These advancements are not only crucial for meeting growing consumer demands for clean-label and functional foods but also for promoting sustainability through reduced energy consumption and more efficient processes. The integration of advanced computational methods ensures a future of increasingly precise and efficient biocatalyst optimization in the dairy sector.

5. Challenges and future perspectives

The dairy industry faces several challenges in enzyme application, as natural enzymes often have suboptimal properties like poor stability and low catalytic efficiency under processing conditions. For instance, heat-stable proteases from psychrotrophic bacteria cause undesirable age gelation and off-flavors in UHT milk. Furthermore, rational design is limited by the need for detailed structural knowledge, while directed evolution struggles with the costly and labor-intensive screening of large mutant libraries.

Future perspectives are highly promising due to the transformative role of AI and machine learning. These technologies will be crucial for predicting beneficial

mutations and designing "smart libraries," significantly reducing experimental effort and cost. This will enable the precise engineering of enzymes like cold-active and thermostable lactases for lactose-free products and regioselective lipases for targeted flavor development. Innovations in immobilization technologies will also improve enzyme stability and reusability. These advancements are critical for developing sustainable practices, such as enzyme-based cleaning systems, and for creating health-promoting products like infant formula enriched with human milk oligosaccharides (HMOs).

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