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REVIEW



Recent developments in enzyme immobilization technology for high-throughput processing in food industries

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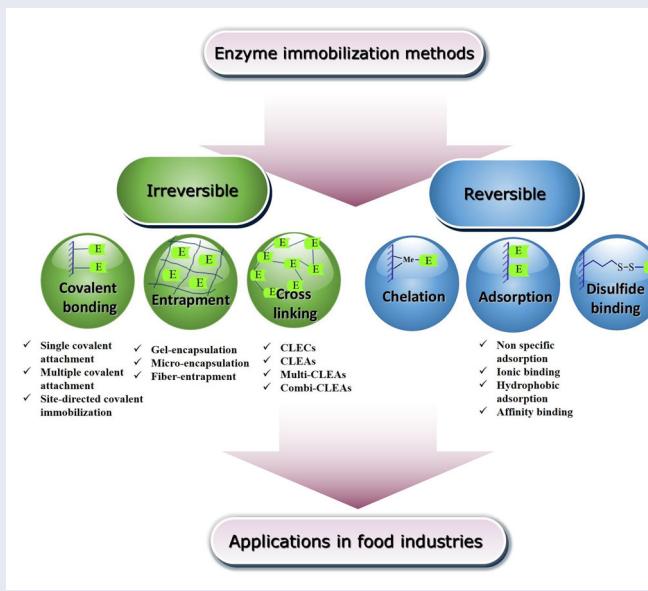
ABSTRACT

The demand for food and beverage markets has increased as a result of population increase and in view of health awareness. The quality of products from food processing industry has to be improved economically by incorporating greener methodologies that enhances the safety and shelf life via the enzymes application while maintaining the essential nutritional qualities. The utilization of enzymes is rendered more favorable in industrial practices via the modification of their characteristics as attested by studies on enzyme immobilization pertaining to different stages of food and beverage processing; these studies have enhanced the catalytic activity, stability of enzymes and lowered the overall cost. However, the harsh conditions of industrial processes continue to increase the propensity of enzyme destabilization thus shortening their industrial lifespan namely enzyme leaching, recoverability, uncontrollable orientation and the lack of a general procedure. Innovative studies have strived to provide new tools and materials for the development of systems offering new possibilities for industrial applications of enzymes. Herein, an effort has been made to present up-to-date developments on enzyme immobilization and current challenges in the food and beverage industries in terms of enhancing the enzyme stability.

KEYWORDS

Immobilized enzymes; food industry; pharmaceuticals; enzyme stability; enzyme reusability

GRAPHICAL ABSTRACT



Introduction

Enzymes are biological catalysts, which enhance the chemical species transformation in living systems and substrate

conversion in various chemical reactions. These molecules are considered as outstanding discoveries in the bioprocess technology field (Brena, González-Pombo, and Batista-Viera

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2013; Datta, Christena, and Rajaram 2013). With the advancement of genetic engineering and protein purification techniques, many widely used and specific novel enzymes have been developed whose new potential fields of applications are still being discovered. The application of enzymes in various industries has been continually increasing over the past few decades. One of the most significant features of enzymes is their capability of increasing the rate of nearly all chemical reactions taking place at the cellular level without being consumed in such reactions (Choi, Han, and Kim 2015; Robinson 2015; Singh et al. 2016). Enzymes are environmentally benign, biodegradable, efficient and cheap and they also have capacity of catalyzing reaction under very moderate conditions with high substrate degree of specificity resulting in the formation of by-products (Brena, González-Pombo, and Batista-Viera 2013). In view of these advantages, enzymes have exhibited great potential in many practical applications in diverse industrial processes including food (baking, dairy products and conversion of starch), processing of beverages (beer, wine and fruit juices), textile, paper, biosensors, wastewater treatment, healthcare and pharmaceuticals (Brady and Jordaan 2009; Homaei et al. 2013; Wohlgemuth 2010). Nevertheless, all these favorable properties of enzymes and their performances are significantly impacted by their instability in long term, surrounding environment and the technical challenges to recover the active enzymes for reuse. One of the most significant and broadly used techniques to overcome these limitations and make the enzyme utilization more favorable in biotechnological processes, is enzyme immobilization (Homaei 2015). The enzymes, which are physically restricted or localized in a certain location while maintaining their catalytic properties and recyclability, are referred to as 'immobilized enzymes' (Brena, González-Pombo, and Batista-Viera 2013). Enzyme immobilization technology has been envisioned as one of the very powerful techniques to tailor and modify a plethora of enzyme's catalytic features such as enzyme activity, specificity, selectivity, and stability under various pH's and temperatures, resistance to inhibitors, and their recyclability over successive catalytic cycles (Bilal, Asgher, et al. 2019; Bilal, Zhao, et al. 2019). Additionally, enzyme attachment to the solid support results in the products with higher purity through the biocatalyst simple removal from the reaction medium (Homaei et al. 2013; Singh et al. 2013; Zdarta et al. 2018; Zhang, Ge, and Liu 2015). Nowadays, due to the aforementioned reasons, immobilized enzymes are preferred over their free counterparts and are used considerably in various large-scale industries ranging from food to pharmaceuticals (Khan et al. 2010). The principle components such systems are the enzyme, the immobilization support, and the mode of attachment. Considering the difference in the type and character of interactions, a series of immobilization approaches are currently been developed for enzyme immobilization varying from reversible physical adsorption and ionic linkages to irreversible covalent bonds. The immobilization methods exploit enzymes bearing amino acids with distinctive features, whereby functional groups in side chains of these amino acids can engage in binding to support

through diverse interactions (Romero-Fernández and Paradisi 2020; Mohamad et al. 2015). Besides immobilization techniques, the selection of appropriate support materials is the most crucial challenge in desirable immobilization, because of their significant impact on the properties of enzyme and catalytic system. The internal geometry, mechanical resistance, specific surface area, pore diameter and activation degree of the support are important parameters to define a suitable immobilization support (Bilal, Asgher, et al. 2019). In recent years, new biopolymers and a variety of materials with organic, inorganic, hybrid or composite origins have been deployed as supporting materials for the immobilization of different enzymes (Bilal, Zhao, et al. 2019). Today, various groups of enzymes have potential utilization in many steps of food industry and the application of enzyme immobilization technology in this industry is gradually increasing (Aggarwal and Sahni 2012). Food technologists usually select the enzymes, which can improve one particular unit operation of food production. In view of the existing art on enzyme immobilization with focus on various strategies, supports and applications (Mohamad et al. 2015; Jesionowski, Zdarta, and Krajewska 2014; Datta, Christena, and Rajaram 2013; Zdarta et al. 2018; Bilal, Asgher, et al. 2019), this contribution comprehensively highlights and discusses the most recent developments of immobilized enzymes applications in food industry including fruit juice, brewing, baking and dairy industries and provides newer insights; especially in food industry. The notable features are:

- Understanding the major problems and the underlying reasons in fruit juice processing, which have created a significant economic problem in this industry.
- Identification of the main groups of enzymes used in fruit juice industry, along with the recent advances in their immobilization.
- Understanding different enzymes involved in each stage of beer processing and the recent efforts in the area of immobilized enzymes to enhance the brewing reactors.
- Use of enzyme immobilization technology in baking and dairy industries.
- Reviewing the immobilized enzymes on various nanosystems and their application in food industries.

Finally, we believe this review will be useful for researchers in the field of (bio)chemistry, (nano)biotechnology, (bio)polymer and biology in the universities, industries and research centers.

Methods for enzyme immobilization

Recently, different protocols have been published in the literature for the enzymes immobilization and several immobilization strategies have been introduced (Sassolas, Blum, and Leca-Bouvier 2012; Garcia-Galan et al. 2011; Bilal, Asgher, et al. 2019; Bilal, Zhao, et al. 2019). A wide variety of interactions such as reversible physical adsorption and ionic linkages to stable covalent bonds are used to attach

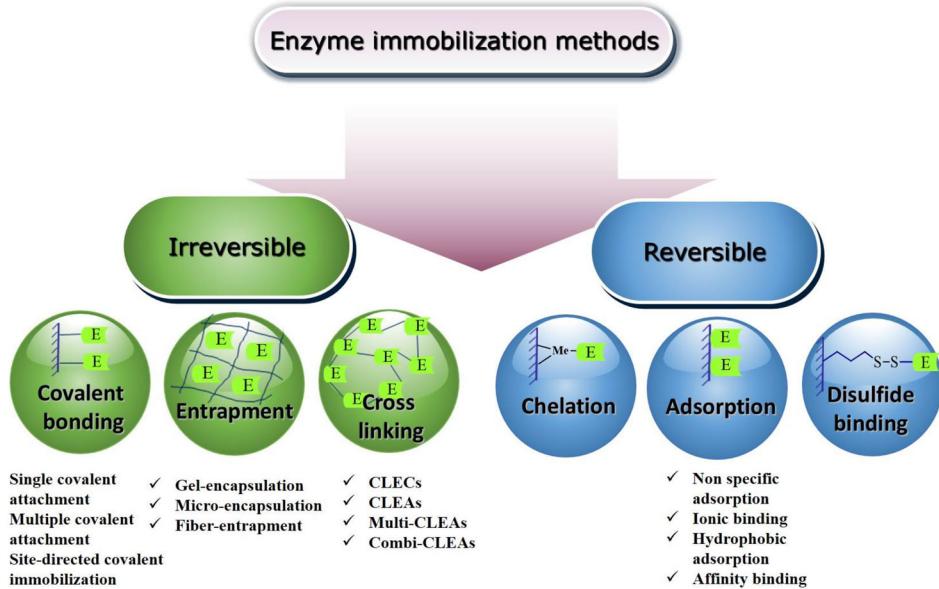


Figure 1. Schemes for major enzyme immobilization methods (E = enzyme).

enzymes to the supports. Enzyme immobilizing is carried out by either reversible or irreversible types of techniques (Brena, González-Pombo, and Batista-Viera 2013) (Figure 1) which may also be categorized based on type of chemical reactions. The facility of reversibility is normally inversely proportional to the strength of the binding.

Methods of irreversible enzyme immobilization

According to the irreversible immobilization mechanism, when a biocatalyst attaches to a support, it cannot be separated unless the biological activity of the enzyme is destroyed. The most extensively applied techniques for irreversible immobilization of enzymes include covalent entrainment (i.e., microencapsulation), coupling, and cross-linking.

Formation of covalent bonds

One of the common techniques for irreversible enzyme immobilization is immobilization of proteins through creating covalent bonds. A benefit of this method is the non-release of the enzyme to the solution during its use considering the higher stability of the bonds created between the matrix and enzyme. Nevertheless, to reach a high-bound activity, the engagement of the amino acid residues required for the catalytic prowess in the covalent attachment to the support must be prevented which is hard to achieve in some situations. A straightforward technique to enhance this activity is the addition of substrate analogs to the reaction media while performing the coupling reaction (Brena, González-Pombo, and Batista-Viera 2013). Covalent techniques are applied for immobilization in case the enzyme does not exist in the final product. With regards to the available functional groups in the matrix, researchers have presented a large number of reactions (Jasti et al. 2014). In a general classification, coupling methods are divided into two major groups: (1) matrix activation by adding a reactive function

to a polymer; (2) polymer backbone modification in order to generate an activated group. Typically, the activation methods are proposed for generating electrophilic groups on a group of supports, which react with strong nucleophiles on the proteins within the coupling step. The main factors, which control the coupling of covalent to the matrices are similar to the ones applied for the proteins chemical modification; several commercially available supports exist which can be used for immobilization. Among these supports, to choose the best one in each case, it is necessary to consider some characteristics of the catalyst and their useful purpose. Nevertheless, in most cases, one needs to try more than one technique for adopting the optimum one with respect to the specific conditions (Santos et al. 2015). In most cases, applying covalent reactions leads to enzymes linking to the support through ether, amide, carbamate, and thioether bonds. Accordingly, a strong bond is created between matrix and enzyme, which is often highly stable. In view of the covalent nature of the bond, as soon as the enzymatic activity starts to decay, it is necessary to discard the matrix together with the enzyme. Despite the advantage of achieving a leak-proof binding between the enzyme and the matrix using these reactions, it is expensive, has a low immobilization activity yield, and is irreversible. However, this immobilization strategy can be modified to overcome its limitations to varying degrees. The activity of the covalently immobilized enzyme depends on carrier material size, shape, composition, and the nature and specific conditions of the coupling reactions (Bilal, Asgher, et al. 2019). Any hydrophobic or hydrophilic support can be used for covalent bond based immobilization technique. During the last two years, several studies have described the use of silica/chitosan-based supports as a suitable carrier for covalent immobilization (Cazaban et al. 2018; Van den Biggelaar, Soumillion, and Debecker 2017; Manzo et al. 2018; Singh, Singh, and Kennedy 2017). Apart from the physical and chemical properties of silica including its chemical resistance and good mechanical characteristics,

high surface area, and the reduced diffusional limitations, the presence of many hydroxyl groups on the surface facilitates enzyme attachment and favors its functionalization with surface modification agents like glutaraldehyde and 3-aminopropyltriethoxysilane (APTES). Chitosan, an abundant natural biopolymer, is a biocompatible, hydrophilic, and biodegradable polysaccharide having preferable structural feasibilities for chemical and mechanical modification and is one of the most frequently used natural organic material for enzyme immobilization. Among the covalent methods, efficient enzyme immobilization has been attained via multiple covalent attachment of amino groups of the enzyme surface onto the cross-linked agarose beads; covalent immobilization may increase the rigidity of enzyme structure, resulting in a greater stabilization against the conformational changes caused by denaturing agents (Romero-Fernández and Paradisi 2020). Covalent immobilization can also be conducted in a site-directed manner to obtain immobilized enzymes with enhanced stability and reactivity. PRECISE (protein residue-explicit covalent immobilization for stability enhancement) system developed by Wu et al. (2014) is a model that deploys non-canonical amino acid incorporation and the Huisgen 1,3-dipolar cycloaddition “click” reaction to enable directed enzyme immobilization at rationally chosen residues throughout an enzyme. This system enables the enzyme immobilization at proximate and distant locations from the active site to study the effect on the activity and stability under harsh conditions (Wu et al. 2015).

Entrapment and cross-linking

The mechanism of entrapment approach relies on the encapsulation of an enzyme within a polymeric network allowing the products and substrate to pass through while retaining the enzyme. This approach is different from the described coupling approaches, in which the enzyme is not limited to the membrane or matrix. Several techniques have been proposed for enzyme entrapment e.g., gel and micro-encapsulation or fiber entrainment (Liu, Chen, and Shi 2018; Livage and Coradin 2018) which improves the enzyme stability, minimizes enzyme leaching and denaturation, and optimizes microenvironment for the enzyme by modifying the encapsulating material to have the optimal pH, polarity or amphiphilicity (Nguyen and Kim 2017). However, the practical application of these techniques is limited by mass transfer restrictions through gels or membranes. It should be pointed out that some newer methods (Tran and Balkus Jr 2011; Galliani et al. 2018) proposed for enzyme immobilization such as cross-linked enzyme crystals (CLECs) and cross-linked enzyme aggregates (CLEAs) are relatively different from the conventional immobilization approaches. While the methodology of these approaches was developed at the turn of the century, it is still very much a method of choice for enzymatic application in the food industry and biorefineries. CLECs are highly active immobilized enzymes of controllable particle size. Their operational stability and ease of recycling coupled with their high catalysts and volumetric productivities, render them ideally suited for industrial biotransformations. However, the extensive protein

purification required for CLECs formation is the severe limitation of this method. The more recently developed CLEAs are an improved version of CLECs produced by simple precipitation of the enzyme from aqueous solution as physical aggregates of protein molecules, by the addition of salts or nonionic polymers (Nguyen and Kim 2017; Homaei et al. 2013). The mechanism of CLEAs is on the basis of multi-point attachment through intermolecular cross-linking between the enzyme molecules and they have emerged as a promising carrier-free immobilization technology due to their simplicity, greater catalytic activity, enhanced operational and storage stabilities, and excellent reusability. In this regard, many studies have been currently undertaken for the successful preparation of CLEAs from several enzymes such as lipases, horseradish peroxidase, penicillin acylases, and laccases (Šulek et al. 2011). A further development of this technique is multipurpose cross-linked enzyme aggregates (multi-CLEAs) and combined cross-linked enzyme aggregates (combi-CLEAs), in which more than one enzyme crosslinked together, creates CLEAs with an enhanced capability to catalyze numerous biotransformation reactions, individually or sequentially as catalytic cascade processes, respectively (Bilal, Zhao, et al. 2019). Recently some examples have been reported on these cross-linked based immobilization approaches. Periyasamy et al. (2016) successfully combined xylanase, cellulase and β -1, 3-glucanase for one-pot cascade saccharification of sugarcane bagasse (SCB). In addition to enhanced temperature and storage properties, the combi-CLEAs could retain 90% activity after six consecutive uses (Periyasamy et al. 2016). A multi-CLEAs of pectinase, xylanase and cellulase was synthesized to perform three different independent catalytic reactions; developed multi-CLEAs was highly thermostable and recyclable (Dalal, Sharma, and Gupta 2007). Stability and reusability of CLEAs have been also improved with their combination with magnetic nanoparticles (M-CLEAs) in food industry, where the ease of separation from the reaction mixture represents a significant advantage for repeated usage and greater potential for further developments in continuous biocatalytic processes (Martins et al. 2018; Nadar and Rathod 2016). Very recently, glucose dehydrogenase- and ketoreductase-based magnetic combi-CLEAs were synthesized, with improved catalytic activity and stability of ensued magnetic combi-CLEAs in both, aqueous and biphasic media than that of original CLEAs (Su et al. 2018). Furthermore, in the last decade, the combination of bio-imprinting technique with CLEAs has been widely pursued to enhance the catalytic performance and stability of numerous enzymes via a novel combinatorial cross-linked imprinting approach named imprinted CLEAs (iCLEAs). The bio-imprinting technique is an attractive tool to manipulate enzyme's catalytic properties, stability, enantioselectivity, and reusability (Cui and Jia 2015; De Winter, Soetaert, and Desmet 2012; Bilal, Zhao, et al. 2019).

Methods involving reversible immobilization

Regarding the type of binding between the support and enzyme, under mild conditions, reversibly immobilized

enzymes can possibly detach from the support. To deal with this issue, reversible methods have received much attention in enzyme immobilization, particularly because of their economic justification. The chief reason for the cost-effectiveness of these enzymes is the decay of their activity, which leads to regeneration and re-loading of the support with the fresh enzyme; generally, support cost is a major factor controlling the total catalyst immobilization cost. Enzyme reversible immobilization is of particular importance for bioanalytical systems and immobilization of labile enzymes (Bilal, Zhao, et al. 2019).

Adsorption (noncovalent interactions)

Nonspecific adsorption. Nonspecific adsorption is the most straightforward method available for immobilization and this technique is mainly established based on ionic binding or physical adsorption (Mohamad et al. 2015). In ionic bonding, the enzymes attach to the matrix via salt linkages. In comparison, in physical adsorption, the enzymes attach to the matrix via forces of van der Waals, hydrogen bonding, and hydrophobic interactions. A process, which can be inverted by altering the conditions controlling the interaction strength (i.e. ionic strength, pH, the solvent polarity, and temperature), is created by the forces engaged in non-covalent immobilization. Adsorption immobilization is a gentle and facile process, which usually preserves the enzyme catalytic activity. Although these approaches are appealing from economic perspectives, they have some disadvantages such as leakage of the enzyme from the matrix in case of relatively weak interactions.

Ionic binding. A commonly used technique for the enzymes reversible immobilization is to employ the protein-ligand interactions through chromatography. For instance, use of ion-exchangers is among the earliest uses of chromatography in the enzyme reversible immobilization (Vaz and Filho 2019). Although this technique is easy and reversible, the conditions required for maintenance of high enzyme activity and strong bonding are generally hard to identify. More recently, interaction between protein and matrix has been modulated using the immobilized polymeric ionic ligands which facilitates to optimize the properties of the derivative. Despite these advantages, problems may arise when using highly charged support in case products or substrates are themselves charged. Through this process, the kinetics is interrupted as a result of diffusion or partition phenomena. Hence, the enzymes properties (e.g. optimum pH and the pH stability range) are prone to variation (Ward, Xi, and Stuckey 2016). Nevertheless, the pH values can be adjusted to reach the optimal conditions for a certain enzyme (Yang, Fan, et al. 2019) as exemplified over the last three years (Benítez-Mateos et al. 2017; Furuya, Kuroiwa, and Kino 2017; Wang, Liu, and Zhou 2017).

Hydrophobic adsorption. Hydrophobic interactions are another group of techniques deployed for the reversible enzyme immobilization wherein an entropically driven interaction occurs rather than chemical bond formation; it has

been employed as a chromatographic principle for over three decades. This approach is based on such common experimental parameters as salt concentration, pH, and temperature (Mohamad et al. 2015). The adsorbent and protein hydrophobicity both determine the interaction strength. The adsorbent hydrophobicity can be regulated by the hydrophobic ligand molecule size and the substitution level of the support. The reversible β -amylase and amyloglucosidase immobilization to hexylagarose carriers have been successfully carried out (Das and Kayastha 2019). Over the past three years, hydrophobic absorption strategy on various supports have been described in literatures; synthetic beads formed by polymethacrylate matrices, silica, and magnetic nanoparticles have been used as support materials for this purpose (Silveira et al. 2017; Castejón et al. 2019; Koutinas et al. 2018; Hüttner 2017; Vescovi et al. 2017; Gao et al. 2019).

Affinity binding. The attraction between complementary biomolecules has been considered as a principle to describe enzyme immobilization. A predominant advantage of this technique is the marked selectivity of the interaction. Despite this advantage, the covalent binding of an expensive affinity ligand (antibody or lectin) is necessitated to be attached to the matrix (Brena, González-Pombo, and Batista-Viera 2013). Affinity tags, that are present or added at a specific position far from the active site in the structure of the native enzymes, could be used to create strong affinity bonds between the protein structure and a surface functionalized with the complementary affinity ligand. The increasing specificity of enzyme adsorption to the support materials results in oriented immobilization instead of random immobilization, where multiple orientations of the immobilized enzyme may be found (Bolivar and Nidetzky 2013). Immobilization of enzyme based on affinity binding between the enzymes and support materials can be performed through the ionic exchange or covalent bonds. An ionic immobilization approach by affinity binding has been described recently; enzymes immobilization with a fused peptide tag containing a polyhistidine chain (His-tag) onto immobilization supports displays metal ions such as Ni^{2+} , Cu^{2+} , Co^{2+} , Fe^{3+} , and Zn^{2+} (Liu et al. 2017; Vahidi, Wang, and Li 2018). Bohmer et al. (2018) co-immobilized an alcohol dehydrogenase (ADH) and a chimeric amine dehydrogenase (AmDH) on controlled porosity glass Fe^{3+} ion-affinity beads by ionic affinity binding. The recyclability of this immobilized dual-enzyme system were demonstrated for five recycles with total turnover numbers of >4000 and >1000 for ADH and AmDH, respectively (Böhmer, Knaus, and Mutti 2018). Another variation is the immobilization of enzymes with a fused discrete protein domain. For instance, fused enzymes to the engineered Z_{basic2} binding domain are attached by ionic interactions onto immobilization matrices displaying anionic surface groups; they exhibit a decisive advantage for oriented immobilization compared to use the small tags like His-tag. The spatial separation between the immobilization matrix and the catalytic activity of enzyme is better attained, thereby the oriented immobilization through



Figure 2. Annual consumption of nonalcoholic beverages worldwide (in billion liters).

Z_{basic2} module is highly preferred and the possible negative effect of surface binding to enzyme activity is reduced (Bolívar Bolívar et al. 2017; Romero-Fernández and Paradisi 2020). Affinity binding based enzyme immobilization can also be realized via the formation of covalent bond between enzyme and immobilization support. In this strategy, like affinity binding via the ionic exchange, a small peptide tag, a discrete protein domain, or a protein is genetically fused to the target enzyme. More recently, an approach for immobilization by covalent affinity binding was developed to attach the horse liver alcohol dehydrogenase fused to a poly-histidine tag onto metal-activated polymethacrylate support that displays epoxy groups (Contente and Paradisi 2018). A recent proposal for covalent immobilization by affinity binding entails a second protein used as spacer for covalent immobilization to improve the catalytic activity of target enzyme by preventing any direct covalent attachment between the enzyme and immobilization matrix. In this study, T4L lysozyme as second protein with naturally rich in lysine amino acid, was fused between the His-tag and different target enzymes and exploited to obtain higher recovered activities of immobilized enzymes (Planchestainer et al. 2017). Moreover, recent developments involve immobilization of enzymes onto the magnetic beads through affinity binding, which improves the performance of immobilized enzyme in analytical assay; magnetic beads provide a relative large numbers of binding sites for biochemical reactions resulting in faster assay kinetics (Badea, Hayat, and Marty 2020).

Chelation or metal binding

Hydroxides or transition metal salts deposited on the organic carrier's surface develop bonds through coordination with nucleophilic groups on the matrix; zirconium and titanium salts being the mostly applied for this purpose. This method is generally referred to as "metal link immobilization" (Singh et al. 2013). Neutralization or heating precipitates the hydroxide or metal salt onto the support (e.g. alginic acid, chitin, cellulose, and silica-based carriers). Due to the existing steric factors, not all the coordination positions in the metal can be occupied by the matrix. As a result, some positions are free for coordination with the

groups in the enzymes. In addition to the facility of the procedure, the enzymes achieve relatively high (30–80%) immobilized specific activities. Nevertheless, the operational stabilities attained are highly inconsistent and the results are not simply reproducible, the reason might be the presence of non-uniform adsorption sites as well as the leakage of metal ions from the support. One strategy to control the creation of the adsorption sites is to immobilize chelator ligands on the solid supports using stable covalent bonds. Thus, the metal ions are bound through coordination. Consequently, the stable complexes prepared can be applied for protein retention. Elution of the bound proteins may be simply carried out via competition with soluble ligands or through decreasing the reaction pH. Next, washing with a strong chelator (e.g. ethylenediaminetetraacetic acid (EDTA)) regenerates the support. These metal chelated supports, referred to as Immobilized Metal-Ion Affinity (IMA) adsorbents, are extensively applied in protein chromatography (Kagedal 2011).

Formation of disulfide bonds

The uniqueness of the techniques presented in this study for irreversible enzyme immobilization is owing to the fact that although the matrix and enzyme form a stable covalent bond, the bond can be degraded upon reacting with an appropriate agent like dithiothreitol (DTT) under moderate conditions. Moreover, since it is possible to regulate the thiol group reactivity by adjusting the pH, the activity yields of the techniques, which involve formation of disulfide bonds is typically high, when using a suitable thiol-reactive adsorbent (Batista-Viera, Ryden, and Carlsson 2011). Enzymes bearing exposed nonessential thiol (SH) groups can be immobilized onto thiol-reactive supports providing reactive disulfide or disulfide oxides under mild conditions. The reversibility of the bonds formed between the thiol-enzyme and the activated solid phase, which can be released with an excess of a low molecular weight thiol, is a potential advantage of this approach. This characteristic is of particular interest when the enzyme degrades much faster than the absorbent, which can be reloaded afterwards (Ovsejevi, Manta, and Batista-Viera 2013).

In conclusion, the covalent reactions commonly stabilize the enzyme conformation. Moreover, because of the strong nature of the irreversible attachment of the enzymes, the matrix has to be discarded together with the enzyme once the enzymatic activity decays for some cost benefits. Although, reversibly immobilized enzymes can be detached from the support under gentle conditions, the use of reversible methods is highly attractive, mostly for the economic reasons; as the enzymatic activity decays, the support can be regenerated and re-loaded with fresh enzyme. Indeed, the cost of the support is often a primary factor in the overall cost of an immobilized catalyst. The reversible immobilization of enzymes is particularly important for immobilizing labile enzymes and for applications in bioanalytical systems.

Immobilization technology in fruit juice industry

Fruit juice component and processing problems

Nowadays, with growing awareness of the vital role of health promoting fruit components in human health and nutrition along with the increasing demand for natural fruit juice consumption (Figure 2), the quality of these products has become a key factor for numerous customers (Cassano 2013; Ferreira et al. 2013); fruit and vegetable juices are one of the fastest growing segments in the food industry. The obtained revenue in this segment has amounted to 94,872 million US \$ in 2018, which is expected to reach 109,977 million \$ in 2022 (<https://www.statista.com>). Appearance is one of the first traits by which consumers evaluate the fruit juice quality (Lozano 2006). Color, clarification, homogeneity, viscosity, yield and shelf life are important characteristics, which can influence the appearance, storage time and finally the quality of industrial juices (Dal Magro et al. 2016). Therefore, the development of novel technologies has attracted remarkable attention with regards to the improved quality of fruit juice. Fresh fruit juices are highly susceptible to microbial, enzymatic, physical and chemical deterioration through the interaction of fluid components with air and environmental microorganisms and not surprisingly, fruit processing is performed to completely reduce these undesirable reactions while still retaining the inherent quality of juices (Mihalev et al. 2018). Various conventional physical and chemical processes including heat treatment, ultrafiltration, and application of chemical additives are applied for processing fruit products. However, the loss of nutrients and formation of unfavorable by-products because of these processes are the negative impacts on the juice quality (Jiménez-Sánchez et al. 2017). Hence, the practical applications of these methods against deterioration have been limited due to reduction in the nutritional value of fruit juices and technical-economic considerations. Juice turbidity, one of the main problems in the fruit industry, is a dominant cause of quality loss during processing and storage. Although fruit juices are considered one of the rich sources of significant components like minerals, vitamins, polysaccharides, antioxidants, dietary fibers and small amounts of proteins and fats, they contain colloids, which can result in problems during the processing and result in turbid and cloudy juices. These colloids are either part of the fruit or are generated by microorganisms during the ripening process (Echavarria et al. 2011; Lozano 2006). The presence of such polysaccharides namely starch, pectin, and hemicellulosic components, which have a tendency to settle during the storage period, is mainly responsible for the turbidity of fruit juices (Shahrestani et al. 2016). Major structural polysaccharides are present in lamellas and primary cell walls, which consist of cellulose-xyloglucan network and pectin matrix and their amount depends on fruit species, climatic conditions, and fruit ripeness (Posé et al. 2018). Pectin, a structural cell wall polysaccharide, is the main substance responsible for cloudiness of most fruit juices; it is a gel forming agent present in fruit juices, the fiber-like structure of which inhibits the clarification process via the reduction of flux and yield. Starch is another potential contributor to a secondary haze in juices, which can lead to

difficulties such as membrane fouling, slow filtration, gelling following concentration and post concentration haze during clarification (Dey and Banerjee 2014). Another significant factor which affects the postharvest bitterness of fruits is the presence of polyphenols in juices. Although polyphenols are a natural source of antioxidants, they cause serious oxidation and cloudiness problems in juice industry (Pezzella, Guarino, and Piscitelli 2015). Usually, the interaction between polyphenols and protein particles obtained from disrupted cell walls leads to the formation of haze suspensions, turbidity intensification, and flavor and aroma alteration during the cold storage (Kammerer, Kammerer, and Carle 2010; Pinelo, Zeuner, and Meyer 2010). Bitterness in citrus fruits is another main problem in fruit juice industry, which creates an important economic problem in this industry. Bitter taste in citrus juices is due to two main types of chemicals; namely flavonoids (primarily naringin) and limonoids (predominantly limonin). In many citrus fruits, the "delayed" bitterness (developed following juice extraction) is caused by naringin, whereas the "immediate" bitterness is caused by limonin (Zhang et al. 2018; Kore and Chakraborty 2015). Naringin, which is the main bittering water soluble component of the fruit's membrane and pulp, is the 7β -neohesperidoside of naringenin, the flavonone glucoside. The concentration of naringin is dependent on the degree of fruit maturity, the lowest concentration being present in ripened fruits. Delayed bitterness is mostly caused by limonin, which is the main lemonoid present in most citrus fruit juices and is a highly oxygenated triterpene derivative, comprising an epoxide group and a furan ring. Therefore, to obtain clear, high quality fruit juices, all these opacity and bitterness factors must be removed before commercialization. The bitterness and cloudiness in fruit juices have been reduced by a number of traditional approaches and physiochemical treatments. However, harsh economic and technical constraints are associated with these which are nonspecific in nature and have often impaired the quality of juices by the partial loss of favorable nutrients during the removal of bitter and turbid components. Novel approaches have been undertaken in recent decades to increase the safety and shelf life of juices and simultaneously maintain their nutritional quality (Rajauria and Tiwari 2017). In association with new apparatus and processing technologies, treatment of fruit juices with industrial enzymes can significantly enhance the yield and storage stability of finished products. While enzymes preserve the nutritional value of juices, they degrade polysaccharide components and avoid undesirable haziness, turbidity, and cloudiness during the shorter process. Hence, the application of enzymes in fruit processing not only minimally affects the fruit quality, but also has low costs, which is important from a commercial standpoint (Ribeiro et al. 2010). Juice clarification process, briefly entails several important steps: mechanical maceration, extraction, and saccharides hydrolysis using enzymes. The initial steps involve peeling, chopping, crushing, and pressing resulting in the extraction of cloudy juices. Juice extraction should be rapidly accomplished to minimize its probable oxidation. A crucial step for efficient clarification is the enzyme treatment of the extracted cloudy juices followed by filtration and

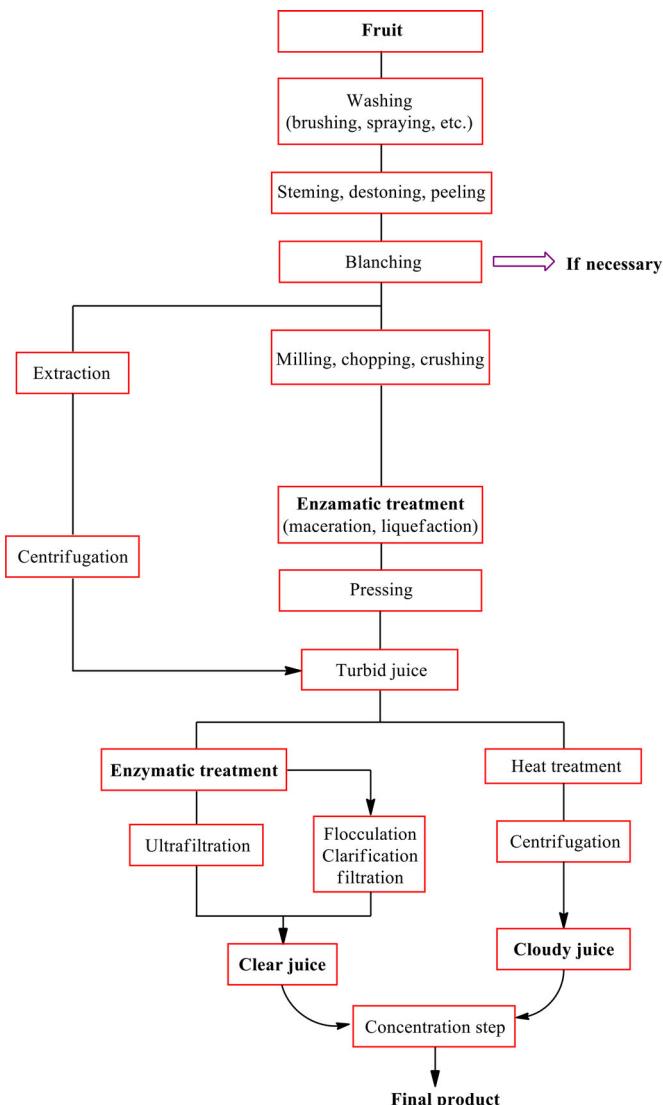


Figure 3. Schematics for processing of fruit juice.

centrifugation steps. Usually, the final step is concentration by evaporation and homogenization to achieve a safe and stable juice (Figure 3) (Lozano 2006; Reyes-De-Corcuera et al. 2014). In summary, it is clear that the fruit juice market is in a state of gradual increase as tracked by the development of per-capita consumption of fruit juices over the years. This offers plentiful opportunities for the fruit juice industry to cater new consumer demands and preferences, which are apparent from the latest trends. By the development of modern processing technologies for improvement of juice quality in a cost-effective manner and meeting the demand for healthy product manufacture, enzymes have emerged as more cost-effective, more environmentally friendly and more sustainable biocatalysts in the juice industry.

Development of immobilized enzymes in fruit juice processing

One of the significant impacts of biotechnology on modern food industry is the use of enzymes in fruit juice industry. Although enzymes resolve the limitations of physicochemical

approaches in fruit juice processing such as inefficiency, undesired alteration of nutrients, lack of high reproducibility, and the necessity of post processing to remove impurities, and natural enzymes are often not desirable for industrial applications (Stepankova et al. 2013). Since industrial process conditions may be unsteady in terms of temperature and pH, enzymes must be strong enough and highly stable to tolerate such conditions. Hence, many attempts have been made to enhance the stability, efficiency and activity of enzymes in industrial processes. In recent years, rendering enzymes insoluble via immobilization has been one the most desirable methods for highly effective and economically efficient biotechnological processes in the fruit industry by increasing the enzyme stability and reusability in continuous processes (Soozanipour, Taheri-Kafrani, and Isfahani 2015; Stepankova et al. 2013; Jiménez-Sánchez et al. 2017; Ferreira et al. 2013). To commercialize the application of immobilized enzymes in fruit juice industry, the cost of finished product must be more economical than the soluble counterparts; cost of an immobilized enzyme depends on the matrix price, enzyme purification, regenerative capability and sanitation requirements (Cowan and Fernandez-Lafuente 2011).

There are mainly three groups of enzymes applied in fruit juice industry including hydrolases, oxidoreductases and cellulases (Cantarelli 2012). A summary of the latest accomplishments regarding the application of several successful immobilized enzymes for application in fruit industry is provided in this section.

Pectinase

Pectinases are one of the first commercial hydrolysis enzymes used as prerequisites for minimizing the impacts of pectic substances on the organoleptic features of final product and attaining clarified juices with higher yields (Lei and Jiang 2011). The resulting juice will also have less viscosity and minimal amount of pectic substances suitable for subsequent filtration. The pectic substances are structural heteropolysaccharides mostly found in the middle lamella of plant cell wall, which contain 1,4- α -D-galacturonan backbone with many partially methoxylated hydrophilic and carboxyl groups (Lara-Espinoza et al. 2018). Despite their numerous applications as a gelling agent; particularly in jams and jellies, stabilizer for fruit juices, and a source of dietary fiber, it may be interlined with other structural polysaccharides and protein particles to form insoluble protopectin, resulting in highly viscous fruit juices (Vanitha and Khan 2019). Pectinases can be categorized into protopectinases or polygalacturonase (PG), pectinesterase (PE), and pectin lyase (PL) on the basis of their mode of action on the degradation of pectin (Garg et al. 2016). The pectinases treatment not only improves the clarification of juices by breaking the polysaccharide pectin structure, but also reduces the viscosity and enhances the yield of the juices (Sandri et al. 2011). Although pectic enzymes were first used in the 1930s for juice clarification, today, they constitute almost one fourths of the world's production of food enzyme (Garg et al. 2016). Pectinolytic enzymes play a significant part in fruit juice

technology and favorably enhance the filterability, clarity and yield of juices (Cerretti et al. 2017; Uzuner and Cekmecelioglu 2015). Despite excellent catalytic properties of free pectinases in fruit industry, they invariably show disadvantages such as poor stability under operational conditions and impossible recyclability in industrial processes; recent publications have mainly focused on utilizing immobilized pectinases in clarification of fresh juices wherein pectinase has been immobilized on various supports with a variety of good properties ranging from high biocompatibility to high durability. There are several reasons why the immobilized form of pectinase is used. Along with the improvement of the enzyme stability in an acidic juice solution, immobilization technology provides a continuous bio-process with reusability for pectin hydrolysis (Cerretti et al. 2017; Rajdeo et al. 2016; Sojitra, Nadar, and Rathod 2017). However, pectinase has been immobilized on a variety of supports including chitin, nylon, silk, ion exchange resins, alginate, polyacrylamide microspheres, silica gel, polyethylene, and magnetic particles using several methods; application of some immobilized enzymes in fruit juice industry has been reviewed (Lei and Jiang 2011; Adalberto et al. 2012; Martín et al. 2019b). A commercial pectinase immobilized on functionalized polyacrylonitrile (PAN), and loaded in a packed bed reactor was applied in the depolymerization of the pectin in apple juice; pectinase immobilized system was used for the successful depectinisation of apple juice (Echavarria et al. 2011). The immobilization of pectinases into polyvinyl alcohol resulted in the immobilized enzyme with good flexibility in an acidic medium such as fruit juice, good reusability and 80% turbidity reduction in three consecutive cycles (Cerretti et al. 2017). A commercial pectinase was successfully immobilized on recyclable polymer matrix. Although the immobilized and free enzymes thermal stability was similar at ambient temperature, the immobilized enzyme was capable of being recycled over 10 times with a catalytic activity loss of less than 5% during the apple juice clarification (Rajdeo et al. 2016). Pectinase has been immobilized on chitosan magnetic nanoparticles using dextran polyaldehyde as a macromolecular cross linking agent. According to thermal kinetic studies, the thermal stability of the immobilized pectinase was doubled compared to the free form. The conformational flexibility of pectinase was maintained following immobilization and a turbidity reduction of up to 74% was observed in apple juice (Sojitra, Nadar, and Rathod 2017). In another study, the immobilization of pectinase on the silica-coated magnetite nanoparticle surface was performed through covalent attachment. The immobilized pectinase exhibited enhanced enzyme activity, improved resistance to pH and temperature variations, and enhanced storage stability in comparison with free pectinase. Apple juice viscosity was decreased by both, the free and immobilized enzymes. However, the immobilized enzyme could only be reused six consecutive cycles and showed a viscosity reduction of only 8.16% (Mosafa, Shahedi, and Moghadam 2014). Recently, adsorption immobilization procedure to immobilize an extracellular fungal exo-polygalacturonase in sodium alginate was illustrated which remarkably improved

the thermal resistance profile; immobilized enzyme preserved > 90% of its original activity after 1 h of heating at 60 °C, while the non-immobilized state of enzyme gets deactivated under this conditions (Amin et al. 2017). Immobilized enzyme retained 41% of residual activity even after seven successive batch reactions. Finally, promising viscosity and turbidity reduction, as well as clarity enhancement in different tested fruit juices suggest a great potential of the immobilized exo-polygalacturonase in food industry (Amin et al. 2017). More recently, a nontoxic and low-cost biocatalyst was developed by immobilizing a commercial enological pectinase within insoluble calcium alginate beads using an entrapment technique. Although the immobilization procedure did not modify the optimal pH and temperature for pectinase activity, entrapped enzyme displayed catalytic activity until six reaction cycles with 40% residual activity; wet entrapped enzyme retained its original activity up to 11 weeks, whereas the lyophilized hydrogels maintained its initial activity after 8 months of storage. Also, the immobilized pectinase significantly deceased the turbidity of grape juice in 120 min at 20 °C (Martín et al. 2019a). In 2020, a commercial enzyme cocktail, comprising pectinolytic and cellulolytic enzymes formulated for fruit juice clarification, was immobilized on chitosan beads activated with glutaraldehyde and deployed for setting-up two different biocatalytic reactor systems including continues packed-bed and fluidized-bed reactors. The immobilized enzyme cocktail retained 80% of its initial activity at 90 °C and pH 4.8, while the free enzyme held only 35%. Clarification analysis of the immobilized biocatalyst provided clearer juices when performed in the fluidized-bed reactor (Dal Magro et al. 2020). Our group has successfully developed an effective strategy to exploit the pectinase in food industry by its covalently immobilization onto polyethylene glycol grafted magnetic nanoparticles via trichlorotriazine. The immobilized pectinase showed enhanced catalytic activity, improved operational stability, excellent reusability and storage stability by retaining up to 55% and 94% of its initial activity after 10 recycles and 125 days storage at 25 °C, respectively. Furthermore, the applicability of this tactic in juice and food processing industries was demonstrated by turbidity reduction up to 59% in pineapple juice treated with immobilized pectinase (Kharazmi, Taheri-Kafrani, and Soozanipour 2020).

Maceration enzymes

Since a common problem in fruit industry is the juice cloudiness, arising from suspended polysaccharides including pectins, cellulose, hemicelluloses, and starchy derivative materials, it has been shown that the use of combined enzymes rather than one enzyme increases extraction yields and improves juice processing without additional capital investments (Heffels et al. 2017; Kermani et al. 2015; Wang, Xu, and Jin 2009). Currently, the commercial enzymes contain a mixture of pectinase, cellulase, hemicellulase, protease, and amylase, known as maceration enzymes. The use of maceration enzymes in two steps (after crushing and the juice extraction steps), increases juice recovery time,

improves yield and decreases viscosity and turbidity without extra costs. In one study, the effect of treatment with 10 various enzymatic macerations on the improvement of anthocyanins and other phenolic materials was investigated in black currant juice; the juice yield, the level of anthocyanins and total phenols as well as the clarity of black currant juice were enhanced by pectinolytic maceration enzymes (Sharma, Patel, and Sugandha 2017). Today, these enzymes have been co-immobilized on a support as a potential tool to enhance the effectiveness of combined enzymes and reduce the costs. A few studies have already been conducted in this field in spite of all the drawbacks of co-immobilized enzymes. The turbidity of apple, grape and pineapple juices was reduced by 41, 46 and 53%, respectively, using pectinase, α -amylase, and cellulase co-immobilized onto functionalized magnetic nanoparticle (Sojitra, Nadar, and Rathod 2016). Recently, co-immobilization of pectinase and glucoamylase was accomplished onto sodium alginate/graphene oxide beads by *N,N'*-dicyclohexylcarbodiimide/*N*-hydroxysuccinimide as activating agents to provide an effective method for improving quality of the pumpkin-hawthorn juice. Reusability studies showed that both enzymes retained over 60% of original activity after consecutive reuse in six cycles. The soluble solids, light transmittance, and reducing sugar contents were significantly increased after treatment of pumpkin-hawthorn juice with co-immobilized enzymes, whereas pectin and total sugar were decreased. These results demonstrated that this approach could be also applied in other juice clarification, which are rich in pectin and starch (Yang, Dai, et al. 2019).

Naringinase

Naringinase, a debittering enzyme, plays a rather significant part in improving the taste of citrus juice and modification of flavonoids in order to give highly bioactive compounds. Naringinase, which is an enzyme complex comprising α -L-rhamnosidase and β -D-glucopyranosides, can hydrolyze many glycosides such as 6-O- α L-rhamnopyranose- β -D-glucopyranosides, hesperidin, naringin, and rutin (Chen et al. 2013; Zhu et al. 2017). This enzyme is able to reduce the bitterness of citrus juice by hydrolyzing naringin, which is the major bitter component of citrus juice, into prunin and its hydrolyzed final product, naringenin (Yadav, Yadav, and Yadav 2013). The application of naringinase in juice debitterization and fruit industry renders it a prominent target for enzyme immobilization technology. Many attempts have been made to date to immobilize naringinase on different supports (Busto et al. 2007; Huang et al. 2017; Lei et al. 2011); as cryogels in poly vinyl alcohol by entrapment being one example wherein entrapped enzyme could be recycled six times while maintaining 36% of its efficiency in naringin hydrolysis in simulated juice (Busto et al. 2007). Moreover, naringinase has been successfully immobilized in electrospun cellulose acetate nanofibers and used for the removal of bitterness in grapefruit juice, removing 22.72% of naringin and 60.71% of limonin. Immobilization of naringinase was also performed onto silica MCM-41 via adsorption with glutaraldehyde. In addition to the excellent thermal and storage

stability of immobilized enzyme in comparison with their free counterparts, this enzyme was able to hydrolyze naringin by about 44.57%, up to 6 runs in the white grapefruits (Lei et al. 2011). In one study, the characteristics and hydrolysis properties of immobilized naringinase on porous silicon materials with different pore diameter of 2 nm (MCM-41), 7.7 nm (SBA-15) and 80 nm (silica gel) were studied to identify a novel naringinase immobilization material with applicability in industrial fruit juice processing. The immobilized naringinase on glutaraldehyde modified SBA-15 showed the highest activity, retained 61.81% of the residual activity after eight consecutive cycles and preserved 80.95% of the initial activity after one month of storage (Luo et al. 2019). Naringinase immobilized on chitosan microspheres activated with glutaraldehyde exhibited good operational stability and retained about 88% of its initial activity after ten runs of naringin hydrolysis from fresh grapefruit juice (Bodakowska-Bocznewicz and Garncarek 2019). Recently, the immobilization of naringinase onto nano-alginate gel beads and nano-chitosan carriers was performed to examine the characteristics of naringinase nano-encapsulated forms; grapefruit rind was used as substrate containing naringin for naringinase production. The immobilized naringinase showed more resistant to changes in environmental parameters such as pH, temperature and inhibitory effect of different compounds. However, it was revealed that the highest enzyme activity was found in the case of chitosan nano-capsule naringinase compared to alginate nano-capsule enzyme (Housseiny and Aboelmagd 2019).

Laccase

One of the most significant effects of the presence of polyphenols in fruit juices is increased susceptibility to enzymatic darkening during storage. Therefore, the removal of phenol derivatives could be desirable for the color and flavor stability of fruit juices. Laccase is an oxidoreductase, which oxidizes polyphenols by reduction of oxygen to water and polymerization of residual oligomers in the juice suspension. Most researchers have known laccase as a special green catalyst because of its requirement for the oxygen as co-substrate, and releasing water as the only by-product, which can reduce product inhibitions (de Souza Bezerra et al. 2015). Furthermore, owing to their high redox potential, laccases can oxidize polyphenols, degrade lignin into the simple carbohydrates (cellulose and hemicellulose) and provide conditions for the activity of other enzymes. Since laccase is able to degrade phenolic compounds and increase color stability, it has considerable industrial potential applications as a clarification factor in fruit juice industry. As with the other enzymes, using the immobilized form of laccase for large scale application is preferred to their free form which leads to the formation of soluble polymeric polyphenolic derivatives rather than insoluble compounds produced by a free enzyme. Hence, it avoids the formation of solid deposit and eliminates the requirement for filtration (Lettera et al. 2016; Stanescu et al. 2012).

It has been shown that the selection of cheap and stable supports has an important effect on improving the operational function and benefits cost ratio in enzyme immobilization process. The covalent immobilization of laccase has been successfully carried out in green coconut fiber and the application of immobilized enzyme has been investigated in apple juice clarification. Based on the findings of this study, immobilized derivatives performed better in juice clarification and efficiently oxidized phenols (de Souza Bezerra et al. 2015). More recently, the use of nanoparticle-laccase conjugate in juice clarification was performed by Narnoliya et al. (2019). In this study, a recombinant laccase was covalently immobilized onto the magnetic nanoparticles of iron oxide (Fe_3O_4) and the immobilized biocatalyst system was recruited for the removal of phenolics and clarification of juice samples; 41–58% of phenol reduction, 41–58% decolorization, and 50–59% turbidity reduction was attained in the extracts of banana pseudo-stem and sweet sorghum stalk, and apple fruit juice. The immobilized enzyme also retained 60% of initial activity after 10 reaction cycles of 2,2'-azinobis (3-ethylbenzothiazoline-6-sulphonic acid) (ABTS) oxidization (Narnoliya et al. 2019).

In recent years, apart from the immobilized enzymes mentioned above, many enzymes including xylanase, mannanase, tannase, and peroxidase were successfully immobilized on a variety of solid supports and applied in fruit industry to develop the clarification of juices; further studies will be needed for the engineering of enzymes and build effective bioreactors which can be utilized in fruit juice industry.

In addition, selection of enzymes for application in this industry depends on the type of fruit and its abundant constituents. However, the use of enzyme mixtures in fruit processing has often yielded better clarification (Shahrestani et al. 2016; Adiguzel et al. 2016; Bilal et al. 2016; Jana et al. 2015); compared to the independent enzymes, mixture of enzymes showed more effective clarification of juice and also improved quality of juice without additional costs.

Application of enzyme immobilization technology in brewing industry

Since 8000 years, brewing process has been considered one of the most important food businesses. Today, brewing industries are rapidly developing with a high contribution to the global markets. With the growing market demands to annual world beer production, the traditional beer brewing process need to be optimized. In general, brewing process is based on the formation of a sweet liquid obtained from a starch source mainly barley, wheat, rye or other grains and followed by fermentation into the beer by means of yeasts. Ordinarily, this process takes place through three main steps including malting, mashing, and fermentation with the interference of stage specific enzymes. Briefly, grains germinate through controlled malting conditions to activate the enzymatic cell machine and disrupt the cell walls (Schmitt, Skadsen, and Budde 2013). Subsequently, mash is produced from malted grains in combination with gelatinized adjuncts

and water. The crucial task of mashing step is the breakdown of starchy content into fermentable sugars mediated by glycolytic enzymes; namely α -amylase, β -amylase, α -glucosidase, and limited dextrinase. The proper degradation of starch occurs in optimum temperatures ranging from 60 to 75 °C and optimum pH of 5.4–5.8; these conditions are required for the maximal activity and enzyme stability involved in this stage (Fox 2018; Hu et al. 2014). Therefore, mashing is aimed at producing a hot sweet wort, which contains simple sugars like maltotriose, glucose, and maltose (Pires and Brányik 2015). Mashing is a significant stage in the process of brewing, which can affect the kind and quality of the beer produced (Langenaeken et al. 2019). After the mashing process, the hot sweet wort is boiled to inactivate the enzymes, sterilize the wort, precipitate the unwanted haze active proteins and condense the wort. Eventually, the fermentation process starts by blending brewing yeasts to the cooled and oxygenated wort in fermenters. During this process, ethanol, CO_2 , and higher alcohols are excreted from the yeasts as a result of anaerobic reactions, known as the “glycolytic pathway” in yeast cells. Although these by-products are intended by breweries, they are toxic for the yeasts at high concentrations. Hence, yeasts are normally removed from fresh beers through settling, centrifugation or filtration (Figure 4) (Wang et al. 2019; Yang 2019).

Nowadays, brewers intend to take the advantages of adding a combination of exogenous enzymes in brewing processes to tune the brew house operations and economize these processes. This approach focuses on describing proper alternatives for naturally endogenous enzymes and presents some effective methods for improving the brewing procedures (Lynch, Steffen, and Arendt 2016); among others, the enzyme immobilization technology in each stage of this process is being pursued which are discussed in the present section. In spite of the significant technological improvements in brewing industry over the last years, the quality of products remains a major challenge. In terms of the factors that impact the quality of beer, brewers have learned not only how the endogenous enzymes contribute to characteristics such as fermentability, foam, flavor, filterability, and clarity, but also how to take advantage of exogenous enzymes in a cost-effective manner.

Malting

During the malting process, endogenous enzymatic machinery plays a vital part in the degradation of β -Glucans, a group of the major components of barley cell wall, which may cause many problems in brewing like low rates of wort separation, high wort viscosity and haze formation. β -Glucans are initially solubilized and degraded by an acidic carboxypeptidase called β -Glucan solubilase. Afterwards, the degradation process continues by several endo- β -glucanases. Among the endo- β -glucanases, commercially available, β -1,4-glucanase, which is commonly derived from fungal and bacterial sources, has preliminary been applied in soluble form to decrease wort viscosity (Bogdan and Kordialik-Bogacka 2017). Subsequently, the majority of studies have

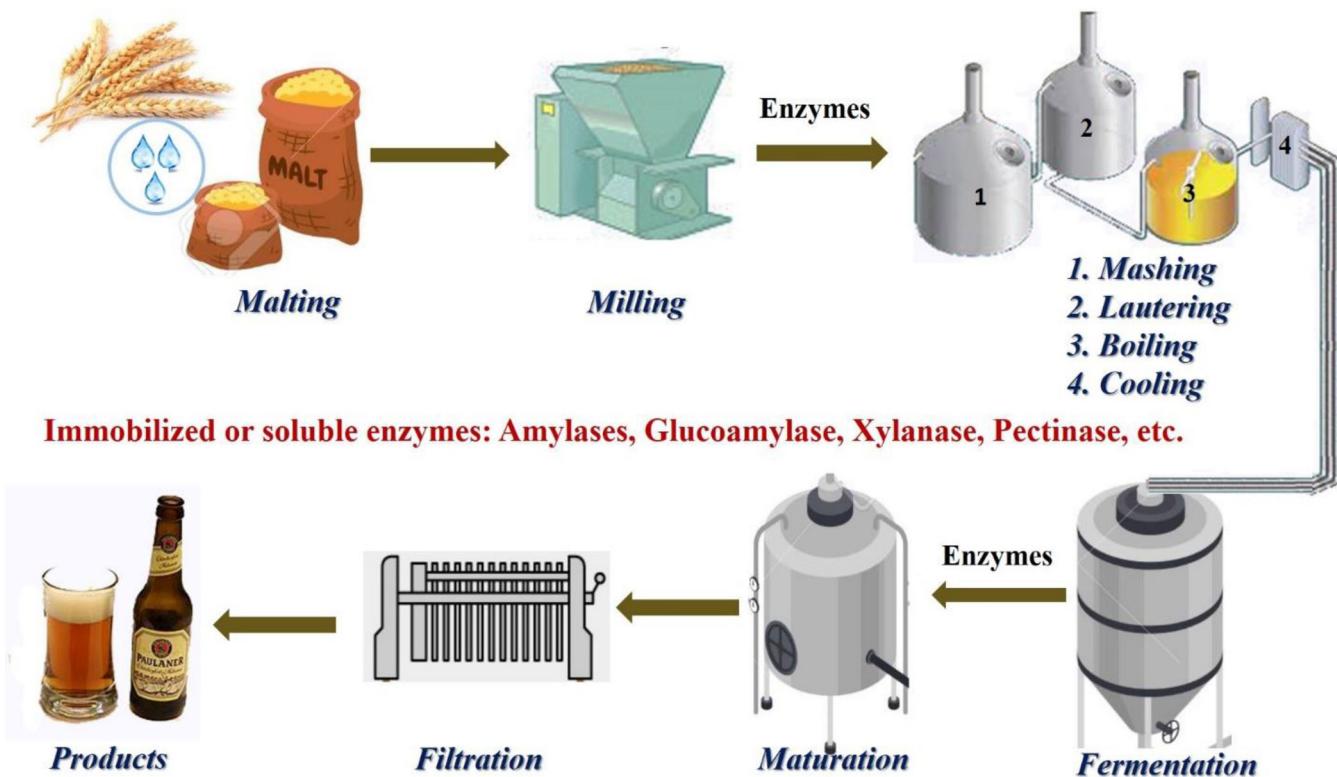


Figure 4. A schematic presentation of brewing process.

been contributed to insolubilize β -glucanase into packed bed reactors for continuous hydrolysis of barley glucans (Schnitzenbaumer and Arendt 2014; Kestwal, Bagal-Kestwal, and Chiang 2011). Covalent immobilization of β -glucanase and some other brewing related enzymes on silanized Spherosil XOB-075 porous silica beads presented a remarkable hydrolysis efficacy (Linko and Linko 1979); application of 11.6 mg immobilized cellulase in a recirculating fluidized bed reactor showed enzyme activity as well as 50 mg soluble enzyme.

Increasing the industrial application of brewing enzymes in large scales over the last decade has attracted major attentions for immobilization of β -glucanase on the surface of new type of supporting materials, namely poly(dimethylsiloxane) (PDMS), Si wafer and indium tin oxide (ITO). Tseng et al. (2015) introduced PDMS and Si wafer as attractive substrates, which provide the highest level of β -glucanase activity at increased temperature and pH compared with the free counterpart. Despite the great binding ability of EglA (an endoglucanase) on the Si wafer, the enzyme immobilized on PDMS had a higher specific activity (921.15 ± 2.60 U/mg) than both Si wafer-EglA (462.64 ± 6.16 U/mg) and soluble enzyme (576.0 ± 13.6 U/mg). The interaction between EglA and Si wafer and exhibited up to six cycles of reusability under optimal reaction conditions (Tseng et al. 2015). In another study, one-step immobilization of a recombinant endoglucanase (CtCelA) on artificial oil bodies (AOBs) provided a new strategy to facilitate large scale and economic use of this enzyme in brewing industries; AOBs are self-assembled particles of triacylglycerol (TAG) in micro scale, surrounded by a monolayer of phospholipids (PLs).

CtCelA was genetically fused with oleosin (Ole) and the fusion product was assembled into AOBs-bound CtCelA construct under optimized conditions; immobilized CtCelA showed optimal glucanase activity at 69°C and pH 6.3 and remained stable after five cycles (Chiang et al. 2013).

Mashing

The degradation of cereal polysaccharides, mediated by means of prepared malt enzymes to convert the polymeric carbohydrates into fermentable simple sugars, is the most important task, which occurs during the mashing process. In this regard, mash tun is considered a real enzyme reactor to convert barley to beer (Bamforth 2009). In order to reach the highest yield in the brewing industries, many exogenous enzymes have been employed to hydrolyze starch and non-starch polysaccharide (NSP) components of grains. Hence, starch hydrolysis using different types of amylases can be accompanied by the limit dextrinase, α -glucosidase, and glucoamylases. As discussed before, immobilization of these enzymes in mashing reactors improves the quality of final products and controls the costs. The following section discusses several enzymes used in mashing stage and reviews the recent advances in enhancing the efficacy of these enzymes in brewery industry.

Alpha-amylase

The α -amylase family is generally deployed to hydrolyze α -glycosidic bonds in amylose and amylopectin, which yields α -anomeric configuration of products (Sindhu et al. 2017). This type of starch hydrolyzing enzymes is more attractive

in brewing industry because of their abundance and thermo-tolerant property. Industrial α -amylase can be obtained on large scales from various plants and microbial sources among which fungal and bacterial α -amylases are more popular (Rana, Walia, and Gaur 2013). Despite their abundance, there are still serious concerns about the loss of enzyme activity under mashing conditions. Therefore, the most of studies have focused on the stabilization of α -amylases on different matrixes for their use in the starch hydrolysis reactors; especially in food industries. Although many promising developments have been made in amylase immobilization, there are not any reports regarding the industrial application of immobilized amylase in brewing industries. Therefore, some of the effective immobilization processes for amylases, which have a great potential for application in large scale brewing processes, will be discussed here.

Immobilization of α -amylase on silica-coated modified Fe_3O_4 NPs resulted in the relatively suitable enzyme activity and thermal stability. The enzymatic reaction of immobilized α -amylase was carried out with reduced K_m value at pH = 6.5 (4.77 compared with 6.27 for the free enzyme), while the V_{max} was increased from 2.44 to 11.58 $\mu\text{mol}/\text{mg min}$ (Sohrabi, Rasouli, and Torkzadeh 2014). Covalent attachment of α -amylase on montmorillonite substrate retained 59% of the free enzyme catalytic activity in the batch reactor, which was improved up to 82% in the fixed bed reactor; immobilized enzyme showed stable activity for 72 h under continuous catalytic conditions with enhanced pH and thermal tolerance, in comparison with the soluble ones. The drop observed in V_{max} and notable increment in K_m value seem to be related to the reduction in the reaction rate of bonded enzyme on porous substrate (Sanjay and Sugunan 2005). To overcome such problems, a number of studies have reported the physical entrapment of α -amylase into calcium alginate beads as an easy, low cost, and non-toxic approach (Zhang, Ge, and Liu 2015). In this method, along with improved enzyme stability, loss of enzyme activity occurred at a minimum level. The entrapment of α -amylase within calcium alginate gel beads was also performed by Yagar et al. (2007) which significantly improved the enzyme stability and K_m value and resulted in the more successful starch hydrolysis compared with the soluble α -amylase. Yagar et al. (2007) provided some evidence for high reduction in the starch hydrolysis ability of entrapped α -amylase during the first 10 minutes; this may be caused by the interference of the matrix with the starch diffusion to the active core of enzyme (Yagar, Ertan, and Balkan 2007). The use of magnetic nanoparticles (MNPs) as supporting agents provides the opportunity to simply recover immobilized enzymes; especially in industrial size reactors (Bilal, Mehmood, et al. 2019). Iron oxide MNPs provide large surface area and considerable loading capacity for α -amylase immobilization, which resulted in over 26 fold increase in the specific enzyme activity. Fe_3O_4 MNP-bound α -amylase also showed improvement in catalytic parameters toward starch hydrolysis and higher thermostability at a specific temperature in mashing reactors. Furthermore, immobilized

α -amylase presented a dramatic increase in storage stability and reusability of the enzyme, as two important factors in industries (Khan, Husain, and Azam 2012; Mukherjee et al. 2010). Production and characterization of cross-linked enzyme aggregate (CLEAs) has opened the path for carrier-free strategies in enzyme immobilization and has resulted in satisfactory catalytic activity for insoluble α -amylase in addition to improved thermal and acidic stabilities. Using the dextran polyaldehyde as the cross-linking increased the activity recovery of cross-linked enzyme aggregates of *Aspergillus oryzae* α -amylase (AoAA) from 21.8% to 60.0% through the lower reaction with amino acid residues in the enzyme active site (Sahutoglu and Akgul 2015). A recent study takes the advantages of α -amylase immobilization on ultrafiltration cellulose membrane to hydrolyze the starchy components in a continuous flow of the substrate (Konovalova et al. 2016). Based on the affinity chromatography techniques, Cibacron Blue F3GA dye was attached on cellulose membrane as an affinity ligand for α -amylase by chitosan intermediate molecules; immobilized enzyme showed 84% starch conversion rate in pH 3.5 ± 0.2 and maintained the highest biocatalytic activity even after three cycles of operation. This system has provided a promising capability in the brewing industry to regenerate the enzymatic layer in a low cost process. In 2020, a carrier-free nanobiocatalyst was fabricated to resolve the two drawbacks of CLEAs usage on industrial scale including mass transfer limitations and enzyme handling, by co-immobilization of α -amylase and maltogenic amylase onto the lysine-functionalized magnetic Fe_3O_4 NPs (NM-Combi-CLEAs). The fabricated nanobiocatalyst was deployed to hydrolyze the corn starch to maltose via nanomagnetic combined cross-linked enzyme aggregates method with a high immobilization activity yield. The NM-Combi-CLEAs also retained 80.4% of its initial activity after 10 cycles and displayed high thermostability at 95 °C, lower K_m value, and higher enzyme affinity to substrate compared to free enzyme (Montazeri and Torabizadeh 2020).

Beta-amylase

β -amylase hydrolyzes the starch molecules from their non-reducing ends and produces β -maltose units. In contrast to the aforementioned enzymes, β -amylase is more thermosensitive and mainly presents in mash barley bound to a blocking protein (Montanuci et al. 2017). Although the optimum hydrolytic activity of β -amylase has been reported around 60 °C, the popular belief is that this enzyme can be degraded under normal mashing conditions. Hence, attempts have been made to improve the β -amylase stability by the enzyme immobilization technology for industrial applications. Martensson obtained the best efficiency of starch hydrolysis at pH 4.8 and 35 °C by dual immobilization of β -amylase-Pullulanase on a polymeric membrane (Mårtensson 1974). However, physical stabilization of a chemically modified form of β -amylase on porous silica supports revealed a suitable activity at increased temperatures of up to 70–75 °C and remained continuously active for 14 days at 55 °C

(Rodrigues, Berenguer-Murcia, and Fernandez-Lafuente 2011).

Glucoamylase

Glucoamylase (GAs or amyloglucosidase) is another important amylolytic enzyme used industrially to improve barely mash for beer production by hydrolyzing the branch points in starch molecules as well as linear glucose units in both, the amylose and amylopectin molecules. A variety of microorganisms are considered as glucoamylase sources for use in the food and beverage industries (Raveendran et al. 2018). Like other soluble enzymes, natural soluble glucoamylases have shown some limitations for application in brewing processing and hence the immobilization technology may be able to overcome the drawbacks of soluble GAs like the low pH and thermal stability and extend the lifetime of enzyme activity for continuous starch conversion in mashing reactors.

Presently, carrier-free strategies such as CLEAs employ the glucoamylase in all starch-based industries. In an experiment, glutaraldehyde and dextran polyaldehyde were separately used for preparing the CLEA of glucoamylase. While glutaraldehyde cross-linking of glucoamylase increased its thermal stability, a low activity recovery for the enzyme (41.2%) was observed. On the other hand, dextran cross-linking inactivates the aggregated enzyme owing to the high affinity of dextran for binding to the glucoamylase active site (Sahutoglu and Akgul 2015; Cui and Zhang 2014). Recently, Xiao-Dong and colleagues modified the conventional CLEA procedures by adding the dextrin or xanthan gum during the cross-linking process, as protecting agents. In comparison with the free enzyme, this modification provided a higher specific activity in broader ranges of pH (3.0–8.0) and temperature (55–75 °C) for the immobilized enzyme; GA-CLEAs also showed excellent recyclability and storability up to three months under usual conditions (Li et al. 2016). Enzyme immobilization onto magnetic hydrogels can have profound effects on large-scale mashing reactors to facilitate the movement of substrate and product through the reactor in an alternative magnetic field. The glucoamylase adsorbed onto a magnetic hydrogel film, prepared by Bayramoglu et al., displayed 59% enzyme activity recovery at the higher temperature (55 °C) and more acidic pH (pH 4.5) in comparison to the optimal condition for the free enzyme (50 °C and pH 5.5) (Bayramoglu, Altintas, and Arica 2013).

A nanostructure of hybrid graphene oxide- Fe_3O_4 -cyanuric chloride (GO/MNP-CC) substrate for enzyme immobilization has been established which showed great capability for supporting the catalytic activity of glucoamylase. However, covalent immobilization of glucoamylase resulting in a slightly higher K_m and lower V_{max} values in comparison to the free enzyme, more than 96% activity recovery at pH 6.5 and 60 °C and its considerable stability over 20 reaction cycles, reinforce the importance of this method (Amirbandeh and Taheri-Kafrani 2016). The superparamagnetic properties of GO/MNP-CC, large surface area, thermo-stability and easy surface modification have made it

advantageous for large-scale application of GO/MNP-CC/GA in brewing industry (Li, Zhang, et al. 2012).

The only commercially available patent regarding the application of immobilized glucoamylase in beer production process is related to 19th century which reports an enzymatic reactor with covalently attached glucoamylases to a rigid, non-particulate ceramic carrier. This invention aimed at producing low caloric beer through a rapid and stable flow of fermenting beer stream over a long period of time (Hassan, Yang, and Xiao 2019).

Fermentation

The main aspect of fermentation stage is the metabolism of wort content into adequate ethanol, carbon dioxide, and other fermentation products and handling of this process is generally applied through regulating behavior of the brewer's yeasts as whole organisms (Stewart 2016). In this regard, enormous studies have focused on the optimization of fermentation bioreactors by using the immobilized yeast bioreactors. On the other hand, genetically modified brewing yeasts have been really considered for producing advanced exogenous fermentation enzymes and accelerating this time consuming process (Steensels et al. 2014; Claus and Mojsov 2018). Although there has been a strong tendency to understand the enzymes involved in the fermentation process, to the best of the authors' knowledge to date, there have been no studies on the immobilization of these enzymes.

Application of enzyme immobilization technology in baking industry

Baking commonly refers to the baked products containing wheat flour as the most necessary component and major source of enzyme substrates for products. Bread is the most popular and traditional food around the world, which is usually made from the wheat flour, water, and baker's yeast and salt. In addition, small quantities of enzymes, sugars, emulsifiers and oxidizing agents are added into the mixture to affect the dough properties and develop the final products quality (Omedi et al. 2019). Wheat flour, as a raw material in bread production, mainly consists of gluten, starch, peptide, polysaccharides and lipids. Starch, which is the essential component of bakery products, is the most abundant form of storage polysaccharides in many plants and operates as a water binder, thickener, emulsion stabilizer and gelling agent (Egharevba 2019). Generally, processing of bread can be classified into three major steps including mixing, fermentation (resting and proofing) and baking. At first, flour is mixed with water and yeast to prepare the dough. Salt, oils and sugars are also common ingredients in bread dough. During the rising, wheat enzymes and yeast catalyze the complex biochemical processes, ferment the sugar into alcohols and carbon dioxide and develop the dough. Rising also improves the flavor and texture of the bread. Finally, the developed dough is converted to bread in the baking phase. The baking process results in various biochemical and physical changes in the products including volume expansion,

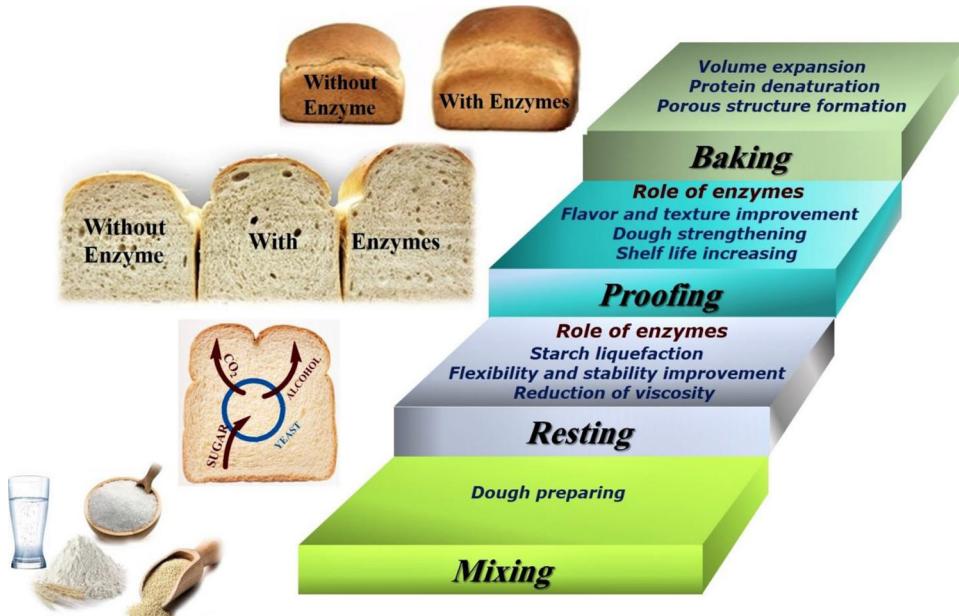


Figure 5. Bread processing and the role of enzymes in baking industry.

water evaporation, protein denaturation, porous structure formation and browning reactions (Figure 5) (Miguel et al. 2013). A series of physicochemical changes gradually occurs in bread during storage, which leads to the quality deterioration; they include losing the pleasant aroma and flavor, increasing the rigidity of bread crumb and water evaporating from the sliced bread surface, collectively referred to as staling. In general, salting implies poor quality and comparatively short shelf-life for fresh bread, which causes the loss of acceptance by consumers. Starch retrogradation; especially in short amylopectin side chains, plays a significant part in loss of moisture and bread staling. In spite of the complexity of the molecular mechanisms involved in staling phenomenon, it has been shown that the application of various additives in baking processes such as chemical agents, enzymes, emulsifiers, sugars and salts delays the staling and improves the bread quality (Fadda et al. 2014). Due to the increasing demand for more natural products, enzymes have been suggested as the most important anti-staling agents to improve bread flexibility and stability. Three sources of enzyme are usually used in bakery industry including endogenous enzymes in flour, enzymes related to the metabolic activity of microorganisms and exogenous enzymes added to the dough. Recently, several enzymes such as α -amylases, amyloglucosidases, xylanase, lipase and peptidase have been used individually or in mixtures as dough and bread improving agents to modify one of the main components of dough (Miguel et al. 2013).

Amylase

As previously described, amylases are a group of enzymes, which hydrolyzes starch into the smaller carbohydrate molecules such as maltose by the addition of a water molecule.

Amylases are divided into two categories, α -amylase and β -amylase, which differ in the way they attack the present bonds in the molecules of starch. α -Amylases are the most frequently used enzymes in bread production which can breakdown α -1,4 glycosidic bonds in the inner part of the amylose chain, culminating in the production of oligosaccharides with varying lengths. β -amylases belong to the exoamylases, which act on the external glucose residues of amylose or amylopectin, producing low molecular weight carbohydrates (Miguel et al. 2013). α -amylases and β -amylases, with complementary functions are added to the dough during the baking and improve the crust color, taste and toasting quality of bread by hydrolyzing the starch, thereby increasing the fermentable compounds level and reducing sugars. These enzymes reportedly function as anti-staling agents and enhance softness retention of the baked products (El-Okki et al. 2017; Bilal and Iqbal 2019). The anti-firming effect of amylase is caused by the starch network quick formation, which contributes to the stabilization of a kinetic texture, and prevents the structure collapse and starch phase rearrangement (Zhou et al. 2019).

They can also reduce the dough viscosity during starch gelatinization which causes an increased loaf volume. Starch gelatinization in dough starts by combination of heat, moisture and time, resulting in the baked bread with typical solid foam structure (Miguel et al. 2013). One of the most effective strategies for improving the performance of amylases in industries; especially baking industry, is immobilization. To date, a wide variety of supports have been used to immobilize amylases.

A simple and economical method, in which iron oxide nanoparticles are used, has recently been applied to immobilize fungal α -amylase. The immobilized enzymes are more stable compared with free enzyme, mainly because of more rigid conformation, less denaturation and less autolysis of

fixed protein. In addition, the magnetically immobilized α -amylase is reasonably stable over cycles and maintains 60% of its initial hydrolytic activity after eight runs. The desirable stability and longer life of the immobilized α -amylase make it potentially valuable in the baking process (He et al. 2017).

The fermentation process in baking takes place in two main reaction steps, which are controlled by amylase activity and consumption of maltose applied as fermentable sugar by the yeast, respectively. In one study, the most suitable immobilization step (enzyme, yeast or both) was investigated on the basis of different reaction rates at various temperatures. The effect of food-grade hydrocolloids on amylase and yeast activity was investigated to characterize the appropriate strategy for immobilization for extending the shelf life of refrigerated dough. While the immobilization of amylase in alginate/gelatin micro-beads effectively reduced enzyme activity (in CaCl_2), it showed the least efficacy due to the high level of enzyme leaching caused by the small size of amylase and the high bead permeability. In contrast, the yeast immobilization was more successful since fermentation was thoroughly stopped under refrigerated and ambient conditions. Despite the advantages of the presented method in bread making, the gelatin impact on different properties of baked bread such as specific volume, crumb firmness and stalling was not measured in this study (Gugerli et al. 2004; Struyf et al. 2017).

In another study, α -amylase was immobilized on alumina by adsorption. Although highest activity was shown by immobilized and both free enzymes at pH 6, the immobilized enzyme was much more stable at higher pH's; V_{\max} values for the absorbed amylase were smaller than that of the free enzyme, which indicates reduced enzyme activity as a result of immobilization. The calculated K_m values were higher than that of the free enzyme probably due to the diffusional resistances or conformational changes in the enzyme due to immobilization (Reshma, Sanjay, and Sugunan 2006). More recently, α -amylase was successfully immobilized onto chitosan-magnetic nanoparticles beads with high immobilization yield, using a method merging the advantages of both physical adsorption and covalent binding. The immobilized enzyme on the polyethyleneimine/glutaraldehyde modified beads was applied in the baking process, presenting enhanced dough-raising of about 2.3 fold and a reusability for 5 cycles with 100% activity (Abdella et al. 2020).

Glucose oxidase

A very important oxidoreductase enzyme belonging to flavoproteins, glucose oxidase catalyzes β -D-glucose oxidation into D-glucono- δ -lactone at its first hydroxyl group. This enzyme not only has wide applications in the food industries like baking products, dry egg powders, beverages, and gluconic acid generation but is also used in pharmaceutical, chemical, textile, and other biotechnological industries (Dubey et al. 2017a). Glucose oxidase has been used effectively to produce bread with enhanced quality, improved loaf

volume and increased shelf life during storage in the baking industry. Studies have shown that the impacts of glucose oxidase alone or in combination with other enzymes on bread quality mostly depend on the quality of the original wheat flour and the amount of enzymes. Recently, the synergistic effects of α -amylase, ascorbic acid and glucose oxidase on the bread quality and wheat dough rheological behavior have been studied which revealed the reduction of bread crumb firmness and chewiness, and enhancement of adhesion, cohesion, elasticity, and volume of bread (Kriaa et al. 2016). It has also been demonstrated that glucose oxidase is significantly capable of improving the specific bread volume for all fiber types (Dubey et al. 2017b). Bonet et al. revealed that gluten and gluten network are significantly changed via the disulfide and non-disulfide crosslinks formation upon the excessive addition of glucose oxidase into the wheat dough, which in turn, results in the negative effects on the dough and bread properties (Bonet et al. 2006).

The potential of immobilization technology in developing the novel methods for utilization of glucose oxidase at large scale, have motivated researches to deploy immobilized enzymes. Nevertheless, problems such as reduced enzyme activity following immobilization and diffusional constraints have restricted the use of immobilized glucose oxidase. Recently, covalent immobilization of glucose oxidase has been shown on modified iron oxide nano-particles. The improved stability of the immobilized enzyme and the minor impact on the diffusion of the substrate and product have been confirmed by the results. However, the glucose oxidase immobilization on nanoparticles has several advantages such as non-chemical separation method, enzyme loading high capacity due to high surface area and acceleration of the rate of enzyme catalyzed reactions and thus cannot be used in baking industry considering the high rate of oxidization and the lack of flour reforming power (Park et al. 2011). The desirable enzyme to replace chemical oxidizing agents such as potassium bromide for improving the bread and dough properties is glucose oxidase which is a fast acting oxidant and reduces the dough elasticity immediately after mixing with it. It has been shown that glucose oxidase loses 25% of its activity in the first 5 min after mixing with the dough. The high rate of oxidization and poor stability of glucose oxidase result in the defective effects in flour (Wang, Zhu, and Zhou 2011). The enzyme was encapsulated in calcium alginate-chitosan microspheres to enhance its stability and catalytic rate in flour; immobilized enzyme in the semi-permeable support keeps the enzyme from denaturing and increases its activity in dough. The highest total activity of immobilized glucose oxidase and encapsulation efficiency was achieved at pH's close to isoelectric point (pI) of glucose oxidase. Storage stability of glucose oxidase was remarkably influenced by the immobilization; as 70.4% catalytic activity of immobilized enzyme was retained after two months in comparison with 7.5% of free enzyme. These results confirmed the considerable potential of encapsulated glucose oxidase as a flour improver (Wang, Zhu, and Zhou 2011). Potassium bromate (KBrO_3) is an oxidizing agent extensively applied in bread

making over long periods of time. It is usually active during the later fermentation and baking processes and improves the crumb structure, loaf volume and texture by oxidizing the free thiol groups. Since the application of $KBrO_3$ has been gradually controlled because of the potential hazard of bromate, fungal glucose oxidase has been considered as one of the most important alternatives for $KBrO_3$ for promoting the disulfide linkage formation in the gluten networks and improving the dough and bread qualities (Ooms and Delcour 2019; Tikhonov et al. 2019). On the other hand, complete replacement of $KBrO_3$ with glucose oxidase has been restricted by the fast oxidation of gluten and its adverse effect on gas retention ability. Hence, to overcome these limitations, immobilization of glucose oxidase was performed in chitosan-sodium tripolyphosphate. The immobilized enzyme showed a high activity recovery (85.1%) when combined with fungal a-amylase and prevented the quick glucose oxidase oxidation during the preparation of the dough. This strategy provided a promising platform for using glucose oxidase as a feasible alternative to $KBrO_3$ in baking industry (Tang et al. 2014).

Xylanase

The cleavage of β 1,4 bonds in main polysaccharide chain of xylan polymers is catalyzed by Xylanases. Xylan, the main constituent of hemicellulose, is a heterogenic polysaccharide consisting of β -D-xylose residues with side chains made up of carbohydrate substituents. These enzymes have been applied in dough making, brewing, baking, and animal feed compositions. Many xylanases have been purified and identified from such different microorganisms as *Streptomyces* sp., *Cochlibolus carbonum*, *Aureobasidium pullulans*, *Penicillium chrysogenum*, *Aspergillus awamori*, *Trichoderma reesei*, *Aspergillus kawachii*, *Aspergillus oryzae* and *Aspergillus tubingensis*. Among them, *Trichoderma* and *Aspergillus* are the most efficient producers of xylanases and are most often applied in industrial sectors. The potential application of xylanases in bakery is due to water content redistribution between pentosan phase and gluten phase of the bread, thereby significantly improving the loaf volume, texture, firmness and shelf life of the bread (Li, Chang, and Liu 2018; Terrone et al. 2018).

The loaf volume of bread is a basic criterion of quality, which can be increased up to 10% by xylanases. The xylanase effect on bread volume may be caused by hydrolyzing the water-insoluble pentosans (Ahmad et al. 2014). Furthermore, the potential application of xylanases in baking process is based on its ability to degrade and debranch xylan and cellulose. Although many attempts already made to immobilize xylanases on a wide range of supports to improve their properties for use on industrial scales, application of these immobilized enzymes in baking industry still needs to be studied. However, the application of most of them has been investigated in various industries (Soozanipour, Taheri-Kafrani, and Isfahani 2015; Kumar et al. 2014; Landarani-Isfahani et al. 2015; Mishra et al. 2017).

In one study, xylanase production was determined by the immobilized spores of *Trichoderma reesei* in nontoxic calcium alginate beads. Immobilization of the microbial cells in a suitable matrix offers increased enzyme productivity, stability and reusability of the cell mass. The production of xylanase was found to reach a maximum amount in a shorter time (48 h) using immobilized cells compared with 72 h in free cell cultivation. Furthermore, xylanase is produced continuously by the immobilized cells up to 10 runs. These characteristics make the immobilized cells on Ca-alginate efficiently applicable for large scale xylanase formation in food industries (Vaz, Moreira, and FerreiraFilho 2016).

Lipase

Lipases are a family of enzymes belonging to the glycerol ester hydrolases, which catalyze the long chain triglycerides hydrolysis and release free fatty acids, diacylglycerols, monoacylglycerols and glycerol (Guerrand 2017; Ray and Rosell 2017).

Although wheat flour is composed of only 2–2.8% lipids, these components greatly affect bread making. The wheat flour lipids are divided into the polar lipids located in the starch granules and the non-starchy lipids called nonpolar lipids. About a half of the lipids is allocated to the polar components and the polar to nonpolar lipid ratio significantly affects dough functionality, by interacting with gluten proteins and holding gas capacity in bread making, thereby enhancing the bread loaf volume and crumb structure (McCann et al. 2009). In baking industry, (phosphorous) lipases can be used as potential substitutes for emulsifiers through hydrolyzing the endogenous lipids in dough such as acylglycerol, phospholipid and galactolipid to surface active lipids. Furthermore, in synergy with other baking enzymes, lipases can prolong the shelf life of most bakery products and improve their softness and texture (Guerrand 2017).

Although publications and patents regarding lipase application in wheat-based bread making are rather scarce compared to those pertaining to other favorite enzymes (amylases and xylanases) in bread industry, they tend to increasingly describe the action mechanisms and functionality of lipases on bread quality with increased recent interest of baking industries in lipases (Gerits et al. 2014). Therefore, despite the presence of abundant explorations on improving the properties of lipases by their immobilization on various supports, there are no reports on the immobilized lipases application in bread industry to date. On the other hand, as these enzymes can be applied in various reaction systems with several applications, different points of views have to be considered concerning this enzyme immobilization (Manoel et al. 2015). Studies showed that the activity of different immobilized lipases is dependent on the binding procedure, support and enzyme structure (A Modenez et al. 2018). According to some studies, lipase immobilization on magnetic cellulose nanocrystals, alkyl silane coated magnetite nanoparticles and hydrophobilized zirconia nanoparticles increases the activity and stability of immobilized lipase

(Cao et al. 2016; Wang et al. 2012; Ortiz et al. 2019). Furthermore, the thermostability, long-term stability and storage life of lipases have been shown to improve by sol-gel immobilization. Tomin et al. have investigated the impacts of surface coverage and adjusting the properties for the sol-gel entrapment of lipase in 2011 and showed that low enzyme loading and thin layer sol-gel entrapment of the lipase significantly increase the activity yields (YA > 200%) for each individual substrate (Tomin et al. 2011).

Protease

Wheat proteins including globulins, albumins and gluten proteins are one of the most significant constituents of bread making industry, which impact the crumb structure and loaf volume. These proteins are broken down into amino acids by the proteases hydrolyzing activity and thus improve the bread quality by increasing the volume, softness and pore size of bread and dough (Ortolan and Steel 2017). Proteases can also contribute to the improvement of crust color and product flavor through the generation of new amino acids caused by the cleavage of peptide bonds (Putseys and Schooneveld-Bergmans 2019).

Proteases are the most useful industrial enzymes after amylases, which are produced from bacteria and fungi. Immobilization of these proteins onto appropriate supports plays a significant part in different fields of technology. Recent effort focused on the immobilization of different types of proteases on a wide range of nanomaterials, reported their stability studies and industrial applications wherein these immobilized enzymes are more efficient than their free counterparts, as they often show better operational stability in batch processes and continuous reactors (Husain 2018).

Application of enzyme immobilization technology in dairy industry

The application of indigenous enzymes (lactoperoxidase, catalase, acid phosphatase, proteinase, lipoprotein lipase, amylase, and xanthine oxidase) is well established in dairy technology; these enzymes play an important part in processing and milk quality for direct or indirect consumption (Campbell and Drake 2013). Proteases, lipases, lysozyme, catalase, glucose oxidase and β -galactosidase are customary exogenous enzymes in dairy processing, which are mostly used to modify the properties and flavors of milk products (Fox and Stepaniak 1993). One of the major uses of these enzymes is in the milk coagulation and cheese processing.

Milk is a complicated biological fluid with high content of soluble proteins (albumin, α -lactalbumin and β -lactoglobulin) and insoluble proteins (α_{S1} , α_{S2} , β , and κ casein), which is subjected to three main groups of biochemical reactions including lipolysis, glycolysis and proteolysis to make cheese. Phe₁₀₅-Met₁₀₆ bond hydrolysis in κ -casein is the primary proteolytic step in cheese making. The κ -casein molecules form small hydrophobic aggregates, which are held together through an amorphous network of calcium and

magnesium phosphate (Fox et al. 2015). In general, several enzymes are always involved in cheese making from milk coagulation to cheese processing and ripening. The two enzymes commonly used in cheese manufacture are rennet (proteinases), a milk coagulant in all cheeses, and lipases, traditionally used in Italian cheeses. Other enzymes with limited applications include lysozyme, which inhibits the growth of *Clostridium tyrobutyricum* as a substitute for nitrate in Dutch, Swiss and other types of cheeses. Catalase reduces hydrogen peroxide as a bactericidal agent, glucose oxidase, produces gluconic acid from glucose for direct acidification and β -galactosidase, which hydrolyzes lactose and accelerates the lactic acid fermentation and cheese ripening in the manufacture of cottage and cream cheese (Fox et al. 2017). In dairy industry, as in other food industries, due to the several advantages of the application of immobilized enzyme systems, many studies have been conducted using a wide variety of supports and bonding methods under different reaction conditions. Green and Crutchfield first attempted to use immobilized enzymes for milk coagulating in 1969 (Green and Crutchfield 1969). Next, the application of several enzymes and their immobilized forms in dairy industry will be appraised (Figure 6).

Rennet

Rennet contains enzymes, which coagulate milk and separate whey into solid curds to make cheese. Rennet is traditionally extracted from the inner mucosa of the fourth stomach of a calf. Today, it can be obtained from different resources including animal, plant and microbes or genetically engineered in various recombinant expression systems such as *Escherichia coli*, *Klaveromyces lactis* and *Aspergillus niger*. Microbial rennets (bacterial and fungal rennets) are produced by microorganisms such as *Rhizomucor miehei* and *Rhizomucor pusillus* (Jacob, Jaros, and Rohm 2011). Most (>90%) of the rennet used in cheese making is lost in the whey, causing an economic loss and creating problems for whey treatment. These problems have been resolved by the immobilization of rennet. Hence, this technology has made it possible to reuse the rennet in the continuous production of cheese. Green and Crutchfield reported the first immobilized rennet in 1969 (Green and Crutchfield 1969). In 1989, Anprung et al. reported that the most active immobilized rennin is attained by covalent bonding on river bed sand as a carrier, thus maintaining 82.6% of its original catalytic activity after four months (Anprung, Chuengsaengsatayaporn, and Thunpitayakul 1989). Because of the importance of rennet application in dairy industry, many researches have already been carried out on this enzyme and its immobilization; newer research explorations are discussed in the following section.

Entrapment in food grade alginate-pectin matrix was investigated as an effective method to immobilize rennet from *Mucor miehei* by Narwal et al. (2016). Alginate is a linear copolymer with homopolymeric blocks of (1-4)-linked β -D-mannuronic acid and α -L-glucuronic acid and pectin is a copolymer of α -(1-4)-linked galacturonic acid residues.

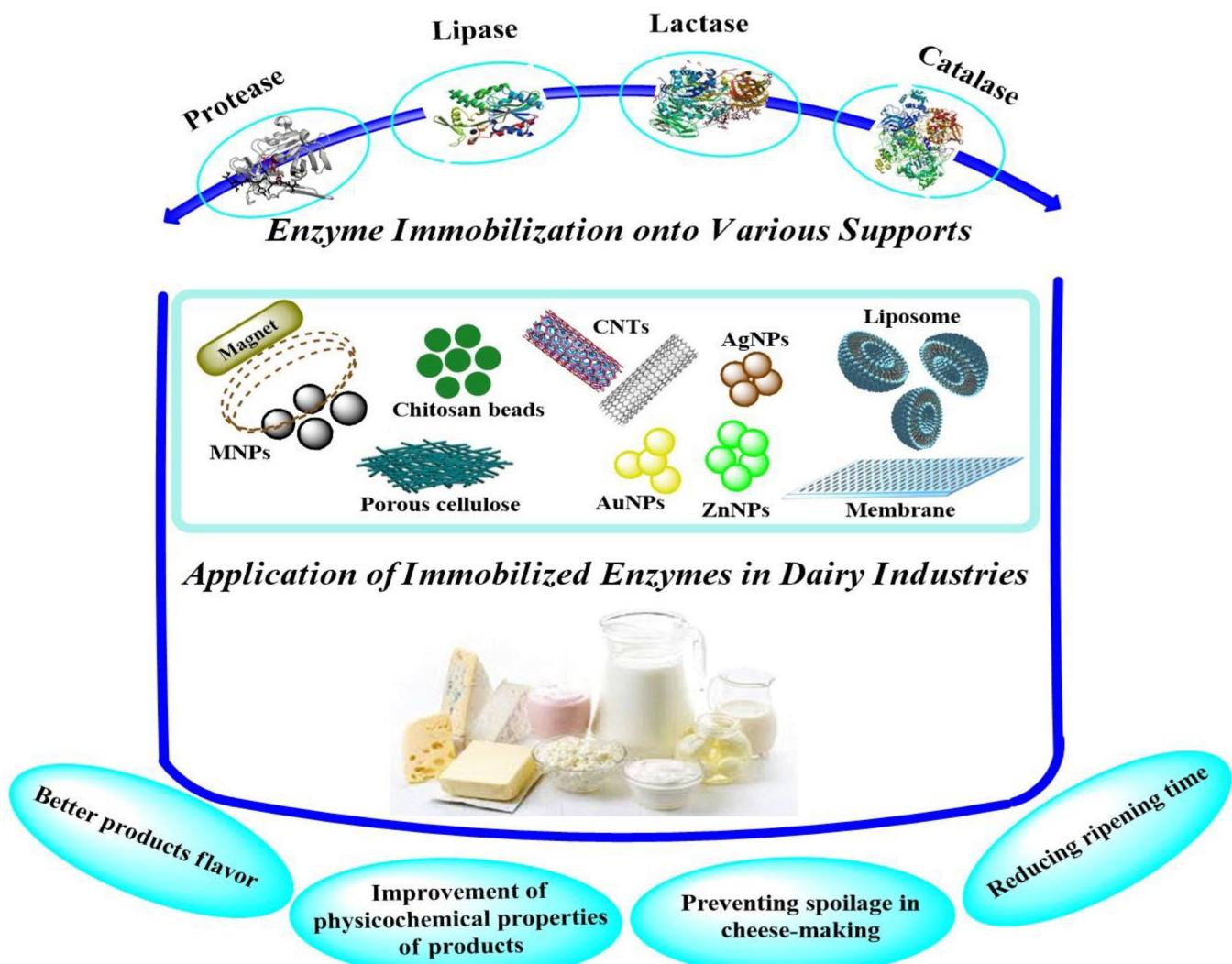


Figure 6. Application of enzyme immobilization technology in dairy industry.

Alginate-pectin mixed gels give hydrogels in the presence of such cations as calcium, copper and zinc and retain molecules whose molecular weight exceeds 5 kDa. In this study, rennet was gel-entrapped by drop wise addition of a mixture of the enzyme, sodium alginate and pectate into a CaCl_2 solution using differently sized needles; concentration of these components determines the cross-linking among sodium alginate, pectin and calcium. Therefore, rennet immobilization was standardized depending on the ratio of sodium alginate, pectin and concentration of CaCl_2 . Encapsulated rennet in interwoven matrix of this carbohydrate polymer preserves the enzyme from the severe effects of temperature and pH variations. Furthermore, the immobilized enzyme is highly stable, maintaining 40% of its catalytic activity after 10 cycles (Narwal et al. 2016).

In another study, rennin was immobilized into tubular cellulose/starch gel (TC/SG) composite by entrapment for Feta cheese making. The aim of this research was continuous coagulation of milk in cheese processing by a filter consisting of TC/SG, which contains the rennin enzyme. The results showed that the TC/SG is an interesting composite tube to create more active enzymatic systems, which have contributed to enhanced stability. In addition, the enzyme

removal from the product, reduction of the rennin enzyme expenses and reusability of the enzyme with retaining the continuous catalytic prowess of the enzyme for 7 days, make this immobilized rennin as promising technology for continuous cheese production. No significant differences were realized in the physicochemical properties compared with the cheese prepared using free rennin enzyme (Barouni et al. 2016).

Recently, carbon cloth was used as a support for rennet immobilization. Pieces of carbon cloth were oxidized using nitric and sulfuric acids to activate it and deployed to immobilize rennet enzyme onto surface carboxylic groups. These carboxylic groups were then reacted with *N*-(3-dimethylaminopropyl)-*N'*-ethylcarbodiimide and *N*-hydroxysulfo-succinimide; rennet was immobilized on chemically modified carbon cloth. The immobilization efficiency of rennet evaluated by calculating the milk-clotting unit (MCU) of the rennet prior to and following immobilization was 60.4%. The milk clotting activity of rennet solution slowly reduced throughout the whole storage period of 32 weeks, final activity being 60%. However, the activity of immobilized rennet was reduced by almost 20% in the primary storage stage (2 weeks) and retained at 70-80% level from 2 to 32 weeks.

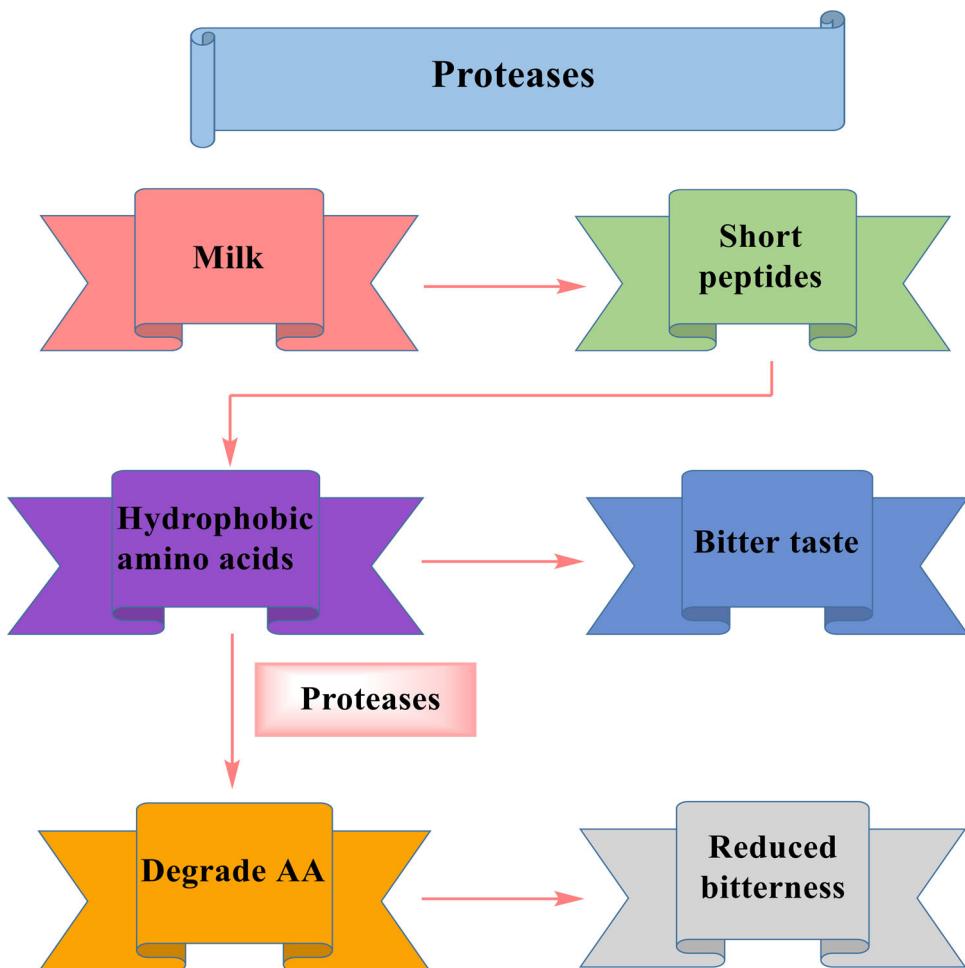


Figure 7. Role of proteases in dairy industries. Reprinted from (Abada 2019) with permission.

These results demonstrated that the rennet immobilized on carbon cloth was more stable than the free rennet over long periods of storage (Jeong, Yun, and Mun 2016).

Proteases

Considering the increased global demand for cheese and relatively high price of calf rennet, several studies have been conducted to find new sources of proteases for milk coagulation. Proteases are naturally produced in all organisms and are involved in many enzymatic industrial processes. These enzymes are used to accelerate cheese ripening, improve physicochemical properties of cheese types and modify milk proteins to decrease the allergenic characteristics of cow dairy products for infants. First, the milk protein is broken down into peptides by proteinases. Peptides are then broken down into amino acids and small peptides by peptidases. Ultimately, the transport system takes charge of the uptake of amino acids and small peptides (Figure 7) (Abada 2019). Generally, cheese production is the main use of proteases in the dairy industry. Although cysteine and serine proteases are able to coagulate milk under proper conditions, aspartic proteases are mostly utilized in milk coagulation (Shah, Mir, and Paray 2014). Aspartic proteases, also known as aspartyl proteases, are a group of proteolytic enzymes with two aspartic acid residues in the active site, which are critical for

their catalytic activity and are optimally active in acidic media. Eukaryotic aspartic proteases include pepsins, cathepsins, and renins. Aspartic proteases play an important role in milk clotting during cheese making because of their high activity and stability in acidic pH (Nair and Jayachandran 2019). *Mucor miehei* aspartic proteases have been used as commercial microbial rennet in several cases; *Mucor miehei* proteases were efficiently immobilized on polyacrylic support, which contained polar epoxy groups. The obtained results showed that the highest activity of *Mucor miehei* aspartic protease for both immobilized and free enzymes occurs at pH 7. Although the immobilized enzyme stability was low, the storage of enzyme at the predicted time for its immobilization (16 h at 4 °C) did not decrease its clotting activity (Esposito 2015). The application of liposomes in cheese production has been described by several studies. In this system, enzyme release in cheese ripening can be induced by surfactants from microcapsules such as liposomes and uniformly dispersed in the milk, subsequently preventing the brine environment in hard cheeses. Liposomes are being developed to reduce ripening time and prevent spoilage in cheese-making (Khanniri et al. 2016). Proteolysis evolution was compared in cheeses prepared using free or liposome encapsulated commercial protease and those supplemented with encapsulated enzymatic extract from *Lactobacillus helveticus*. A very fast proteolysis along

with a strong bitterness was observed in cheeses with added free enzymes, while a lower primary proteolysis in cheese with added *Lactobacillus helveticus* enzymatic extracts resulted in better cheese flavor (Jahadi and Khosravi-Darani 2017; Khattab et al. 2019). It has also been shown that most “plant rennets” are unsuitable for application in cheese making because of their strong proteolytic activities, which causes dairy products to taste extremely bitter. However, there is an exception in the leaves of *Cynara scolymus* and the flowers of *Carduus defloratus*, which contain different aspartic proteases. These enzymes were successfully used for cheese production in some countries like Spain and Portugal (Shah, Mir, and Paray 2014). Immobilization of the protease extracted from the flowers of *Carduus defloratus* was successfully carried out by using an epoxy support wherein higher stability was shown by immobilized enzyme compared with the commercial rennet with the same immobilization condition. Epoxy components are polymers containing epoxide groups which are suitable for multi-point covalent attachment with various nucleophilic groups on the surface of the enzyme to generate highly strong linkages with minimal modification of the enzyme (Barbosa et al. 2013). This polymer was also used to immobilize the plant proteases for application in milk coagulation, by Esposito et al. In this study, two different aspartic proteases were extracted from both *Cynara scolymus* leaves and *Carduus defloratus* flowers, the latter being covalently immobilized on a polyacrylic support, which contained epoxy groups; immobilization of *Carduus defloratus* aspartic protease exhibited higher stability than *Cynara scolymus* aspartic protease. The immobilization of *Carduus defloratus* aspartic protease was performed successfully at pH 7.0 with about 68% yield and the enzyme recovery of about 54%. The use of this immobilized enzyme is suggested to produce cheese and other processed dairy products because the optimum pH of this enzyme was similar to that usually used for milk clotting during cheese making (Esposito et al. 2016).

Lactase

Lactase (β -galactosidase) hydrolyzes β -(1-4) linkages in lactose, produces galactose and glucose and then improves the solubility and sweetness of various dairy products. Lactase can be obtained either in extracellular or intracellular form, from various sources including plants, microorganisms and animals. The application of β -galactosidase for hydrolyzing lactose in whey and milk is one of the favorable enzymatic uses in dairy industries such as lactose intolerance and whey treatment. Lactose transglycosylation to synthesize galactooligosaccharides (non-digestible oligosaccharides with prebiotic effects) can also be carried out using β -galactosidase. This enzyme can be used as both soluble form in batch systems and immobilized form in batch and continuous processing systems. Various methods have been applied to immobilize β -galactosidase in many investigations (Husain et al. 2011; Vasileva et al. 2016; Vieira et al. 2013; Souza, Garcia-Rojas, and Favaro-Trindade 2018; Zhang, Gao, and Gao 2010).

Lactose intolerance

Lactose is the major type of sugar in milk as well as other dairy products. Many lactose intolerant people, who have low levels of lactase for digesting lactose, show unpleasant symptoms after consuming milk and dairy products. Lactose-free milk production by directly adding the β -galactosidase enzyme to whole milk can help such people drink milk and eat dairy products. β -galactosidase is deactivated by heat treatment after hydrolysis of lactose at a desired level. Thus, the free enzyme cannot be recycled and the resulting operation is not economical. To overcome this disadvantage, immobilized β -galactosidase is applied for skim milk hydrolysis.

Physical adsorption mechanism has been used to compare *Aspergillus oryzae* β -galactosidase immobilization on native zinc oxide (ZnO) and zinc oxide nanoparticles (ZnO-NP); immobilization yield of the enzyme on ZnO and ZnO-NP were 60 and 85%, respectively. ZnO-NP immobilized β -galactosidase maintained 53% of its catalytic activity after 1 h incubation. The corresponding values for the native ZnO and soluble β -galactosidase were 35 and 28%, respectively, under identical conditions. In addition, native ZnO and ZnO-NP immobilized β -galactosidase retained 61 and 75% of their initial enzyme activity, respectively, after seven runs. Soluble ZnO and ZnO-NP immobilized β -galactosidase reportedly hydrolyzed 54, 63 and 71% of milk lactose in the batch process after 9 h while hydrolyzing 61, 68 and 81% of whey lactose under identical experimental conditions, respectively. Since ZnO-NP bound β -galactosidase is more stable against several chemical and physical denaturants compared with the soluble enzyme and native ZnO adsorbed β -galactosidase, the nanoparticle adsorbed enzyme may be applied for the hydrolysis of milk and whey lactose in batch and continuous systems (Husain et al. 2011).

Stabilization of multi-meric enzymes such as β -galactosidase, in which the dissociation of the subunits inactivates the enzyme, is difficult. Immobilization of such enzymes on a solid support with active groups, which allow the linkage to the enzyme support and preserve the multi-meric structure of enzyme, is an important strategy for the successful stabilization of multimeric enzymes and their industrial applications. In one study, a stable and active immobilized β -galactosidase from *Kluyveromyces fragilis* was prepared for hydrolysis of lactose in whole milk in a batch system. Chitosan-alginate beads, coagulated with KOH and activated with glutaraldehyde were used as supports for lactase immobilization. Ionic interactions between the chitosan amine groups and the alginate carboxylic groups yield the chitosan-alginate beads. Lactose immobilization on activated hybrid hydrogels (chitosan-alginate) significantly improves enzyme catalytic activity and thermostability. In addition, immobilized enzymes may be applied for a wide range of pH values. Epoxy-modified chitosan-alginate allows covalent bonding between the enzyme and the activated support by stable epoxy groups and physical adsorption by ionized groups (carboxylate from alginate and amino from chitosan). β -galactosidase immobilized via covalent bonding on chitosan, coagulated at 50 °C in 500 mM KOH and activated

with low glutaraldehyde concentration, gave the best immobilization yield and recoverability. Lactose was effectively hydrolyzed the immobilized biocatalyst in the whole milk under normal conditions (25°C , pH 6.75) with a conversion of over 95% within 3 h after four runs (Vieira et al. 2013).

In another study, Concanavalin A layered Celite 545 was applied as the immobilization support for *Aspergillus oryzae* β -galactosidase with 71% activity yield of cross-linked enzyme; immobilized β -galactosidase maintained 90% of its catalytic activity after 1 month of storage at 4°C and 71% after the seventh consecutive run. In addition, cross-linked β -galactosidase was more resistant to glucose and galactose mediated product inhibition (Ansari and Husain 2012b).

Ansari et al. reported that the silver nanoparticles (Ag NPs) functionalized with glutaraldehyde can be used as supports to immobilize *Aspergillus oryzae* β -galactosidase in high yield; they showed improved pH stability and increased temperature resistance in comparison with soluble and adsorbed β -galactosidase unmodified Ag NPs. The catalytic activity of surface modified Ag NPs enzyme for lactose hydrolysis in milk was retained even after the fourth successive reuse (Ansari et al. 2012).

Whey treatment

Whey, which is produced in large quantities and causes major environmental pollution, is the main by-product of the cheese or casein production processes. Lactose is the main component in whey, which causes serious problems in the treatment of wastewaters from cheese manufacturing industry by increasing the Biochemical (BOD) and Chemical Oxygen Demand (COD), due to its low solubility and biodegradability. As β -galactosidase operates with high selectivity under mild conditions of temperature and pH, lactose hydrolysis using β -galactosidase is preferred to convert the whey into more useful components. The extent of lactose hydrolysis for whey treatment is extensively improved by using immobilized forms.

The commercial *Kluyveromyces lactis* β -galactosidase enzyme immobilization into calcium alginate spheres and gelatin was evaluated using glutaraldehyde and concanavalin A (ConA) as modifying agents for hydrolyzing the lactose in cheese whey. The results showed that the enzyme encapsulation, complexed with ConA in alginate-gelatin spheres without glutaraldehyde in the immobilization support, greatly enhanced the lactose hydrolysis rate to 72% (Mörschbächer, Volpato, and Souza 2016). β -galactosidase was covalently immobilized onto a modified polypropylene membrane, using glutaraldehyde in Vasileva et al.'s study. They reported that the activity and stability of lactose hydrolysis by this immobilized β -galactosidase is 1.6 and 2 times, respectively, larger than that of the free enzyme. Glucose-galactose sirup was obtained from waste whey using immobilized β -galactosidase on polypropylene membrane and the yield of bioreactor for lactose hydrolysis was retained as 69.7% after 20 cycles (Vasileva et al. 2016).

Galactooligosaccharides synthesis

Galactooligosaccharides (GOSs) are widely recognized non-digestible oligosaccharides, which are resistant to gastrointestinal digestive enzymes, but are fermented selectively by beneficial bacteria (probiotics) in colon. In the presence of higher amounts of lactose, galactooligosaccharides can be produced by lactase via transferring galactosyl residues to lactose.

Lactulose is an industrially relevant GOS. Immobilized β -galactosidase was used by Song et al. (2013) in the synthesis of lactulose from the whey lactose in batch and continuous forms. This enzyme was pretreated with lactose before immobilization on silica gel; immobilized β -galactosidase maintained 52.9% of its catalytic activity after 10 recycles in lactulose synthesis. Lactulose was continuously synthesized at a 0.5 ml/min flow rate. Based on the results of this study, the inhibitory effects of galactose and glucose in transgalactosylation reaction are greater than that in lactose hydrolysis. Furthermore, the inhibitory effect of inhibitors on the immobilized enzyme was reduced by immobilization compared to those of the soluble enzyme (Song et al. 2013).

A packed-bed reactor filled with chitosan-immobilized β -galactosidase has also been used for the synthesis of galactooligosaccharides by continuous hydrolysis of lactose. Reusability of the enzyme, continuous production, facility of substrate handling, long term runs and industrial scale operations are some of the salient advantages of the mentioned strategy. Moreover, the low cost and easy accessibility of chitosan macroparticles as well as their pH and temperature ranges of operation activity make them suitable for galactooligosaccharides synthesis. In this study, the pH and temperature ranges were increased by lactase immobilization. Furthermore, the enzyme thermal stability was significantly enhanced by lactose. The hydrolysis of lactose was carried out at 2.6 mL min^{-1} rate at 37°C for both whey and lactose solution. Maximum GOS concentration obtained at a flow rate of 3.1 mL min^{-1} was 26 g L^{-1} and lactose hydrolysis activity was $186 \text{ g L}^{-1} \text{ h}^{-1}$. Steady-state operation of the reactor stability for continuous lactose hydrolysis was retained for 15 days (Klein et al. 2013).

Lipase

Lipases are commonly found in all animals, plants and microorganisms. Lipases act on the bonds of carboxylic esters and triglycerides are hydrolyzed into diglycerides, monoglycerides, fatty acids, and glycerol by them. Esterification, interesterification, and transesterification reactions in non-aqueous media are also catalyzed by these enzymes. This versatility makes lipases suitable for use in the food industry.

Lipases are mainly deployed in cheese ripening to develop lipolytic flavors in special cheeses. The traditional sources of lipases for improving the cheese flavor are animal tissues; especially pancreatic glands and pre-gastric tissues from kid, lamb and calf. Cheese production process from *Mucor miehei*, *Aspergillus niger*, *Aspergillus oryzae* and several others has been carried out using microbial lipase (Raveendran

et al. 2018). Lipases from *M. miehei*, and *A. niger* were used before the rennet to give Italian cheeses stronger flavors by a moderate lipolysis to increase the quantity of fatty acids. *Penicillium roqueforti* lipase produced peppery flavor in blue cheese by generation of short chain fatty acids and methyl ketones (Salum, Erbay, and Selli 2019). Milk fat hydrolysis of butter fat modification are other applications of lipase enzymes in dairy industry (Jooyandeh, Amarjeet, and Minhas 2009).

Since the activity of lipase is influenced by the inhibitory and stimulatory action of various milk constituents upon the enzyme, there are limited reports on the application of immobilized lipase in dairy industry. However, some basic works on the immobilization of lipase in dairy process are beginning to appear in the literatures.

The potential of immobilized lipase from *Chinook salmon* (a species of fish) for generating the flavor compounds in milk was investigated. Hydrophobic resin was used as a support and the immobilized lipase hydrolyzed milk lipids in a batch reactor. Large surface area, small particle size and large pores give resins high protein adsorption capacity. The immobilization of *Chinook salmon* on the resin was carried out by digestive lipase via hydrophobic interactions; immobilized lipase showed an activity of 85.7 U/g of resin with 45% of applied activity. This immobilized enzyme showed the highest specificity for butanoic and hexanoic acids (short chain fatty acids), the main flavor compounds in dairy products. In this study, before the rate of hydrolysis significantly decreased, eight cycles of lipase hydrolysis were achieved by the immobilized lipase (Kurtovic et al. 2016).

In another study, anhydrous and buffalo milk fat were hydrolyzed by lipases immobilized on *Thermomyces lanuginosus*; higher quantities of butanoic/hexanoic acids and other flavor compounds were prepared by both of these milk fats after lipolysis (Omar et al. 2016).

Catalase

Hydrogen peroxide is used as an efficient sterilizing chemical to treat raw milk instead of heat pasteurization. Catalase, used at the end of the milk processing to remove the remaining hydrogen peroxide, breaks down hydrogen peroxide to oxygen and water. It has been shown that this method has no significant effect on the properties and taste of milk.

Physical and chemical immobilization of catalase from *Aspergillus niger* was investigated by Akertek and Tarhan. Chemical immobilization of catalase was performed on porous SiO₂ modified with γ -aminopropyltriethoxysilane, followed by glutaraldehyde. In addition, this enzyme was physically entrapped in the alginate and γ -carrageenan gel. The immobilized catalase was applied in the decomposition of H₂O₂ in the cold pasteurization of milk in discontinuous batch reactors. The retained activity of chemically immobilized catalase was 20.17% while the corresponding value for the entrapped enzyme in alginate gel was 1.54%. Although the amount of physically immobilized catalase was higher than the other one, its retained activity was lower. This can be attributed to the interactions between catalase and

alginate and the problems involved in the diffusion of hydrogen peroxide into gel (Sooch, Kauldhara, and Puri 2014). Recently, some novel immobilization methods were applied to increase the operational activity of catalase. In one study, chitosan (CTS) and HEMA were used to prepare synthetic-natural copolymer in nanosize. Immobilized metal-chelate affinity chromatography (IMAC) material was produced by chelation of Cu(II) ions and used for the catalase immobilization. Catalase was successfully absorbed onto p(HEMA-CTS)-Cu nanospheres and the immobilized catalase exhibited higher activity with regard to the free catalase in acidic pH region and at low temperature. Not only comparatively high immobilization capacity using rather low amounts of IMAC nanospheres was an important merit of this immobilization strategy, the operational and storage stabilities of the immobilized catalase presented the evidence of being suitable for industrial applications (İnanan 2019).

Food enzymes and nanotechnology

Today, advances in enzyme immobilization have allowed using materials in micrometric dimensions and nanomaterials including nanofibers, nanospheres, nanopores, and nanotubes. Both micrometric and nanometric support materials are required to have a great surface area and functional groups as well as provide biocompatibility and water solubility (Ansari and Husain 2012a). In comparison to conventional supports, nanostructured materials have a larger ratio of surface area to volume, which leads to their higher immobilization (Misson, Zhang, and Jin 2015).

Recent advancements in nanotechnology have led to the introduction of several new options for enzyme immobilization in different nanoscaffolds (Husain 2010). Nanostructures are considered more suitable alternatives compared to conventional matrices, because of their lower mass transfer rate, higher diffusivity, and smaller surface area. Nanobiocatalysts are generated by the integration of nanomaterials with enzymes as biocatalysts and these catalysts are preferred over chemical catalysts because of the greener technique employed in their synthesis (Agustian, Kamaruddin, and Aboul-Enein 2016; Meyer et al. 2013). These immobilizations have been investigated in nano-sized fibers, spheres, tubes, and similar structures. To date, a large number of immobilization processes each with unique features have been investigated; e.g., mesoporous materials, self-assembled monolayers, polymeric matrices, gold nanoparticles, magnetic and nonmagnetic nanoparticles, and thermally evaporated fatty lipid films (Arsalan and Younus 2018; Zdarta et al. 2018). Several enzymes have been immobilized on various nanosystems and many immobilizations have been performed. For example, α -amylase has been immobilized on carboxylated magnetic, aminated magnetic, and cellulose-coated magnetic nanoparticles (Khan, Husain, and Azam 2012); lipase immobilized on magnetic Fe₃O₄-chitosan shows a marked ability in the synthesis of ascorbyl palmitate using palmitic and ascorbic acids. Conversion of 52% was attained after a 12 h reaction and the enzyme illustrated increased recyclability, facile recovery, and enhanced

thermal stability. These developments make immobilized lipase potentially applicable in industry (Wang et al. 2015). One of the other systems involving polydopamine-coated magnetic-chitin, enhanced the optimal temperature and pH for starch hydrolysis to provide multiple interactions between surface and enzyme. Dopamine was self-polymerized on the magnetic-chitin surface to immobilize biomolecules (Sureshkumar and Lee 2011). Other methods for immobilization of enzymes at the nanoscale level and their applications (Table 1) are discussed in the next section.

Magnetic nanoparticles

Nanoparticles are small particles with sizes of <100 nm in all three dimensions with interesting properties, which are very different from their bulk state. Cobalt, nickel, iron, and their chemical compounds are among the most important magnetic nanoparticles. A major issue in using these materials in food applications is their toxicity and safety. Accordingly, in food systems, it is preferred to use iron oxides such as superparamagnetic Fe_3O_4 , which have a high biocompatibility and are generally free from toxicity (Cao et al. 2012). Additionally, iron oxide magnetic nanoparticles have garnered tremendous interest due to ease of their preparation, good capacity for transfer of charge, desired physical properties of unique size and shape, capability of surface modification, high loading capacity, and strong magnetic characteristics which facilitates their recovery from the reaction mixture. However, the surface of nanoparticles need to be modified by techniques using silica, amino compounds, composites, carbohydrates, and polymer functionalization to protect against oxidation, to improve stability under acidic conditions, and prevent agglomeration caused by magnetic dipole moments between the nanoparticles and high chemical reactivity (Roto 2018; Khoshnevisan et al. 2019; Muley, Mulchandani, and Singhal 2020). Another relevant example is the study which described a tri-enzyme mixture of cellulase, pectinase and xylanase co-immobilized on magnetic nanoparticles by cross linking with glutaraldehyde; ensuing tri-enzyme co-immobilized magnetic complex was catalytically active up to fourth reusability cycle with more than 85% activity recovery of each co-immobilized enzyme (Muley et al. 2018). A shift in optimum temperature from 55 to 60 °C with improved pH tolerance and enhanced thermodynamic parameters was observed in this study; co-immobilized enzymes were successfully used for the papaya juice clarification. More recently, a novel heterofunctional support, divinyl sulfone (DVS) superparamagnetic nanoparticles (SPMNPs) functionalized with polyethyleneimine (PEI), was used to immobilize a lipase from *Thermomyces lanuginosus* by multipoint covalent attachment. The nanobiocatalyst characterization showed that the SPMNPs@PEI is a good immobilization support capable of condensing with the enzyme's negative charges. Improved enzyme properties, enhanced operational stability, and easy and rapid recovery of the immobilized lipase by applying an external magnetic field, bodes well for its industrial applications (Bezerra et al. 2020).

Nanofibers

Fibers are nanomaterials, which have achieved increased significance due to their unique properties. Electrospun nanofibers, which are long, have a uniform diameter and diverse compositions, are preferable and simple for application in biocatalysts (Xue et al. 2019). Nanofibers prepared by electrospinning exhibit a high surface area, porosity and inter-connectivity, giving them remarkable capability of operating for various applications from drug delivery to water filtration systems (Huang et al. 2016). Nanofiber-enzyme composites show modified activity three times more than those of native enzymes (Hwang and Gu 2013). Electrospun nanofibers function in enzyme immobilization by different methods. One technique consists of layer by layer (LBL) deposition of materials, which supply powerful non-covalent integration and a suitable set of oppositely charged molecules to give nanoscale structures with significant functionalities (Xiang, Lu, and Jiang 2012). Recently, Huang et al. (2017) removed the naringin and limonin components of grapefruit juices by naringinase-immobilized cellulose acetate nanofibers using layer by layer self-assembly process; amount of naringinase could be tuned by altering the number of nanofibers layers. This system removed 22.72% of naringin and 60.71% of limonin from grape juices by naringinase hydrolysis and adsorption on cellulose acetate nanofibers, respectively (Huang et al. 2017). To develop an easy, efficient, and economically viable immobilization procedure for *Aspergillus oryzae* β -galactosidase on electrospun gelatin nanofiber, two simple methods were applied for the immobilization by Sass and Jördening (2020). First, β -galactosidase was covalently immobilized on activated gelatin nanofiber mats with a hexamethylenediamine bifunctional linker. Second, the immobilization of β -galactosidase was performed through entrapment into the support during the electrospinning process. Both immobilized enzymes showed an excellent long-term operational stability. However, the optimum conditions of immobilized β -galactosidase shifted from pH 4.5, 30 °C to pH 3.5, 50 °C for covalently immobilized enzyme and pH 3.5, 40 °C for entrapped enzyme. The results also indicated that the maximum velocity (V_{max}) of entrapped enzyme was about 20 times higher than that of the covalent one, while the calculated Michaelis constant (K_m) was about 10 and 7.8 mmol/L for entrapped enzyme and covalently immobilized enzyme, respectively (Sass and Jördening 2020).

Nanosheets

Increased applications of carbon family materials have led to several approaches for producing carbon-based functional nanomaterials. Graphene is a polymorph of carbon utilized in immobilization of enzyme because of its interesting electronic, thermal, mechanical, and optical properties. Graphene consists of a dense layer of carbon atoms, which make a 2D dense structure resembling a honeycomb (Novoselov et al. 2004). Graphene has several applications in drug/gene delivery because of forming a perfect matrix for immobilization, which maintains enzyme activity. In a study, graphite oxide thermal exfoliation was used to prepare functionalized graphene utilized for immobilization of

Table 1. Immobilized enzymes in food industry.

Enzyme	Support	Immobilization method	Application in food industry	Ref.
Pectinase	PVA gel	Entrapment	Turbidity reduction of apple and pomegranate juices	(Cerretti et al. 2017)
	Dextran-PEI modified acrylate polymer	Covalent	Red apple juice clarification	(Rajdeo et al. 2016)
	Dextran-chitosan MNPs	Covalent	Apple juice clarification	(Sojitra, Nadar, and Rathod 2017)
	Chloropropyl-silica-PVP-MNP	Covalent	Viscosity reduction of apple juice	(Mosafa, Shahedi, and Moghadam 2014)
	Celite	Physical adsorption	Pineapple juice clarification	(Chauhan et al. 2015)
	Sodium alginate beads	Entrapment	Clarification of apple and umbu juices in packed bed reactor	(de Oliveira et al. 2018)
	Glutaraldehyde-calcium alginate beads	Covalent	Cashew apple juice clarification	(de Carvalho Silva et al. 2019)
	Sodium alginate nanoparticle	Nano-capsulation	Viscosity reduction and turbidity elimination of orange juice	(Mahmoud, Abo-Elmagd, and Housseiny 2018)
	Calcium alginate hydrogel	Entrapment	Color improvement of grape musts in wine industry	(Martín et al. 2019b)
	PVA cryogel	Entrapment	Debittering of citrus juice	(Busto et al. 2007)
Naringinase	Cellulose acetate nanofiber	LBL self-assembly	Increasing of grapefruit juice sweetness	(Huang et al. 2017)
	Silylated mesoporous MCM-glutaraldehyde	Covalent	Debittering of white grapefruit juice	(Lei et al. 2011)
Laccase	Graphene oxide	Covalent	Production of citrus flavonoids prunin and naringenin from grapefruit wastes	(Carceller et al. 2019)
	Chitosan and sodium alginate nanoparticles	Nano-encapsulation	Citrus juice debittering	(Housseiny and Aboelmagd 2019)
	Green coconut fiber-glyoxyl PVA cryogel	Covalent	Apple juice clarification	(de Souza Bezerra et al. 2015)
	Poly methacrylate beads-epoxy	Covalent	Oxidation of apple juice phenolic compounds	(Stanescu et al. 2012)
	Silica based matrix	Physical adsorption	Phenolic compound reduction and juice clarification in pomegranate, cherry, peach, apricot and orange juices	(Lettera et al. 2016)
Xylanase	CC-APTES-SiO ₂ @MNPs	Covalent	Phenolic removal in must and wine	(Brenna and Bianchi 1994)
Mannanase	Aluminum oxide pellets-glutaraldehyde	Covalent	Clarification of orange, pineapple, and apple juices	(Shahrestani et al. 2016)
	Chitosan@MNPs-glutaraldehyde	Covalent	Enrichment of orange and grapefruit juices	(Kumar et al. 2014)
Peroxidase	Gelatin hydrogel-glutaraldehyde	Encapsulation	Clarification of peach, orange, pomegranate, grape, kiwi and apple juices	(Nadaroglu and Sonmez 2016)
β-glucanase	Duolite S-761	Physical adsorption	Quality improvement of apple and orange juices by turbidity and phenolics reduction	(Bilal et al. 2016)
Endoprotease	Chemically activated surface of κ-carrageenan	Covalent	Viscosity reduction of barley wort and improvement of filterability in packed bed reactor	(Linko and Linko 1979)
	Amino-functionalized methacrylate carrier	Covalent	Reduction of gluten (gliadin) content from wort in beer	(Hiseni, Galaev, and Edens 2019)
Tyrosinase	PEDOT/Sonogel-carbon electrode	Electrodeposition layer	Development a biosensor for the determination of polyphenol index in beers and wines	(García-Guzmán et al. 2019)
Glucoamylase	—	CLEA	Hydrolysis of starch into soluble sugars	(Hassan, Yang, and Xiao 2019)
Fungal β-glucosidases	—	—	Enhancing the flavor of wine by aroma compounds releasing	(Ahumada et al. 2016)
Amylase	Acrylic bead-epoxy (Eupergit C)	Covalent	Aroma enhancement in wines	(González-Pombo et al. 2014)
	Gelatin and alginate micro-beads	Adsorption	Extension the shelf life of refrigerated dough	(Gugerli et al. 2004)
	Gelatin	Covalent	Detergent additive	(Jaiswal and Prakash 2011)
	Beeswax	Micro-encapsulation	(Haghigat-Kharazi et al. 2018)	

(continued)

Table 1. Continued.

Enzyme	Support	Immobilization method	Application in food industry	Ref.
Trypsin	SiO ₂ @MNPs-glutaraldehyde	Covalent	Retarding gluten-free bread staling and improvement of bread quality Detection of milk allergens in baked food	(Qi et al. 2019)
Glucose oxidase	Chitosan-TPP microparticles	Physical adsorption	Improvement of breadmaking quality	(Tang et al. 2014)
Rennin	Nano-porous cellulose-starch gel composite Alginate-pectate interwoven gel	Encapsulation	Continuous coagulation of milk in cheese production Enhancement of catalytic properties of enzyme in cheese making	(Barouni et al. 2018) (Narwal et al. 2016)
	Tubular cellulose-starch gel composite	Entrapment	Feta cheese making	(Barouni et al. 2016)
	Aminated magnetic particles-glutaraldehyde	Covalent	Continuous milk coagulation process to produce soft cheese	(Liburdi et al. 2018)
β -galactosidase	Zinc oxide nanoparticle	Adsorption	Enhancement of lactose hydrolyzes in milk and whey	(Husain et al. 2011)
	Chitosan beads Agarose-glyoxyl beads Epoxy-chitosan-alginate composite AgNP-glutaraldehyde	Encapsulation Covalent Covalent Covalent	Lactose hydrolyzes in whole milk Production of lactose free dairy products	(Vieira et al. 2013) (Ansari et al. 2012)
	Polypropylene membrane-glutaraldehyde	Covalent	Production of glucose-galactose sirup from waste whey	(Vasileva et al. 2016)
Lipase	Hydrophobic resin beads	Physical adsorption	Generation of flavor compound in milk	(Kurtovic et al. 2016)
	MNPs-MWCNTs	Physical adsorption	Synthesis of 1,3-dioleoyl-2-palmitoylglycerol-rich human milk fat substitutes	(Zheng et al. 2017)
Pectinase/cellulose complex	APTES@MNPs-glutaraldehyde	Covalent co-immobilization	Improvement of lycopene extraction of tomato peels	(Ladole et al. 2018)
Pectinase/cellulose complex	Glutaraldehyde-activated magnetic particles	CLEA co-immobilization	Grape juice clarification	(Dal Magro et al. 2018)
Pectinase/glucoamylase complex	Sodium alginate/graphene oxide composite beads	Covalent co-immobilization	Quality improvement of the pumpkin-hawthorn juice	(Yang, Dai, et al. 2019)
Pectinase/ α -amylase/cellulose complex	APTES@MNPs-glutaraldehyde	Covalent co-immobilization using glutaraldehyde	Clarification of apple, grape, and pine apple juices	(Sojitra, Nadar, and Rathod 2016)
β -mannanase/endo-xylanase/ α -galactosidase/ β -xylosidase/ β -glucosidase complex	Aluminum oxide pellets	Covalent co-immobilization	Clarification of apple, kiwi, orange and peach juices and enhanced their reducing sugar content	(Suryawanshi et al. 2019)

Abbreviations: PVA: polyvinyl alcohol; PEI: polyethyleneimine; PVP: polyvinylpyrrolidone; APTES: 3-aminopropyltriethoxysilane; LBL: layer-by-layer; CC: Cyanuric chloride; PEDOT: poly(3,4-ethylenedioxythiophene); TPP: tripolyphosphate; MWCNT: multi-walled carbon nanotube.

α -galactosidase extracted from the white chickpea (*Cicer arietinum*). The graphite oxide thus formed was heated again quickly under an Ar atmosphere. In the next step, it was further heated up to 1050 °C, followed by cooling to ambient temperature. Immobilization of the enzyme was performed by glutaraldehyde and cysteamine cross-linking agents wherein glutaraldehyde, which contains the -CHO group, binds to the NH₂ group from cysteamine. In addition, the other -CHO group binds to the enzyme via the lysine residue amino group. According to the results, the immobilized enzyme was more thermally stable than the soluble enzyme. Moreover, it was found that this enzyme was rather stable as it maintained 60% of its catalytic activity after 10 consecutive cycles (Singh et al. 2014).

Nanotubes

Carbon nanotubes (CNTs) are among the important nanomaterials used to immobilize enzymes. These nanomaterials

have received much attention because of their large specific surface as well as outstanding mechanical, electrical, structural, and chemical traits. Single and multi-walled carbon nanotubes (SWCNTs and MWCTs, respectively) are the two most important CNT types (Homaei and Samari 2017). Recently, enzyme immobilization on CNTs has been used for enhancing the enzymes stability and activity, particularly when exposed to denaturing environments. CNTs also have a large surface area, high enzyme loading, and low mass transfer resistance (Saifuddin, Raziah, and Junizah 2012; Silva et al. 2014). Enzyme binding to nanotubes can be performed using both non-covalent and covalent approaches. Among these techniques, the non-covalent method maintains the structure of the native enzymes such as pectate lyase obtained from a psychrophile and supplemented with calcium hydroxyapatite nanoparticles (NP-PL) (Calvaresi and Zerbetto 2013). These particles, in turn, can be used in cationic activation as a calcium source. In this study, further entrapment of the enzyme was carried out in SWNTs.

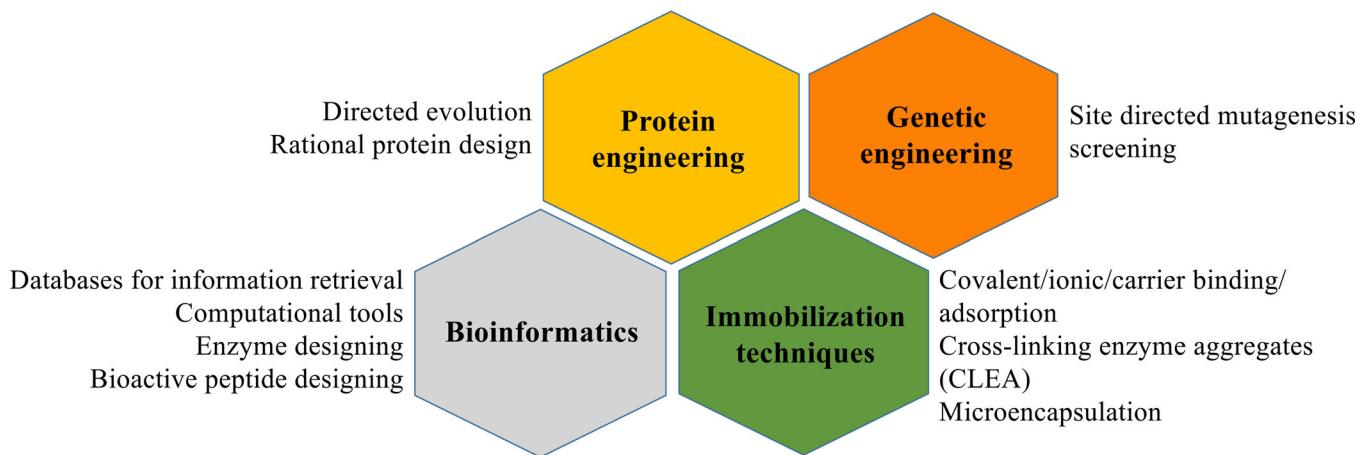


Figure 8. Summary of the methods used in enzyme technology and future dimensions. Reprinted from (Rastogi and Bhatia 2019) with permission.

Hence, immobilized pectate lyase demonstrated increased stability and activity at both 80 and 4°C. The maintained activity of this enzyme lasted even after performing several freeze-thaw cycles. Given the strong potential of these SWNTs in activating and stabilizing cold active enzymes at temperatures over their optimum, they can be considered proper candidates for processes at both low and high temperature bioconversions. Challenges for use of CNTs, caused by enzyme leaching (physical adsorption) and perturbation (covalent attachment), have been resolved by special metal-organic frameworks, namely Zeolitic imidazolate frameworks (ZIFs). Costa et al. (2019) developed an effective approach to anchor large-substrate enzymes on the surface of CNTs using ZIFs in one-pot synthesis; system was illustrated by two T4 lysozyme and amylase enzymes. Both immobilized enzymes displayed enhanced stability, reduced leaching, and retained the catalytic activity under neutral and acidic conditions; leaching problem of physically adsorbing enzymes to CNTs was solved by this effort. Also, the orientation of enzymes on both CNTs and CNT-ZIF supports was characterized to understand the enzymes behavior on these matrices. The structural investigations exhibited enhanced enzyme exposure to the solvent compared to the enzymes in the ZIF crystal alone, indicating that the encapsulating enzymes on CNTs via ZIF is an effective means to immobilize enzymes on crystal surfaces to improve large-substrate contact thereby enhancing catalytic efficiency (Costa et al. 2019).

Despite numerous nanotechnology-based applications of the products in enzyme immobilization, risk assessment studies and further research is warranted to critically evaluate the adverse effect of nanoparticles on human health.

Challenges and prospects

Enzymes are natural catalysts used to change the way foods are processed. Enzymes have been applied in several food industries like juice and drinks, meat, brewing, dietary products and supplements, fats, vegetable processing, and oils (Robinson 2015). The favorable features of enzymes such as fast reaction rate, thermostability, the capability of functioning over wide pH and pressure ranges, a large number of substrate utilization, nonmetal ion dependency, and the ability to

function when exposed to common inhibitory molecules are among the main advantages of these biological catalysts. Such single or integrated techniques as screening, protein engineering, and rDNA technology are employed to harness the properties of enzymes (Figure 8). The immobilization ability of enzymes has made them more economically justified and as a result, they can be reused with low or even no activity loss (Rastogi and Bhatia 2019). With the progress made in genetic engineering strategies, several gene-encoding enzymes can be possibly transferred to and expressed in the desired host microbes for mass production. Nowadays, gene technology is widely used for producing several industrial enzymes through the discovery of novel enzyme and optimization of the present proteins. Using enzyme subsidies has several advantages in the food industry. For example, they can improve the quality and preserve the product, lower the dependence on raw materials for processing, serve as alternatives for chemical food additives, and prevent dangerous potential by-products (Li, Yang, et al. 2012).

Numerous benefits have been reported for the immobilization of enzymes. For instance, it enhances operational, thermal, and storage stability of enzymes functioning under variable and harsh environmental conditions. Moreover, it can be used for regulating the extension of the reaction. Another feature is the simplification of the downstream application of product purification and its removal from the biocatalyst. Through the enzymes immobilization, it is possible to prevent enzyme denaturation in the autolysis process and load it at higher amounts. Using immobilized enzymes facilitates the bioreactor operation automated. Moreover, depending on the applied instruments, it can be a continuous or batch mode operation on a drain-and-fill basis. However, this can be observed when the enzyme immobilization encapsulates the enzyme with no activity loss as immobilized biocatalysts can be readily removed and recycled. As a result, their productivity is enhanced per enzyme unit and substrate inhibition is minimized (Bilal, Zhao, et al. 2019; Bilal, Asgher, et al. 2019; Zhang, Ge, and Liu 2015). Some common applications of immobilized enzyme reactors in the food industry are as follows: (1) isomaltulose synthase for the producing isomaltulose; (2) biocatalysts filled with immobilized glucose isomerase used for

preparation of high-fructose corn syrup; (3) invertase for producing inverted sugar sirup and aminoacylase needed for generating amino acids; (4) lactase production of tagatose and whey hydrolysates; (5) lipases for the edible oils interesterification and producing cocoa butter equivalents and trans-free oils and modification of triacylglycerols; and (6) β -fructofuranosidase for producing fructooligosaccharides (Bilal and Iqbal 2019).

So far, there are no efficiently accessible approaches for optimizing the juice production process that is of palatable quality without affecting the natural properties. It appears that the attention to green technology would make consumers more willing to use these food products. The progress in enzyme technology could open new pathways to obtain higher yield and productivity over the use of chemical materials while providing mild and safe operational conditions and generating more added value to the final productions. With potential advantages of enzymes, it is better to separate biocatalysts for recycling and ensure enhanced food safety.

In the past decades, significant attempts have been made in the immobilization of enzymes while the recent studies show that immobilized enzymes could be largely applied in various industries including food industry. The treatment of fruit juices with immobilized enzymes has many benefits on industrial scale, which can provide more precise control of the process. Until now, several researchers have studied enzyme immobilization on diverse supports for juice clarification. Notwithstanding the successful application of the commercial soluble enzymes, most data regarding the application of immobilized enzymes is limited to laboratory experiences. Therefore, it is difficult to choose a suitable matrix and immobilization method for designing an efficient reactor with good yield and reasonable cost. In addition to the economic issues, there are some legislative scientific-technological problems as well. For example, the problems associated with kind of fruit, which can shift the optimum pH of the enzymes, or even presence of adsorbed high molecular weight compounds on the immobilized enzyme increase the viscosity of the juice and also occlude the pores especially in the porous supports. Thus, it is important to find more advanced non-porous catalysts in this case.

Generally, challenges exist to further control the immobilization techniques due to the lack of long-term operational stability, surrounding environment and technical bottlenecks in terms of recyclability. Therefore, despite the advantages of enzymes, the development of simple and more efficient methods for their immobilization with higher stability and reusability aspects is essential in continuous processes and their application in food industries. Thus, future research should focus on addressing the problems pertaining to the industrial scale application of enzymes.

Although the integration of enzymes is generally considered an accepted technique in the food and feed sectors, it is clearly evident that researchers are making consistent efforts to apply biological agents via a variety of pathways more effectively. These efforts have led to novel techniques and activities to design novel biocatalysts encompassing useful advantages, including more stability (against pH and

temperature), less dependency on metal ions, and less susceptibility to inhibitory agents and to aggressive environmental conditions. This is particularly significant in food and feed processes, wherein the performance is enhanced under operational conditions, and the risk of microbial contamination is minimized. Furthermore, the concerted application of enzymes having different requirements for efficient use results in facilitating the process integration. The aforementioned advances can be attained by ever-continuing progresses in molecular biology, the accumulated evolutionary enzyme engineering expertise, the (bio)computational instruments, and by conducting high-throughput approaches, with high parallelization level in which the effective and timely screening of the biocatalysts are possible. Furthermore, to render these proteins appropriate for industrial targets, enzymes immobilization can be implemented in which their catalytic characteristics can be simultaneously improved. Nevertheless, although significant advances have been made in this specific field, the shortage of a set of unanimously appropriate rules of selecting the carrier and approach of enzyme immobilization is evident. To overcome this setback, both economic and technical requirements are essential. The economic requirements can be especially limiting in the food and feed sector due to the low added value of the majority of products. Hence, no particular approach can be adopted for enzyme immobilization as applied in food and feed. On the other hand, the immobilized biocatalyst appropriate for a particular product and process can be entirely inappropriate for another. Since the applications and enzyme's nature are diverse, the mentioned pattern is unlikely to be reversed. Therefore, it is expected that immobilized biocatalyst with appropriate physical, chemical, and geometric properties need to be developed. It is feasible in several reactor configurations under a mild condition, which is in accordance with the economic necessities for large-scale use. The mentioned approaches, either isolated or preferably appropriately integrated, have been implemented to modify the available processes or to conduct new ones. The latter is mostly performed by combining the output of new goods, obtained by novel enzymatic activities. The trend is expected to continue owing to the recent advances in this field.

It can be foreseen that processes engaging multienzyme systems will have a substantial prospect, when combined with cofactor regeneration in the production of high-value products. The productivity may be increased by integrating the bioprocess with the downstream processing using the immobilized enzyme methodology while the product recovery cost is minimized. In nonaqueous enzymology, the immobilized enzyme method is an effective approach, particularly to develop continuous bioreactors, a promising option in the immobilized enzymes field. It is expected that various practices will be replaced by immobilized systems as they will have technical and commercial feasibility.

Authors contributions

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