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Recent Innovative Techniques for Enzyme Immobilization

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Abstract:
Enzyme immobilization, a critical strategy in biocatalysis, offers substantial improvements over soluble enzyme systems, particularly in industrial and dairy biotechnology. This review articulates the core concepts of enzyme immobilization, elucidating the benefits of augmented stability, enhanced reusability, and improved operational efficiency. A systematic analysis of diverse immobilization techniques, encompassing adsorption, entrapment, encapsulation, covalent conjugation, and cross-linking, is presented, with a focus on their respective merits and limitations. The application of immobilized enzymes in dairy processes, such as lactose hydrolysis and cheese maturation, and in broader industrial contexts, including biofuel synthesis and wastewater remediation, is critically examined. Furthermore, this review explores contemporary approaches to enhance enzyme stability and reusability, including the utilization of nanomaterials, biomimetic matrices, and genetic modification. These advancements aim to mitigate challenges associated with enzyme leaching, thermal denaturation, and economic viability, thereby facilitating the development of sustainable and cost-effective bioprocesses.

Keywords: Enzyme immobilization, Recent enzyme immobilization, Dairy products

Introduction:

Enzymes that are physically restricted to certain solid supports and may be utilized again and continuously while retaining their catalytic capabilities are known as immobilized enzymes. [4]
The majority of chemical reactions in living things are carried out by enzymes, which act as catalysts. [1] Biomolecules that the cell produces to trigger certain biochemical responses in moderate environments. Specific substrates are affected by enzymes or biocatalysts, which ultimately transform or converts substrates into products. [3]
There are several benefits of using immobilized enzymes in biotechnology, including Initially, batch of enzymes could be used repeatedly or in many batches. Compared to mobile enzymes, immobilized enzymes are typically more stable. the enzyme might be extracted from the reaction solution to quickly control the reaction. Another benefit is that the enzyme may be easily separated from the product, preventing contamination. Additionally, a multi-enzyme reaction system can be developed through the use of immobilized enzyme. [4]
Rennet, a general term for commercial preparations containing acid proteases isolated from animal tissues, is the most well-known dairy enzyme preparation.

The milk products and their enzymes were reported by the study:

Acid proteinases:
coagulation of milk, Neutral peptidases and proteinases: accelerated ripening, debittering, enzyme-modified cheese, creation of milk-based hypoallergenic meals, Lipases: accelerated ripening of cheese, cheese that has been enzyme- or flavor-modified, structurally altered milk fat products, Whey products that have been decreased in lactose by β-galactosidase Lactoperoxidase: Cold-sterilizing milk, calves' milk substitutes, Lysozyme: A nitrate substitute for washed-curd and eye-shaped cheeses (like Emmental), Transglutaminase has been demonstrated to enhance the emulsifying qualities of milk proteins and raise the yoghurt's viscosity and water-holding capacity. Lipases are employed in the technology of cheese flavor. LMF, or lipolyzed milk fat, the short to medium chain fatty acids and fatty acid chemical derivatives that lipases release from milk fat give LMF its creamy, buttery, and cheesy scent. [11],[13]
Aldolase, Lysozyme, a-amylase, Peroxidase, amylase, Phosphatase (acid), Carbonic anhydrase, Phosphatase (alkaline), Catalase, Protease (quite specific), Cytochrome C reductase, Rhodanese, Diaphorase, Ribonuclease, Esterase, Salolase (arylesterase), Lactase b (galactosidase), Xanthine oxidase, and Lipase were among the 19 enzymes identified in the milk, according to the study.[12],[15]
Enzyme immobilization techniques include adsorption, covalent binding, cross-linking, microencapsulation, and entrapment are used to immobilize all enzymes.

Immobilization methods in milk enzyme immobilization :
Frequently employed immobilization methods for milk enzyme immobilization include:

Adsorption:
It is a straightforward carrier-bound method for achieving reversible immobilization. Physical adsorption is the primary premise of this approach. Alumina, activated carbon, ion-exchange resins, and many other materials are used to facilitate adsorption. 33 This approach has a weak binding force (i.e., ionic bonds, hydrophobic bonds, salt linkage, hydrogen bonds, etc.) between the enzyme and the carrier, despite being somewhat inexpensive and simple to use. [7]. Benefits include minimal or nonexistent harm to cells or enzymes, simple, inexpensive, and quick, Reversible; no alterations occurred to the carrier, enzyme, or cells. Leakage of cells or enzymes from the support is a drawback. Product separation is difficult. binding that is not specific. [7],[14] as shown in Figure 1 [9]

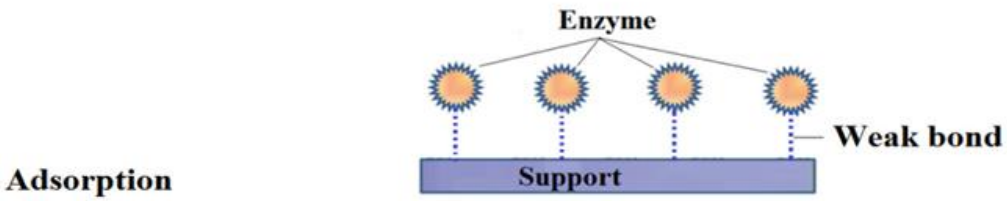

Figure 1. Representation of Adsorption process in Enzyme Immobolization.

Covalent Binding:
This well-known method of immobilizing enzymes involves joining the enzymes to support materials (such as porous silica, polyacrylamide, agarose, porous glass, etc.) via extremely strong and stable

bonds[7]. A few examples of enzyme functional groups that could be used in covalent coupling are hydroxyl, carboxylic, phenolic, sulfhydryl, thiol, imidazole, and indole groups [8]. For various surfaces (natural or synthetic polymers, membranes, or inorganic material) and immobilization techniques (directly onto the transducer surface or onto a thin membrane attached to the transducer), different linkers are employed [8]. One drawback is that the enzyme's active site (an amino group) could be used to carry out the reaction. This indicates that the enzyme is no longer active. Therefore, it is essential to select the appropriate immobilization technique for each unique reaction .[9],[14] as shown in Figure 2 [9].

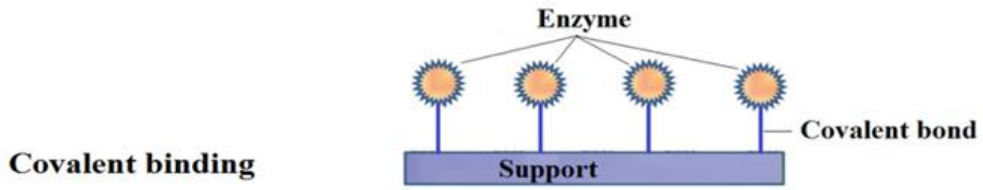

Figure 2. Representation of Covalent Binding process in Enzyme Immobolization.

Cross-linking:

Enzymes are joined by covalent bonds without carriers in cross-linking immobilization. The presence of linker agents, which act as bridges between two neighboring enzyme molecules, enables intermolecular cross-linking [7]. The bonds that develop between molecules of enzymes cannot be broken. A multipurpose reagent that serves as a linker to join enzyme molecules into three-dimensional cross-linked aggregates facilitates the process. The reaction mixture contains the immobilized enzyme, which is not attached to any support. In cross linking immobilization, there are two methods: using cross linking enzyme aggregate (CLEA), and crystals of cross-linking enzymes (CLEC). In order to cross-link enzyme molecules, both approaches call for the application of a cross-linking agent, such as glutaraldehyde [8]. This approach has the benefit of maintaining enzyme activity. Additionally, the enzyme makes direct touch with the substrate. One drawback is that certain cross-linking chemicals have the potential to damage cells or enzymes, and they may denature the enzyme.[9],[14]] as shown in Figure 3 [9].

Figure 3. Representation of Cross-linking process in Enzyme Immobolization.

Entrapment:

It is a process of immobilization that cannot be reversed. It is simply defined as the encapsulation of enzymes via covalent or noncovalent connections within a network of fibers [7]. The process of entrapment immobilization is carried out in two steps: (1) adding an enzyme to a monomer solution, then (2) having the monomer solution polymerized by an altering experimental conditions or a chemical reaction [8]. Since there is no chemical contact between the enzyme and the support material, enzyme

entrapment can increase the stability of an enzyme without reducing its activity.[9],[14] as shown in Figure 4 [9].

Figure 4. Representation of Entrapment process in Enzyme Immobolization.

Micro-encapsulation:

Enzymes are encased in a spherical, semi-permeable membrane during immobilization by encapsulation, an entrapment technique. The membrane can be non-ionic, lipoidal, polymeric, or based on lipoproteins [8]. The creation of specialized membrane reactors, the creation of emulsions, and the creation of microcapsules from these emulsions are the three primary techniques for microencapsulation [9]. The benefit is By trapping multiple enzymes inside the membrane, a multi-enzyme system might be created. The drawback is In order to prevent enzyme leakage, the technique requires precise control of membrane pore size. As a result, the membrane porosity needs to be precisely modified based on the size differences between the enzyme and substrate molecules.[8],[14] as shown in Figure 5 [9].

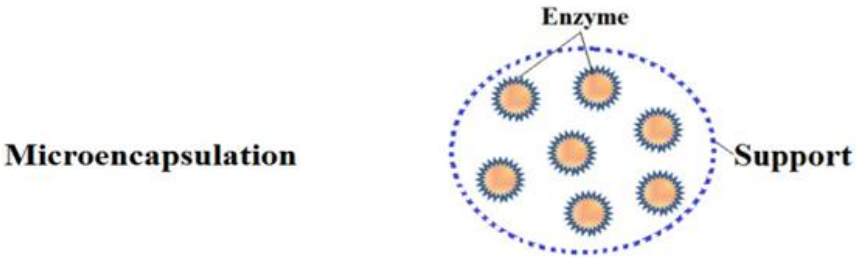

Figure 5. Representation of Micro-encapsulation process in Enzyme Immobolization.

RECENT INNOVATIVE ONGOING TECHNIQUES:

Crosslinked Enzyme Aggregates (CLEAs), Covalent Organic Frameworks (COFs), Metal-Organic Frameworks (MOFs), 3D printing, electrospinning, electrospraying, hybrid nanoflowers, Pickering Emulsion Enzyme Encapsulation, and Peptide-Guided Immobilization are some recent, cutting-edge methods that are still in use. [10],[17]

Crosslinked Enzyme Aggregates (CLEAs):

A simple and efficient technique for immobilizing enzymes is the use of crosslinked enzyme aggregates, or CLAEs. This method enables the rapid combination of several enzymes while maintaining the structure and activity of the individual enzymes. Because it don't require strong supports, they are more stable, effective, and active. The enzyme is precipitated and rendered immobile by the use of substances such as polymers or ammonium sulfate. Covalent bonds are created between the crosslinking agent and the enzyme by the addition of glutaraldehyde. Higher glutaraldehyde levels increase efficiency and stop leaching, whereas lower levels facilitate enzyme fixation. This are frequently utilized for a variety of

enzymes in chemical manufacturing and are economical because they don't require costly carriers. [10] as shown in Figure 6 [28].

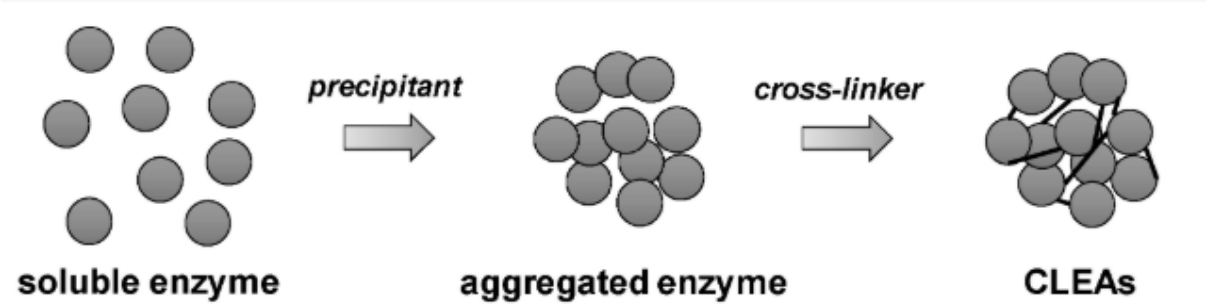

Figure 6. Representation of Crosslinked Enzyme Aggregates (CLEAs) process in Enzyme Immobolization.

Covalent Organic Frameworks (COFs):

These unique porous materials are composed of organic building blocks with crystalline, stable structures. Their excellent thermal stability and resistance to hostile environments like acids and bases make them valuable in a variety of applications, including drug detection, gas storage, and catalysis. Additionally, it may function efficiently at high temperatures and have huge surface areas. The solvothermal process used to create it necessitates high pressures and temperatures. It is possible to modify their structures to enhance their qualities. They have recently demonstrated promise in the immobilization of enzymes due to their stability, wide surface areas, and well-defined pore diameters. The catalytic performance of enzymes can be enhanced by using this to physically or covalently bind them. According to recent research, it may efficiently immobilize enzymes. For example, lipase can be immobilized for the generation of biodiesel by utilizing a magnetic COF composite. Compared to free enzymes, this approach offers greater outcomes by allowing the enzyme to be reused efficiently. Because of their stability, porous structure, and functional groups that promote improved molecule adsorption through hydrogen bonding, it facilitate enzyme immobilization. Enzyme physical adsorption in this has benefits over covalent bonding. Multi-enzyme systems can be produced for industrial usage, and it can be printed with enzymes to provide flexible and selective materials. Because it is enable effective, sustainable biocatalytic processes that may be reused numerous times, they are perfect for a wide range of applications, including medicine manufacture and environmental remediation. [10]as shown in Figure 7 [10].

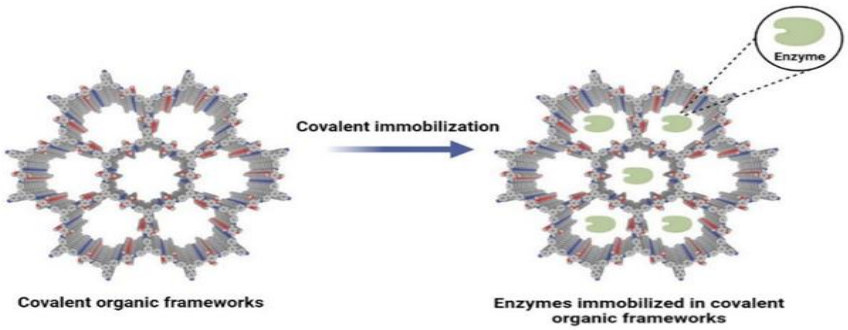

Figure 7. Representation of Covalent Organic Frameworks (COFs) process in Enzyme Immobolization.

Metal-Organic Frameworks (MOFs):

Materials composed of metal ions and organic molecules are known as metal-organic frameworks, or MOFs. They are beneficial for catalysis, storage, and purification because of their special qualities, which include high porosity, pore size adjustment, and flexibility. They can be tailored to perform better in a variety of applications. Through metal centers or functional groups affixed to their structure, it aid in catalyzing processes. They can also serve as size-selective supports and stabilize nanoparticles or enzymes. Compared to conventional materials, their enormous surface area enables the loading of more enzymes, and their structure shields enzymes from adverse environments. . There are three methods for immobilizing enzymes in MOFs: encapsulation, surface bonding, and pore trapping. This technique increases the stability of enzymes under challenging circumstances, such as high temperatures or organic solvents. It have demonstrated potential in applications such as the generation of biodiesel, providing an economical and environmentally beneficial alternative. They are useful for many industrial processes because of their capacity to increase enzyme activity. [10],[19] as shown in Figure 8 [10].

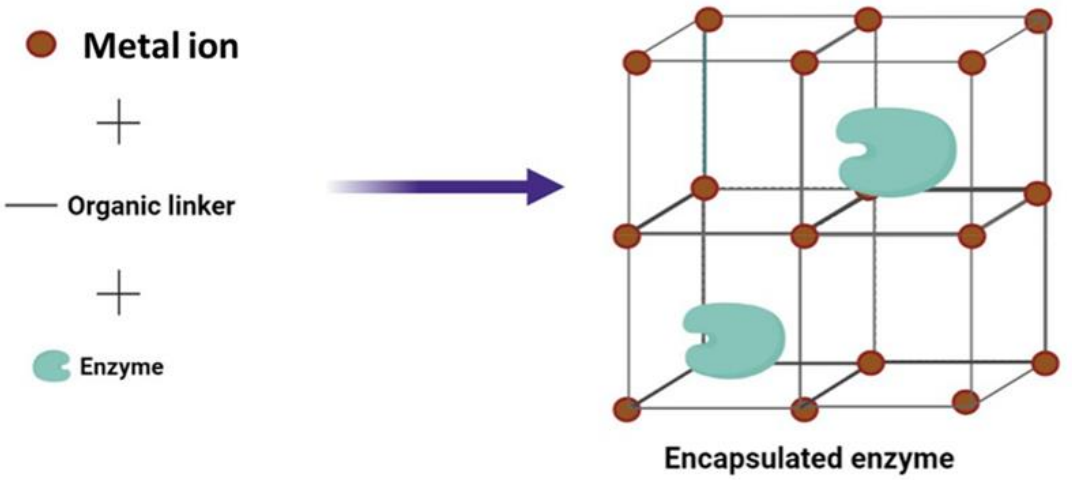

Figure 8. Representation of Metal-Organic Frameworks (MOFs) process in Enzyme Immobolization.

3D printing:

Using computer-aided design (CAD), 3D printing, sometimes referred to as additive manufacturing, is a method that builds objects layer by layer. Aerospace, biology, and catalysis are among the fields that benefit from its affordability, speed, and ability to produce intricate shapes with extreme precision. Custom hydrogel structures that trap enzymes, shield them from adverse environments, and regulate reaction rates can be made via 3D printing in the process of enzyme immobilization. Additionally, these printed materials can be chemically altered to directly bind enzymes, increasing their lifespan and effectiveness. Research has indicated that 3D-printed materials are effective at immobilizing enzymes. To produce biodiesel, for instance, a lipase enzyme was immobilized using geopolymers, which retained high activity after reuse. 3D-printed PLA scaffolds were utilized in another investigation to enhance enzyme performance throughout a number of cycles. All things considered, this provides an affordable, adaptable option for industrial enzyme immobilization.[10] as shown in Figure 9 [10].

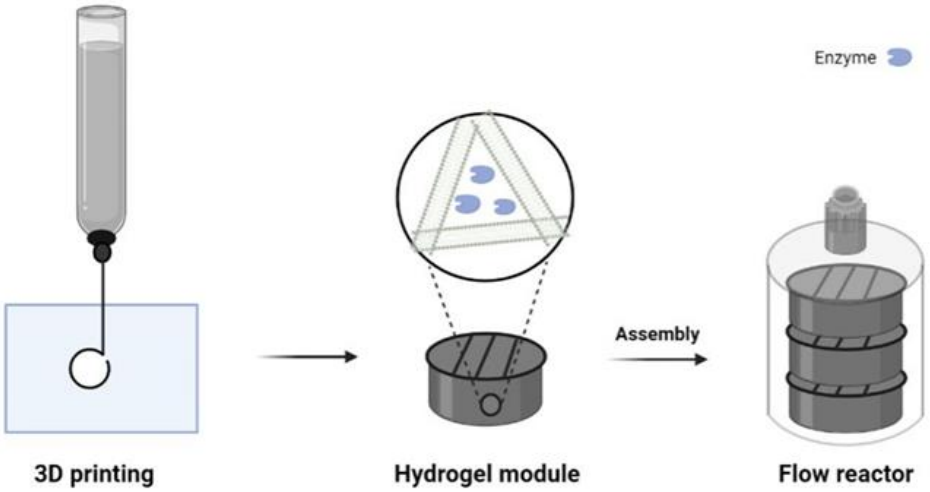

Figure 9. Representation of 3D printing process in Enzyme Immobolization.

Electrospinning:

Since it can address typical enzyme problems, enzyme immobilization is gaining popularity. Enzyme-trapping micro fibers with high surface area and porosity are produced by the efficient process of electrospinning. Enzyme activity is increased and their separation from the reaction environment is facilitated by this procedure. High stability in heat, pH, and solvents is just one of the numerous advantages of electrospun fibers. They enhance enzyme stability, mass transfer, and recyclability, which makes them beneficial for enzyme immobilization. Enzymes can be immobilized in two primary ways: either by embedding them inside the fibers or by adsorbing them onto the fiber surface. Burkholderi cepacia lipase, for instance, was found to improve enzyme loading and stability in electrospun fibers. The enzyme activity stayed high after multiple usage, indicating that electrospinning is a viable option for sectors like medication manufacturing, wastewater treatment, and cosmetics. In conclusion, it is a flexible and affordable technique for immobilizing enzymes, which makes it perfect for a range of industrial applications.[10]as shown in Figure 10 [10].

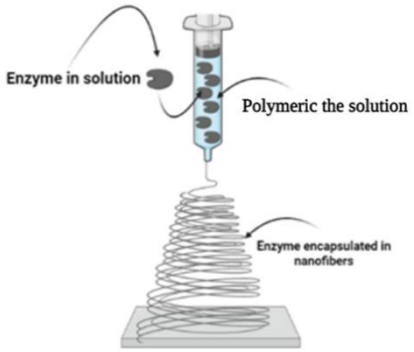

Figure 10. Representation of Electrospinning process in Enzyme Immobolization.

Electrospraying:

Electrospraying, sometimes referred to as electrohydrodynamic atomization (EHDA), is a method that uses electrical forces to produce bioactive materials or polymeric nanoparticles. In contrast to electrospinning, electrospraying aims to atomize liquids by varying variables such as voltage, viscosity,

and solution concentration. This procedure creates uniformly sized micro- and nanoparticles with a high loading capacity. Coaxial electrospraying is a more recent technique that enables the production of nanoparticles with core and shell materials that may be customized for certain applications. Because the process parameters may be changed to alter the size and form of the nanoparticles, this approach is helpful for immobilizing enzymes. The enzymes are stabilized and their effectiveness is enhanced by the nanoparticles' enormous surface area and improved porosity. Studies have demonstrated the potential for enhanced stability, heat resistance, and reusability of lipases immobilized on electrospray fibers with additional materials. Despite these benefits, because electrospraying is still a relatively new method, there is still little study on its use for enzyme immobilization. To enhance the procedure, scale it up, and make it more financially feasible for industrial application, more research is required.[10] as shown in Figure 11 [10].

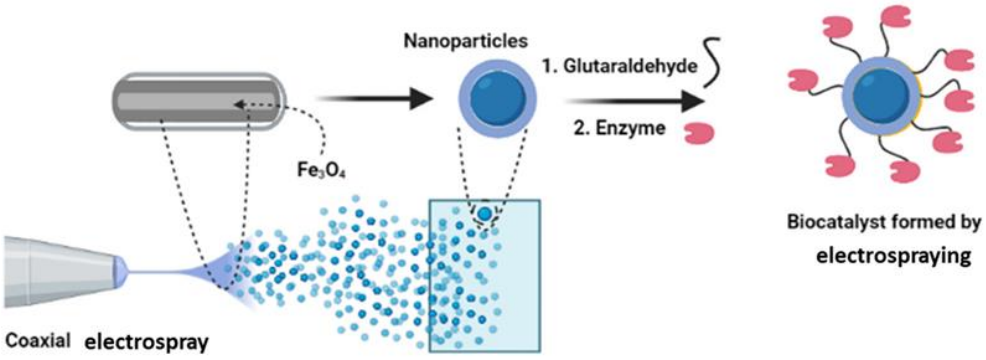

Figure 11. Representation of Electrospraying process in Enzyme Immobolization.

Hybrid Nanoflowers:

Materials with a flower-like structure that are composed of both organic and inorganic components are known as hybrid nanoflowers (UFHs). Due to their huge surface area, high thermal stability, and environmental friendliness, these materials are widely used for enzyme immobilization. They are therefore helpful in fields including pollution control, chemical analysis, and biocatalysis. One of the main advantages of employing hybrid nanoflowers is that they increase tolerance to organic solvents and enhance enzyme stability, particularly at high temperatures. Additionally, they facilitate the repurposing of the enzymes in other cycles of reactions. Enzymes and metal ions are combined in a straightforward procedure to create these nanoflowers. The production time has been shortened from several days to a few minutes due to recent improvements. According to studies, these nanoflowers may effectively encapsulate enzymes like lipase, preserving their high activity even after numerous applications. To sum up, hybrid nanoflowers show promise as an enzyme immobilization solution, particularly when used at high temperatures. To completely understand how enzymes work with these materials to enhance their performance in industrial settings, additional study is expected. [10],[20] as shown in Figure 12 [10].

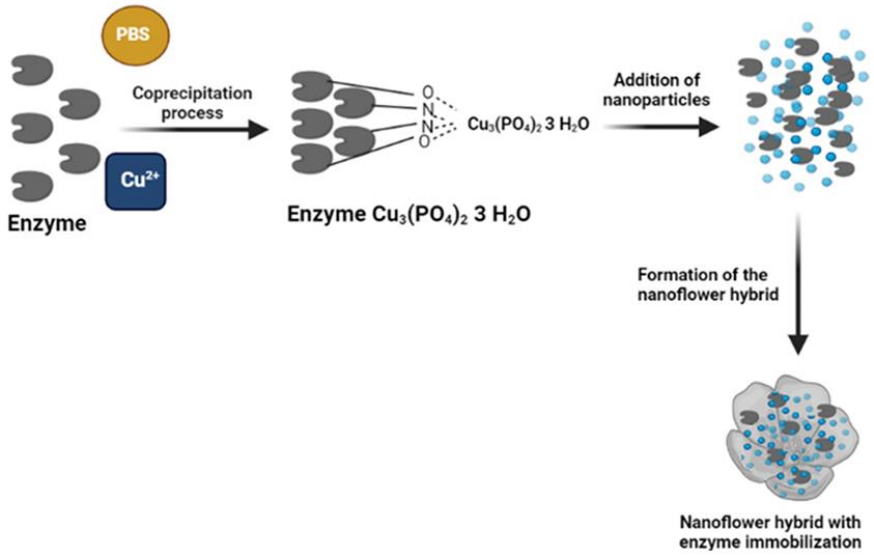

Figure 12. Representation of Hybrid Nanoflowers process in Enzyme Immobolization.

Emulsion Pickering Enzyme Encapsulation:

Two liquids are mixed in an emulsion process, and one of them creates microscopic droplets in the other. Food, medicine, and cosmetics are just a few of the businesses that employ it. The distinctive feature of a Pickering emulsion is the employment of solid particles to stabilize the droplets and keep them from combining. Because of its high efficiency, low toxicity, and ability to function well in challenging conditions, this approach is helpful in catalysis. Pickering emulsions are becoming more popular in enzyme biocatalysis due to their high catalytic activity and affordability. For instance, the emulsion may trap lipase enzymes, so enhancing their potency. By encouraging greater interaction between the enzyme and the emulsion, ultrasound can enhance the procedure. By creating stable and reusable enzyme platforms, this technique has decreased the amount of enzymes lost during processes. Reinforcing particles improve the enzyme's ability to interact with the emulsion and increase its efficacy. All things considered, Pickering emulsions are a viable and reasonably priced method for producing commercial enzyme biocatalysts. Although there is certainly room for development, this approach has enormous potential for widespread use across numerous industries.[10] as shown in Figure 13[10].

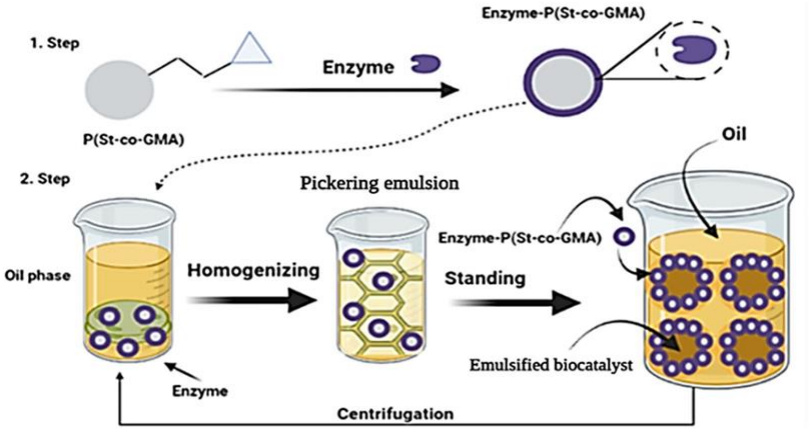

Figure 13. Representation of Emulsion Pickering Enzyme Encapsulation process in Enzyme Immobolization.

Peptide-Guided Immobilization:

A range of techniques are being developed to improve the process of enzyme immobilization, which enhances the performance of enzymes. To improve efficiency, peptides are employed in enzyme immobilization. Because these peptides are hydrophobic, they function effectively in both oily and watery conditions. They are adaptable to a variety of substrates because they can be altered to change their hydrophobic or hydrophilic characteristics. In non-aqueous settings, peptides often take on an alpha-helical shape, which can help with enzyme interaction. Peptides and proteins work together to ensure a tight attachment to the support and preserve the biological activity of the enzyme. One intriguing characteristic is that low pH can be used to undo the peptide attachment. enabling the recycling of resources for further uses. Because of their size and sequence characteristics, peptides are particularly helpful in lipase immobilization. However, developing stable and effective enzymatic biocatalysts requires an understanding of how peptides interact with proteins. Optimizing these systems to make them affordable for industrial application presents another difficulty.[10] as shown in Figure 14[10].

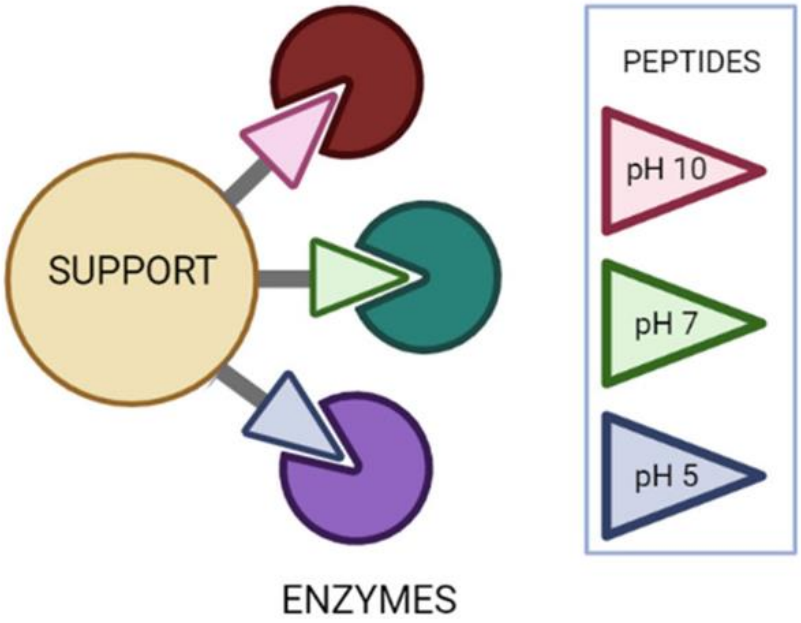

Figure 14. Representation of Peptide-Guided Immobilization process in Enzyme Immobolization. Peptide-Guided Immobilization

When applied to the processing of milk and dairy products, these innovative enzyme immobilization techniques provide an array of benefits over conventional methods:

Enhanced Stability - Techniques such as Metal-Organic Frameworks (MOFs) and Crosslinked Enzyme Aggregates (CLEAs) greatly increase the stability of enzymes against changes in pH, temperature, and storage conditions, guaranteeing extended action in milk processing. [21], [22]
Higher Enzyme Loading Capacity - Because of their porous structure, Covalent Organic Frameworks (COFs) and MOFs enable a high enzyme loading, which can result in enhanced efficiency in casein breakdown, lactose hydrolysis, and other enzymatic activities in dairy.[23]
Improved Reusability — Methods like Peptide-Guided Immobilization and Pickering Emulsion Enzyme Encapsulation provide improved enzyme recyclability, which lowers manufacturing costs in milk processing facilities. [24]

Greater Enzyme Activity Retention: Electrospinning and 3D printing produce well-structured enzyme carriers that reduce enzyme leaching and preserve high enzymatic activity over time.[25]
Enhanced Control Over Enzymatic Reactions: Dairy products like yogurt and cheese have better texture and consistent lactose breakdown thanks to electrospinning and hybrid nanoflowers, which provide exact control over enzyme dispersion.[26]
Eco-Friendly Approach: By enabling the reuse of enzymes, several of these techniques, including CLEAs and COFs, lessen chemical waste and their negative effects on the environment, which is in line with the objectives of sustainable food processing.[27]

Summary:

Immobilized enzymes are enzymes fixed to solid supports, allowing them to be reused while retaining their activity. Enzymes are proteins that act as catalysts for chemical reactions in living organisms. They convert specific substrates into products. Using immobilized enzymes in biotechnology has several advantages: they can be reused in multiple batches, are generally more stable than free enzymes, allow for easier extraction from reaction mixtures, prevent contamination, and can support multi-enzyme systems.
Rennet is the most recognized dairy enzyme preparation, composed of acid proteases from animal tissues. Various dairy products and enzymes are highlighted, including acid proteinases for milk coagulation, neutral peptidases for cheese ripening, lipases for cheese flavor enhancement, and β-galactosidase for lactose reduction in whey. Other enzymes like lactoperoxidase, lysozyme, and transglutaminase also play roles in dairy processing.
Immobilization techniques include adsorption, covalent binding, cross-linking, entrapment, and microencapsulation. Each method has pros and cons. For instance, adsorption allows for reversible binding but may cause enzyme leakage. Covalent binding offers strong attachment but can compromise enzyme activity. Cross-linking involves bonding enzyme molecules without a support, maintaining their activity but potentially causing damages. Entrapment encapsulates the enzymes in a network, preserving their stability and activity. Microencapsulation forms a surrounding membrane for enzymes, increasing stability but requiring precise control of membrane properties.
Recent techniques, such as crosslinked enzyme aggregates (CLEAs), covalent organic frameworks (COFs), and metal-organic frameworks (MOFs), utilize advanced materials for enzyme immobilization. Techniques like 3D printing, electrospinning, and electrospraying create structures that enhance enzyme stability and activity. Hybrid nanoflowers and Pickering emulsions also show promise for improving enzyme functionality while being cost-effective for industrial applications. Peptide-guided immobilization techniques utilize peptides to enhance enzyme performance and ensure reuse, but they require further study for optimization.

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