LOFOTECH: Journal of Local Food Technology
Vol. 1, No. 1, 2025, p. 023-041

Utilization of Alginate-Chitosan Matrix in Protease Immobilization for Fish Protein Hydrolysate Production

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Article Information

Article History

Received: November 16, 2025
Revised: November 24, 2025
Published: December 8, 2025

Keywords:

Alginate;
Chitosan;
Enzyme immobilization;
Fish protein hydrolysate;
Protease.

ABSTRACT

Protease enzymes are widely used in protein hydrolysis to produce fish protein hydrolysates (FPH) with functional and bioactive properties. However, large-scale application remains challenging due to high enzyme costs and instability under industrial conditions. Enzyme immobilization offers an effective solution by improving enzyme stability over wider temperature and pH ranges, enabling recovery and reuse, and lowering operating costs. This review focuses on the utilization of alginate and chitosan as immobilization matrices for protease enzymes in FPH production. Entrapment, adsorption, and covalent binding techniques are examined with respect to their practicality, advantages, and limitations. Alginate provides a low-cost, biocompatible support with good thermal stability, whereas chitosan improves mechanical strength and minimizes enzyme leakage. Combined alginate–chitosan systems demonstrate excellent performance in retaining enzyme activity and enhancing operational stability. Immobilized proteases can be reused in multiple cycles while maintaining high catalytic efficiency, making the process more economical for industrial implementation. Additionally, FPH produced using immobilized enzymes exhibits desirable functional properties and bioactivities, including antioxidant and antihypertensive effects. Overall, alginate–chitosan immobilization represents a promising strategy for sustainable and cost-effective FPH production.

INTRODUCTION

Proteases (EC 3.4) are one of the most important classes of industrial enzymes (Tavano, 2013). These hydrolytic enzymes catalyze the cleavage of peptide bonds in proteins, yielding peptides and free amino acids with diverse applications across multiple industries, including food processing, pharmaceuticals, detergents, leather tanning, and biotechnology (Fernández-Lucas et al., 2017; Li et al., 2013). The was estimated at USD 2.9 billion in 2020 and is expected to reach USD 4.5 billion by 2027, reflecting the growing demand for these biocatalysts in various industrial sectors including fish protein hydrolysate production (Contesini et al., 2020). The global FPH market shows significant growth potential is projected to show continued growth driven by increasing protein demand and sustainability trends (Vaishnav et al., 2025). Large-scale FPH production requires process efficiency, particularly in the use of proteolytic enzymes, making enzyme immobilization critically important. One sustainable and low-cost method for immobilization is the alginate-chitosan matrix (Dutta

et al., 2003; Taqieddin & Amiji, 2004; Wang et al., 2011). Proteases are essential for protein hydrolysis in the food industry, resulting in the production of bioactive compounds and functional ingredients.

Fish protein hydrolysate (FPH) has received significant attention owing to its high nutritional value, beneficial properties, and bioactive potential (Chalamaiah et al., 2012; Kristinsson & Rasco, 2000). In addition to these functional and bioactive properties, the method used to produce FPH greatly influences its quality and composition. Fish protein hydrolysates exhibit better solubility, emulsifying capacity, foaming properties, and water-holding capacity than native proteins, making them valuable ingredients in food formulations (Halim et al., 2016). According to (Salampessy et al., 2017; Slizyte et al., 2016), FPH also contains bioactive peptides that have been shown to have antioxidant activity, angiotensin-I converting enzyme (ACE) inhibitory effects, and potential anticancer effects. Owing to its specificity, mild reaction conditions, lack of hazardous residues, and capacity to maintain the nutritional value of the resultant hydrolysates, enzymatic hydrolysis is favored over chemical methods (Valencia et al., 2014).

Despite the advantages of enzymatic protein hydrolysis, there are several significant challenges associated with the industrial use of protease enzymes. The primary limitations include high enzyme cost and acquiring enzymes, which can account for as much as 30-40% of the total production cost (Sheldon & van Pelt, 2013). Additionally, free enzymes in solution are often unstable under industrial operating conditions, susceptible to denaturation by temperature fluctuations, pH changes, and organic solvents, and difficult to recover and reuse after the reaction (Rodrigues et al., 2021). These enzymes also exhibit limited operational stability and and therefore cannot be efficiently separated from the reaction products, necessitating disposal after a single-use cycle (Mohamad et al., 2015). Furthermore, the narrow optimal pH and temperature ranges of free enzymes restrict process flexibility and necessitating additional process control systems to maintain ideal reaction conditions (Singh et al., 2011).

Enzyme immobilization has emerged as a powerful strategy for overcoming these limitations and enhancing the economic viability of enzyme-catalyzed processes. Immobilization involves attaching enzymes to or within solid support matrices, converting them from soluble to insoluble forms, while retaining their catalytic activity (Cao et al., 2003). This technique offers multiple advantages, including (1) enhanced enzyme stability against thermal denaturation, pH variations, and proteolytic degradation; (2) facilitated enzyme recovery and reusability for multiple reaction cycles, significantly reducing operational costs; (3) improved process control and continuous operation capability; (4) easier separation of enzymes from products, eliminating contamination concerns; and (5) extended operational pH and temperature ranges compared to free enzymes (Jesionowski et al., 2014; Zucca & Sanjust, 2014). Studies have demonstrated that immobilized proteases can retain up to 70-90% of their initial activity after 10-15 reuse cycles, whereas free enzymes are typically discarded after a single use (Kumari et al., 2015; Y. Wang et al., 2014). The enhanced thermal stability of immobilized enzymes can extend their half-life by factors of 5 – 100 folds compared to their free counterparts (Rodrigues et al., 2021).

Among the various support materials used for enzyme immobilization. Naturally derived biopolymers have gained significant attention owing to their biocompatibility, biodegradability, non-toxicity, and regulatory acceptance for food applications. Alginate and chitosan are two of the most promising naturally derived matrices for enzyme immobilization in food-grade applications (Bilal & Iqbal, 2019). Alginate, a linear polysaccharide extracted from brown seaweeds, forms stable hydrogels in the presence of divalent cations, such as Ca^{2+}, and has been widely used for cell and enzyme encapsulation (Skjåk-Bræk & Draget, 2012). Its advantages include low cost, mild gelation conditions that preserve enzyme activity, high porosity allowing substrate diffusion, and Generally Recognized as Safe (GRAS) status for food use (Illanes et al., 2013). Chitosan, obtained through deacetylation of chitin from

crustacean shells, possesses unique properties including natural antimicrobial activity, film-forming ability, and abundant reactive amino and hydroxyl groups suitable for enzyme attachment (Datta et al., 2012). The combination of alginate and chitosan has shown synergistic effects, where chitosan coating on alginate beads prevents enzyme leakage while maintaining mechanical stability and permeability (Taqieddin & Amiji, 2004).

The immobilization of proteases using alginate–chitosan matrices is particularly relevant to the production of fish protein hydrolysates (FPH), where enzyme stability, reusability, and controlled hydrolysis conditions directly influence product functionality and bioactivity. Although numerous studies have investigated protease immobilization and FPH production separately, comprehensive reviews integrating both aspects, with a focus on alginate-chitosan systems, remain limited. Understanding the relationship between immobilization strategies, enzyme performance, and FPH quality characteristics is essential for developing economically viable and sustainable processes for industrial-scale FPH production.

This review aims to provide a comprehensive analysis of the utilization of alginate and chitosan as immobilization matrices for protease enzymes in fish protein hydrolysate production. Specifically, this review discusses (1) what are the characteristics and classification of protease enzymes relevant to protein hydrolysis; (2) which enzyme immobilization methods—entrapment, adsorption, and covalent binding techniques convenient for FPH production; (3) what properties and advantages of alginate and chitosan as immobilization supports, including their combination strategies; (4) how the application of immobilized proteases in FPH production, including effects on functional properties and bioactive characteristics; and (5) what is the current challenges and future research directions for advancing this technology toward industrial implementation. This work highlights opportunities and challenges for developing economically viable and sustainable biocatalytic processes within the fish protein valorization industry.

RESEARCH METHODS

This study was conducted through a comprehensive literature search using the Web of Science, Scopus, PubMed, ScienceDirect, and Google Scholar databases. Publications from 2000 to 2024 inclusive were considered. The search strategy applied Boolean operators and included combinations of keywords such as "protease", "immobilization", "alginate", "chitosan", and "fish protein hydrolysate".

Peer-reviewed journal articles published in English were prioritized. Review papers and selected high-quality book chapters were also included when relevant to protease immobilization, alginate–chitosan matrices, and fish protein hydrolysate production. Studies were screened based on methodological rigor, quality of evidence, and relevance to the objectives of this review. Information regarding protease types, immobilization techniques, performance stability, and effects on FPH physicochemical and functional properties was systematically extracted. The collected data were critically analyzed and synthesized to identify research trends, evaluate outcomes, and highlight existing knowledge gaps. The synthesized information was organized to ensure comprehensive coverage from fundamental principles to industrial application prospects within the field.

RESULTS AND DISCUSSION

Protease Enzymes for Protein Hydrolysis

Classification and Catalytic Mechanisms

Protease enzymes belong to the hydrolase class (EC 3.4) and are responsible for catalyzing the hydrolytic cleavage of peptide bonds in proteins and in polypeptides. According to the International Union of Biochemistry and Molecular Biology (IUBMB) classification system, proteases are categorized into seven major classes based on their catalytic

mechanism and the key amino acid residues or cofactors present in their active sites: serine (EC 3.4.21), cysteine (EC 3.4.22), aspartate (EC 3.4.23), metalloproteases (EC 3.4.24), threonine (EC 3.4.25), glutamate (EC 3.4.23), and asparagine peptide lyases (EC 3.5.1) (Fernández-Lucas et al., 2017; Tavano, 2013).

The catalytic mechanisms of these protease classes differ fundamentally in how they activate the nucleophile that attacks the peptide bond. In serine and cysteine proteases, the hydroxyl group of serine or the thiol group of cysteine acts as a nucleophile through a charge relay system involving histidine and aspartate residues. In contrast, aspartate and metalloproteases activate water molecules to serve as nucleophiles, with aspartate residues or metal ions (typically Zn^{2+}) coordinating and polarizing the water molecules (Erez et al., 2009). Despite these mechanistic differences, all protease classes achieve the same overall result: hydrolytic cleavage of peptide bonds to produce shorter peptides and free amino acids.

For food applications of FPH, the choice of protease significantly influences the characteristics of the resulting hydrolysate, including molecular weight distribution, amino acid composition, functional properties, and bioactive potential. The proteases most commonly employed in FPH production can be categorized based on their origin as microbial (e.g., Alcalase, Neutrase, and Flavourzyme), plant (e.g., papain, bromelain, and ficin), and animal (e.g., pepsin, trypsin, and chymotrypsin) enzymes.

Characteristics of Proteases Relevant to Fish Protein Hydrolysis

The selection of appropriate protease for FPH production requires consideration of several key characteristics that influence the hydrolysis efficiency, product quality, and process economics. Table 1 summarizes the fundamental properties of the major protease classes used in protein hydrolysis applications.

Table 1. Characteristics of Protease Enzyme Classes Relevant to Protein Hydrolysis (adapted from (Alagarsamy et al., 2006; Tavano, 2013))

Protease Type	EC Num.	Molecular Weight (kDa)	Opt. pH	Opt. Temp. (°C)	Key Cofactor /Ions	Active Site Residues	Common Inhibitor	Representative Enzymes
Serine proteases	3.4.21	18-35	6-11	50-70	Ca^{2+}	Ser, His, Asp	PMSF, DIFP, EDTA	Alcalase, trypsin, subtilisin
Cysteine proteases	3.4.22	34-35	2-3	40-55	-	Cys, His	Indoacetamide, p-CMB	Papain, bromelai, ficin
Aspartate proteases	3.4.23	30-45	3-5	40-55	Ca^{2+}	Asp	Pepstatin	Pepsin, chymosin
Metallo proteases	3.4.24	19-37	5-7	65-85	Zn^{2+}, Ca^{2+}	Glu, His	EDTA, EGTA	Thermolysin, neutral proteases

Alcalase (from *Bacillus lichenifromis*) is the most widely utilized microbial protease for FPH production because of its broad substrate specificity, high catalytic efficiency, and stability at alkaline pH (pH 8-10) and elevated temperatures (50-70 °C). Alcalase is an endopeptidase that preferentially cleaves internal peptide bonds at hydrophobic amino acid residues and belongs to the serine protease family (Cox & Nelson, 2000). Hydrolysis using Alcalase typically exhibits high degrees of hydrolysis (DH 15-30%), adequate solubility over a broad pH range, and potent antioxidant activity (Chalamaiah et al., 2012). Flavorzyme, an aminopeptidase/endopeptidase complex, is frequently combined with Alcalase to produce hydrolysates with enhanced flavor profiles and higher free amino acid contents (Rezvankhah et al., 2021).

Plant-derived cysteine proteases, particularly papain (from *Carica papaya*), bromelain (from pineapple), and ficin (from *Ficus carica*), are valued for their activity across neutral to slightly acidic pH ranges and their ability to hydrolyze diverse protein substrates. These enzymes demonstrate optimal activity at temperatures between 37-60°C and pH 4.5-7.0 (Holyavka et al., 2021). Papain has been reported to exhibit the highest protein hydrolysis efficiency among plant proteases, achieving up to 34.8% under optimized conditions (Domokos-Szabolcsy et al., 2024). The relatively mild operating conditions of plant proteases make them suitable for preserving heat-sensitive bioactive peptides during hydrolysis. However, their broader specificity can cause extensive hydrolysis, potentially leading to bitterness duet o the fromation of hydrophobic (Fernández-Lucas et al., 2017).

Animal-derived proteases, such as pepsin (aspartate protease) and trypsin (serine protease), are highly specific enzymes with well-characterized substrate preferences. Pepsin operates optimally at acidic pH (pH 2-3) and preferentially cleaves peptide bonds adjacent to aromatic amino acids, whereas trypsin functions at neutral to slightly alkaline pH (pH 7-9) and specifically cleaves at the carboxyl side of lysine and arginine residues. This high specificity is advantageous for generating hydrolysates with defined peptide sequences and targeted bioactivities. However, the requirement for extreme pH conditions (particularly for pepsin) and their relatively high cost of animal-derived proteases compared with microbial proteases limit their industrial applications (Kristinsson & Rasco, 2000).

Influence of Protease Source on Hydrolysate Characteristics

The protease source significantly impacts the structural and functional characteristics of fish protein hydrolysates (FPH). Different proteases generate hydrolysates with distinct molecular weight distributions, amino acid profiles, and bioactive properties, even when applied to the same protein substrate. Ktari et al. (2012) demonstrated that crude protease extracts from different sources yielded significantly different amino acid concentrations when hydrolyzing *Salaria basilisca* fish proteins, as confirmed by RP-HPLC. Similarly, Ambigaipalan et al., (2015) reported that Alcalase, Flavourzyme, and Thermolysin produced raisin seed peptide hydrolysates with markedly different antioxidant activities and ACE-inhibitory capacities.

The degree of hydrolysis (DH), defined as the percentage of cleaved peptide bonds relative to the total peptide bonds in the protein substrate, is a critical parameter that strongly correlates with functional and bioactive properties. Proteases with broader substrate specificity (e.g., Alcalase and papain) generally achieve higher DH values and yield hydrolysates containing lower molecular weight peptides with improved solubility and enhanced antioxidant activity. In contrast, proteases with narrow specificity (e.g., trypsin) tend to produce hydrolysates with higher molecular weight peptides, which may exhibit superior emulsifying and foaming properties (Chalamaiah et al., 2012).

The amino acid composition of hydrolysates is also strongly dependent on the protease employed. Hydrolysates produced using alkaline proteases are typically enriched in hydrophobic amino acids such as leucine, phenylalanine, and valine, whereas hydrolysates generated using acidic proteases contain higher proportions of acidic amino acids (Taheri et al., 2013). This compositional variation directly influences functional properties such as solubility, emulsification capacity, and sensory characteristics. For FPH applications, enrichment in specific amino acids including arginine, lysine, histidine, and leucine is particularly desirable because of their nutritional value and contribution to bioactive peptide sequences (Thiansilakul et al., 2007).

Operational Stability and Process Considerations

A critical challenge in utilizing proteases for large-scale FPH production is maintenaning enzyme activity and stability throughout processing. Free proteases are susceptible to thermal denaturation, autolysis, pH-induced conformational changes, and inhibition by reaction products or impurities in protein substrates. The operational stability of proteases varies

depending on their source and structural characteristics. Thermostable metalloproteases from thermophilic bacteria, such as *Bacillus stearothermophilus,* can function optimally at temperatures of 70-90°C and pH 7-8.5, making them suitable for processes simultaneous microbial inactivation (Sookkheo et al., 2000). However, most commercially available proteases for food applications operate optimally at moderate temperatures (40-60°C) and require careful process control to prevent loss of catalytic activity.

The stability limitations of free proteases necessitate the development of strategies to enhance their operational performance and economic feasibility. Enzyme immobilization has emerged as one of the most effective approaches to overcome these limitations by providing physical protection, reducing autolysis, enabling enzyme recovery and reuse, and often broadening the operational pH and temperature ranges. The following sections discuss immobilization technologies and their applications to protease enzymes in FPH production, with particular emphasis on alginate and chitosan-based support systems.

Enzyme Immobilization: Principles and Methods

Fundamentals and Advantages of Enzyme Immobilization

Enzyme immobilization refers to the confinement or localization of enzymes within a defined space while retaining their catalytic activity, thereby allowing their recovery, reuse, and continuous operation (Sheldon & Van Pelt, 2013). This approach converts soluble enzymes into heterogeneous biocatalysts that can be easily separated from substrates and reaction products. The concept was commercialized in the 1960s with the introduction of immobilized aminoacylase for L-amino acid production and has since become a cornerstone technology in industrial biocatalysis (Cao et al., 2003).

Immobilization offers multiple advantages for protease applications in FPH production. Economically, enzyme reusability significantly reduces processing costs; immobilized proteases can typically be reused for 10–50 cycles while maintaining 50–80% of their initial activity, unlike free enzymes which are generally single-use (Kumari et al., 2015; Rodrigues et al., 2021). For example, Wang et al., (2014) demonstrated that alginate–chitosan-immobilized Alcalase and trypsin retained 70% activity after 15 reuse cycles, translating to an estimated 85–90% reduction in enzyme cost per unit of product. Operational advantages include continuous processing capability, simplified downstream separation, minimization of enzyme contamination in final products, and reduced reactor volumes through higher enzyme loadings (Mohamad et al., 2015).

The greatest benefit of immobilization lies in its ability to enhance enzyme stability. Confinement or attachment to solid supports can substantially increase resistance to thermal denaturation, pH extremes, organic solvents, and proteolytic degradation. These improvements arise through several mechanisms: (1) increased conformational rigidity that restricts unfolding and helps maintain the native structure; (2) formation of a protective microenvironment that buffers changes in pH and ionic strength; (3) spatial separation that minimizes aggregation-induced denaturation; and (4) physical protection that prevents protease autolysis (Jesionowski et al., 2014; Rodrigues et al., 2021). For instance, De Queiroz et al., (2006) reported that alginate-immobilized protease from Myceliophthora sp. exhibited enhanced thermal stability at 55 °C compared with 50 °C for the free enzyme. while Zanphorlin et al., (2011) observed that the same immobilized enzyme retained 50% activity after 5 hours at 50–65 °C, whereas the free enzyme was completely inactivated under identical conditions.

Immobilization Methods and Mechanisms

Enzyme immobilization techniques are generally classified into three main approaches based on the nature of enzyme–support interactions: physical adsorption, covalent binding, and physical entrapment (or encapsulation). Each method presents distinct advantages and limitations in terms of enzyme loading capacity, activity retention, stability enhancement, and compatibility with various enzyme–support systems (Cao et al., 2003; Mohamad et al., 2015).

Physical adsorption involves the attachment of enzymes to support surfaces through weak non-covalent interactions such as van der Waals forces, hydrogen bonding, hydrophobic interactions, and electrostatic attraction. This is the simplest and most economical immobilization strategy, requiring minimal chemical modification of either the enzyme or the support (Jesionowski et al., 2014). The procedure typically consists of mixing the enzyme solution with the support under controlled pH and ionic strength conditions, followed by washing to remove unbound enzyme. Advantages include operational simplicity, minimal loss of enzymatic activity, reversibility that enables support regeneration, and broad applicability across enzyme–support combinations. Trypsin immobilized on magnetic iron oxide (Fe_3O_4) nanoparticles via adsorption exhibited improved durability and reusability, with easy magnetic recovery (Aslani et al., 2018). Similiarly, Wu et al., (2006) reported efficient trypsin immobilization on macroporous chitosan–silica supports with good activity retention and operational stability. However, weak enzyme–support interactions may result in enzyme leaching under high ionic strength, extreme pH, or solvent exposure. Although reversible adsorption facilitates support reuse, gradual enzyme desorption can cause product contamination. In addition, surface saturation limits loading capacity, and random enzyme orientation may lead to partial active-site obstruction (Talbert & Goddard, 2012).

Covalent binding involves the formation of stable chemical bonds between functional groups on the enzyme surface (commonly ε-amino groups of lysine, N-terminal amino groups, carboxyl groups, or thiol groups) and reactive groups on the support. This method produces irreversible linkages that enhance enzyme stability and prevent leakage, even under harsh reaction conditions (Rodrigues et al., 2021; Zucca & Sanjust, 2014). Single-point covalent attachment maintains relatively high specific activity while improving enzyme rigidity. Common coupling chemistries include glutaraldehyde crosslinking, carbodiimide-mediated coupling (EDC/NHS), and epoxy activation. For example, Yazid et al. (2016) immobilized protease on iron oxide nanoparticles via glutaraldehyde crosslinking, while Benucci et al. (2018) used glutaraldehyde and direct coupling approaches for bromelain immobilization on chitosan.

Multipoint covalent attachment further increases rigidity by forming multiple bonds between the enzyme and the support, resulting in superior thermal stability. Adriano et al. (2008) demonstrated that multipoint covalent immobilization of penicillin G acylase on chitosan enhanced enzyme stability fivefold at 50 °C. However, excessive rigidification may distort the active site, decreasing catalytic performance by 20–40% if not properly controlled. Although covalent binding prevents enzyme leaching and supports prolonged operation—especially in continuous-flow reactors—disadvantages include potential activity loss due to structural alteration, more complex processing requirements, the need for specific surface chemistries, and the irreversibility of enzyme–support bonding (Mohamad et al., 2015; Singh et al., 2011).

Entrapment immobilization physically confines enzymes within three-dimensional polymer matrices or hollow capsules while allowing substrates and products to diffuse freely through the structure (Cao et al., 2003). Gel entrapment is the most common form, where enzymes are mixed with polymer precursors before gelation, resulting in enzyme molecules being trapped within the gel network. Alginate is the most widely used polymer for gel entrapment because of its mild gelation conditions and biocompatibility. The simple mixing of enzyme-alginate solution with calcium chloride solution forms hydrogel beads within seconds without requiring harsh chemicals or extreme conditions that could denature the enzymes (Illanes et al., 2013). Other entrapment matrices include agar, carrageenan, polyacrylamide, and silica-based mesoporous materials. Kumari et al., (2015) demonstrated successful protease entrapment in mesoporous silica (SBA-15) and zeolite (nano-ZSM-5), while Guleria et al., (2016) entrapped alkaline protease in a chitosan-agar-polyacrylamide composite matrices.

Entrapment offers advantages such as high activity retention (>80%), protection against denaturation and proteolysis, prevention of enzyme aggregation, applicability to multi-enzyme systems, and straightforward processing. The confined microenvironment protects enzymes from mechanical shear, liquid–air interfaces, and hydrophobic solvents (Datta et al., 2012). However, mass-transfer limitations can significantly reduce observable reaction rates (by 30–70%) due to diffusional constraints (Bezbradica et al., 2011). The severity depends on pore size, enzyme loading, substrate molecular weight, and reaction kinetics. Mechanical weakness of some matrices can restrict enzyme loading capacity and may allow leakage of smaller enzymes or degradation over time. Table 2 provides a systematic comparison of the three immobilization strategies, highlighting their respective advantages in protease immobilization for FPH production.

Table 2. Comparative Evaluation of Enzyme Immobilization Methods for Protease Application

Parameter	Physical Adsorption	Covalent Binding	Physical Entrapment
Enzyme-support interaction	Weak, reversible	Strong, irreversible	Physical confinement
Preparation complexity	Simple	Moderate to complex	Simple to moderate
Activity retention (%)	70-95	40-80	75-90
Operational stability	Low to moderate	High to very high	Moderate to high
Enzyme leakage	High risk	Negligible	Low to moderate
Reusability (cycles)	5-10	20-50	10-30
Support regeneration	Possible	Not possible	Not possible
Best suited for	Preliminary screening, mild conditions	Harsh condition, continuous operation	Large substrates, multi-enzyme systems.

The selection of the optimal immobilization method depends on the specific application requirements. For FPH production, where the substrates are relatively large (fish proteins and protein fragments), the products need to be enzyme-free, and economic viability is critical. Entrapment methods, particularly those using alginate-chitosan systems, offer an attractive balance of high activity retention, adequate stability, ease of preparation, and cost-effectiveness (Datta et al., 2012; Wang et al., 2014).

Alginate and Chitosan as Immobilization Matrices

Alginate is a naturally occurring anionic polysaccharide extracted primarily from brown seaweeds (Phaeophyceae), including Laminaria, Macrocystis, and Ascophyllum species, which collectively produce approximately 30,000 tons of alginate annually for industrial applications (Skjåk-Bræk & Draget, 2012). It is composed of linear copolymers of β-D-mannuronic acid (M) and α-L-guluronic acid (G), linked by (1→4) glycosidic bonds and arranged in homopolymeric G-blocks, homopolymeric M-blocks, and alternating MG-blocks. Variations in block composition and sequence—affected by the seaweed source and extraction conditions—strongly influence alginate's mechanical strength and gel-forming behavior (Lee et al., 2000).

One of alginate's most distinctive characteristics is its ability to form thermally stable hydrogels in the presence of divalent cations, particularly Ca^{2+}, through ionic crosslinking described by the "egg-box" model (Figure 1). In this mechanism, G-blocks from adjacent polymer chains cooperatively bind Ca^{2+} ions, creating junction zones that form a robust three-dimensional network (Tordi et al., 2025). Gelation occurs rapidly under mild conditions (room temperature, neutral pH) and is reversible using chelating agents such as EDTA or sodium citrate. These properties make alginate highly suitable for enzyme entrapment without

exposure to heat or harsh chemicals that could cause enzyme denaturation (Skjåk-Bræk & Draget, 2012). Key advantages of alginate as an immobilization matrix include: (1) biocompatibility and non-toxicity— granted GRAS status for food uses; (2) mild gelation conditions— preserving enzyme structure and activity; (3) high porosity— facilitating diffusion of substrates and products; (4) low cost—due to abundant natural availability; (5) ease of scale up— simple bead formation using $CaCl_2$; and (6) biodegradability (Bilal & Iqbal, 2019; Illanes et al., 2013; Skjåk-Bræk & Draget, 2012).

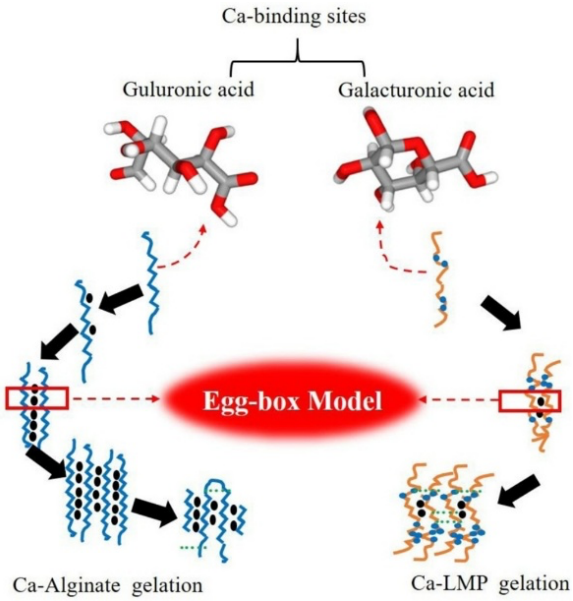

Figure 1. Egg box model or ionic cross-linking of alginate (Cao et al., 2020).

Applications in protease immobilization have shown consistent improvements in stability and reuse. Proteases immobilized in calcium alginate demonstrated enhanced thermal resistance, retaining activity at 55 °C versus 50 °C for the free enzyme (de Queiroz et al., 2006). The immobilized protease retained 50% of initial activity after 5 h at temperatures between 50-65°C, while the free enzyme was completely inactivated under these conditions (Zanphorlin et al., 2011). Abdella et al., (2023) successfully immobilized alkaline protease in calcium alginate/dextrose beads, displayed good reusability as it retained 92.7 and 52.4% of its activity and demonstrating stable operation over 8 and 12 consecutive cycles. These studies consistently show that immobilization enhances protease thermal stability by 10°C and extends the operational lifetime by 1.47 fold compared to free enzymes.

However, alginate matrices also have limitations. Prolonged operation in acidic or low-ionic-strength environments can cause excessive swelling, enlarged pore size, enzyme leakage, and mechanical weakening—particularly in the presence of calcium-chelating agents. Furthermore, the anionic nature of alginate can lead to electrostatic repulsion when interacting with negatively charged substrates or products. To address these issues, composite and coated formulations—most notably those incorporating chitosan—have been developed to enhance mechanical strength and structural stability (Datta et al., 2012; Taqieddin and Amiji, 2004).

Chitosan is a linear cationic polysaccharide derived by alkaline deacetylation of chitin-the second most abundant biopolymer after cellulose and the structural component of crustacean shells, insect exoskeletons, and fungal cell walls. Seafood processing generates over 100,000 tons of chitin annually, providing an inexpensive and renewable raw material for chitosan production (Dutta et al., 2003; Ravi Kumar, 2000). Chitosan consists primarily of β (1→4)-linked D-glucosamine units with varying fractions of N-acetyl-D-glucosamine, depending on the degree of deacetylation (typically 70–95%).

Chitosan's cationic nature—arising from protonated amino groups (pKa ~6.5) under acidic conditions—enables strong electrostatic interactions with negatively charged molecules. As a result, it readily forms polyelectrolyte complexes with alginate, producing composite structures with enhanced mechanical strength (Ravi Kumar, 2000). The presence of multiple amino and hydroxyl groups also supports diverse immobilization chemistries, including covalent coupling, crosslinking, and surface functionalization.

Advantages of chitosan for enzyme immobilization include: (1) high mechanical strength and structural reinforcement; (2) chemical versatility for controlled immobilization methods; (3) antimicrobial properties—intrinsic antimicrobial activity reduces contamination risks in bioreactor operations; (4) pH-responsive behavior enabling controlled loading and release; (5) excellent biocompatibility and biodegradability; and (6) renewability supporting circular bioeconomy principles (Bilal & Iqbal, 2019; Mohamad et al., 2015).

Chitosan has been applied in several immobilization configurations. Covalent immobilization is especially effective for improving durability and reusability. Papain and cysteine proteases have been immobilized on chitosan supports with strong stability enhancement (Hayashi & Ikada, 1991; Singh et al., 2011). Glutaraldehyde-activated systems are frequently used and have been shown to improve resistance to harsh conditions and increase reuse cycles—for example, pepsin immobilization by Altun & Çetinus, (2007) and bromelain by Benucci et al., (2018). Chitosan-immobilized bromelain retained 50% activity after five reuse cycles (Colmenares and Cuellar, 2020).

Despite these benefits, chitosan also has limitations. pH-dependent solubility restricts stability under acidic conditions unless crosslinked or chemically modified. Its relatively hydrophobic character may reduce substrate accessibility compared with highly hydrophilic alginate. Additionally, the use of chemical crosslinkers (e.g., glutaraldehyde) may raise toxicity concerns in food applications, requiring extensive washing or the adoption of food-grade alternatives (Datta et al., 2012).

Alginate-Chitosan Composite Systems: Synerginistic Benefits

The combination of alginate and chitosan represents a strategic immobilization approach that leverages the complementary strengths of both polymers while minimizing their individual limitations. In the most common configuration, alginate beads or hydrogels are coated with chitosan through polyelectrolyte complexation. Here, negatively charged carboxyl groups of alginate interact electrostatically with the positively charged amino groups of chitosan, forming a stable polyelectrolyte complex membrane on the bead surface (Datta et al., 2012; Taqieddin & Amiji, 2004).

Chitosan coatings markedly enhance the mechanical strength and operational stability of alginate beads. The chitosan layer functions as a semi-permeable barrier that regulates mass transfer and reinforces the bead structure, reducing swelling and preventing disintegration under processing conditions. Tan et al. (2016) reported that alginate–chitosan microcapsules exhibited a 2–3-fold increase in compressive strength compared with uncoated alginate beads. Moreover, the chitosan coating prevents calcium ion leaching and thus stabilizes the calcium–alginate network, particularly in low-ionic-strength environments or in the presence of chelating agents. Accordingly, Wang et al. (2014) showed that chitosan-coated alginate beads maintained structural integrity after 15 reuse cycles, whereas uncoated beads began to fragment after only 8 cycles.

In same study, Wang et al., (2014) demonstrated the effectiveness of immobilizing Alcalase and trypsin in alginate–chitosan beads for fish protein hydrolysis. The immobilized enzymes showed: (1) high activity retention—78% (Alcalase) and 73% (trypsin); (2) excellent reusability—70% retaining activity after 15 cycles; (3) enhanced pH stability— Alcalase remained active over pH 6–10 versus pH 7–9 for the free enzyme; (4) improved thermal stability— maintaining 60% activity after 6 h at 60°C, while free enzyme lost 80% activity; and (5) efficient hydrolysis performance— comparable degrees of hydrolysis to free-enzyme systems, yet with substantially reduced enzyme consumption.

Optimization Strategies for Alginate-Chitosan Protease Immobilization

Several formulation variables influence the performance of alginate–chitosan immobilization systems, including chitosan molecular weight (50–300 kDa), degree of deacetylation (70–95%), concentration (0.1–1.0% w/v), and coating duration (15–60 min). Low–molecular-weight chitosan (50–150 kDa) within the range of 0.2–0.5% (w/v) typically provides the most effective coating, achieving adequate enzyme retention while minimizing mass-transfer resistance. Stability can be further enhanced through the application of multiple coating layers; however, additional layers increase diffusion barriers, which may reduce the apparent reaction rate (Datta et al., 2012; Taqieddin & Amiji, 2004). Table 3 summarizes recent studies utilizing alginate, chitosan, and alginate–chitosan composite systems for protease immobilization, demonstrating the versatility of these materials and the variability in performance outcomes.

Table 3. Selected Studies on Alginate- and Chitosan-Based Protease Immobilization

Enzyme	Support Matrix	Immobilization Method	Key Performance Indicators	Reference
Alcalase, Trypsin	Alginate-chitosan beads	Entrapment and coating	78% activity retention; 70% after 15 cycles; pH 6-10 stable	(Y. Wang et al., 2014)
Alkaline protease	Chitosan-agar-polyacrylamide	Entrapment	Enhanced thermal stability; 8 reuse cycles	(Guleria et al., 2016)
Bromelain	Chitosan	Direct linking, glutaraldehyde	65% activity after 8 cycles; pH 4-8 active	(Benucci et al., 2018)
Protease (*Myceliophtora* sp.)	Alginate beads	Entrapment	Stable at 55°C; 50% activity after 5 hours at 50-65°C	(De Queiroz et al., 2006; Zanphorlin et al., 2011)
Pepsin	Chitosan	Glutaraldehyde cross-linking	Enhancedd pH stability; improved storage stability	(Altun & Çetinus, 2007)
Papain	Chitosan	Covalent attachment	Good activity retention; enhanced thermal stability	(Hayashi & Ikada, 1991)
Cycteine protease	Chitosan	Covalent coupling	Stable immobilization; recyclable	(Singh et al., 2011)

Substantial evidence indicates that alginate–chitosan composite systems outperform single-polymer matrices for protease immobilization, offering superior activity retention, improved operational stability, and enhanced enzyme containment with practical ease of handling. These features make alginate–chitosan composites particularly advantageous for industrial-scale fish protein hydrolysate production, where enzyme reusability, process robustness, and food-grade safety are essential requirements.

Application in Fish Protein Hydrolysate Production

Fish protein hydrolysate (FPH) is a value-added product derived from the enzymatic hydrolysis of fish proteins, offering both functional and bioactive properties that support applications in the food, nutraceutical, and pharmaceutical sectors (Chalamaiah et al., 2012). FPH can be produced from whole fish, edible muscle tissues, or fish processing by-products such as heads, frames, viscera, and skin — contributing to waste valorization and circular-economy initiatives within the seafood industry (Aspmo et al., 2005). Globally, fish processing generates an estimated 20–30 million tons of by-products annually, yet only 25–30% is currently utilized, highlighting a major opportunity for expanded FPH production (Aspmo et al., 2005).

The desirable functional properties of FPH include: (1) high solubility across a wide pH range (pH 2-12), owing to reduced molecular weight and increased hydrophilicity, facilitating incorporation into liquid food systems;; (2) emulsifying capacity, driven by amphiphilic peptide structures that stabilize oil-in-water emulsions; (3) foaming capability, resulting from viscoelastic interfacial film formation, useful in aerated products; (4) water-holding and fat-binding capacities, improving texture and moisture retention in processed foods; and (5) enhanced digestibility, making FPH suitable for clinical nutrition and early-life dietary formulations (dos Santos et al., 2011; Halim et al., 2016).

In addition, FPH has gained significant research interest due to its bioactive properties. Documented activities include: (1) antioxidant effects via radical scavenging and metal chelation, reducing oxidative stress and lipid oxidation (Senphan & Benjakul, 2014; Slizyte et al., 2016); (2) antihypertensive effects through angiotensin-I converting enzyme (ACE) inhibition (Elavarasan et al., 2014; Ketnawa et al., 2017; Salampessy et al., 2017); (3) antimicrobial activity against foodborne and spoilage microorganisms (Ngo et al., 2012); (4) immunomodulatory effects that support host defense mechanisms (Chalamaiah et al., 2012); and (5) anticancer properties, including the inhibition of tumor cell proliferation and induction of apoptosis (Hung et al., 2014; Picot et al., 2006).

The nutritional advantages of fish-derived protein hydrolysates over other protein sources are notable. FPH typically contains higher proportions of essential amino acids, particularly arginine, lysine, histidine, and leucine, which are critical for growth, immune function, and metabolic health (Taheri et al., 2013; Thiansilakul et al., 2007). The amino acid profiles show approximately 35-45% hydrophobic amino acids, contributing to superior solubility and bioavailability compared to plant or terrestrial animal protein hydrolysates (dos Santos et al., 2011).

Challenges and Future Perspectives

Despite significant advances in enzyme immobilization technology and the growing recognition of FPH value, several challenges must be addressed to facilitate the broader industrial adoption of immobilized protease systems for FPH production. Mass transfer limitations remain the primary technical constraint in immobilized enzyme systems. The requirement for substrates to diffuse through polymer matrices to reach enzyme active sites and for products to diffuse out creates concentration gradients that reduce apparent reaction rates by 30-70% compared to free enzyme systems. This limitation is particularly significant for FPH production, where the substrates are large protein molecules and aggregates. Although optimization strategies, such as reducing bead size, increasing agitation, and optimizing enzyme loading, can mitigate mass transfer effects, they cannot be completely eliminated without compromising bead mechanical stability or process practicality. Advanced matrix designs that incorporate hierarchical pore structures or channels can improve substrate accessibility while maintaining the retention of enzymes (Rodrigues et al., 2021).

Structural instability can also limit operational performance. Alginate–chitosan beads may undergo gradual degradation due to calcium ion leaching under low-ionic-strength conditions, mechanical abrasion during agitation or pumping, and microbial interference during extended processing. While chitosan coatings substantially enhance durability, further

improvements via controlled cross-linking, composite material engineering, or novel gelation strategies could extend bead performance beyond the currently reported 15–20 reuse cycles (Datta et al., 2012). Additionally, gradual activity loss during storage and repeated use can occur due to slow enzyme denaturation, fouling within the support matrix, microbial colonization, and polymer structural fatigue. Advancements in enzyme regeneration techniques, food-grade antimicrobial solutions, and mechanistic insights into performance decline are needed to improve long-term stability (Mohamad et al., 2015).

Another challenge is the need for substrate-specific optimization. Differences in protein composition, lipid levels, endogenous enzyme activity, and rheological properties across fish species and tissues require re-optimization of immobilization and hydrolysis conditions for each raw material source. Developing flexible, broadly applicable processing protocols would considerably enhance industrial scalability and technology transfer (Aspmo et al., 2005).

Looking ahead, several key trends are expected to drive innovation in immobilized protease systems for FPH production. Growing consumer interest in functional foods and personalized nutrition creates opportunities for the development of targeted FPH formulations addressing health priorities such as cardiovascular wellness, immune modulation, metabolic regulation, and sports performance. Immobilized protease systems—offering controlled hydrolysis and improved process efficiency—are well positioned to support such product specialization (Ngo et al., 2012). Additionally, increasing emphasis on sustainability reinforces the relevance of FPH as a waste-valorization strategy. Integrating immobilized-enzyme hydrolysis into circular biorefinery frameworks that recover proteins, lipids, minerals, and chitin from seafood by-products represents a promising pathway toward resource-efficient and environmentally responsible processing (Ketnawa et al., 2017).

CONCLUSION

This review highlights the application of alginate and chitosan as immobilization matrices for protease enzymes in fish protein hydrolysate (FPH) production, emphasizing both their considerable advantages and the challenges that remain. The complementary characteristics of these biopolymers make them particularly well suited for immobilization systems: alginate offers excellent biocompatibility, mild gelation conditions, and high porosity, while chitosan provides enhanced mechanical strength and superior enzyme retention. When combined, alginate–chitosan systems demonstrate robust performance, achieving 15–20 reuse cycles while maintaining 60–80% residual activity, thereby substantially lowering enzyme costs and improving process sustainability.

Importantly, immobilized protease systems can produce hydrolysates with functional and bioactive properties comparable to those generated using free enzymes, confirming that product quality is preserved. Enhanced thermal and pH stability further increases operational flexibility, while the reduced need for enzyme separation simplifies downstream processing.

Nevertheless, several technical challenges persist, including mass-transfer limitations, long-term mechanical degradation of immobilization matrices, and limited validation at industrial scale. Future progress is expected through innovations in matrix architecture, process optimization, and multi-enzyme immobilization strategies, as well as through the integration of advanced tools such as AI-driven process monitoring and control. These developments will help overcome current limitations and enable tailored FPH products aligned with emerging trends in personalized nutrition and the circular bioeconomy. With continued research and refinement, immobilized protease technology offers a promising and sustainable pathway for producing high-value marine protein ingredients, supporting both economic competitiveness and responsible utilization of seafood resources.

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