



## Microbial Aspartic Protease: Bio-synthesis, Characteristics, and Emerging Applications: A review

Sadeq Fenjan Hasnawi <sup>1,2</sup>, Alaa Jabbar Abd Al-Manhel <sup>1</sup>, Zena Kadhim AL-Younis <sup>1</sup>, Ammar B. Altemimi <sup>1\*</sup>, Tarek Gamal Abdelmaksoud <sup>3</sup>, Mohammad Ali Hesarinejad <sup>4\*</sup>

<sup>1</sup> Department of Food Science, College of Agriculture, University of Basrah, Basrah 61004, Iraq.

<sup>2</sup> Department of Animal Production, College of Agriculture, University of Misan, Misan 62004, Iraq

<sup>3</sup> Food Science Department, Faculty of Agriculture, Cairo University, Giza 12613, Egypt; tareekgamal.

<sup>4</sup> Food Processing Department, Research Institute of Food Science and Technology (RIFST), Mashhad, Iran.

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\*Corresponding Author E-Mail:  
ammar.ramddan@uobasrah.edu.iq;  
ma.hesarinejad@rifst.ac.ir

### ABSTRACT

Aspartic proteases play a vital role in diverse biological processes, with microbial variants garnering considerable attention for their commercial potential across various industries. This comprehensive review delves into the intricate aspects of microbial aspartic proteases, encompassing their biosynthesis, genetic regulation, secretion mechanisms, post-translational modifications, characteristics, and emerging applications. The biosynthesis of these proteases involves the translation of corresponding genes into precursor proteins, which undergo a series of processing steps culminating in the formation of mature enzymes. The efficiency of aspartic protease production and commercialization relies heavily on understanding the mechanisms governing their secretion. Notably, post-translational modifications, such as glycosylation and phosphorylation, play a significant role in influencing the activity and stability of these enzymes. The review further explores the substrate specificity and catalytic properties of microbial aspartic proteases, along with their potential applications in the food industry, pharmaceuticals, and biotechnology sectors. In the food industry, these proteases find utility in enhancing flavor, texture, and nutritional value. In the pharmaceutical realm, they contribute to drug discovery and development. Additionally, in biotechnology, microbial aspartic proteases exhibit versatility in applications such as protein engineering, peptide synthesis, and bioremediation. This comprehensive examination provides valuable insights into the multifaceted nature of these enzymes, shedding light on their biosynthesis, genetic regulation, secretion mechanisms, post-translational modifications, characteristics, and promising commercial applications.

## 1- Introduction

Proteases, also known as peptidases or proteinases,<sup>1</sup> are enzymes that break down proteins by cleaving peptide bonds. They play a critical role in numerous biological processes,<sup>2</sup> including digestion,<sup>3</sup> blood clotting,<sup>4</sup> and immune responses.<sup>5</sup> Proteases are also used in the regulation of cell growth and differentiation.<sup>6-7</sup> Therefore, they are essential for the normal functioning of living organisms.

Aspartic proteases belong to a group of proteases that are characterized by their dependence on two aspartic acid residues for catalytic activity.<sup>8</sup> These enzymes can be observed across all domains of life, spanning bacteria, fungi, plants, and animals.<sup>9</sup> Aspartic proteases have been extensively studied due to their involvement in various pathological conditions such as Alzheimer's disease,<sup>10</sup> cancer,<sup>11</sup> and viral infections.

Microbial aspartic proteases (APs) constitute a group of enzymes that play a vital role in catalyzing the hydrolysis of peptide bonds within proteins and peptides.<sup>12</sup> These enzymes are characterized by the presence of aspartic acid residues at their active sites. The historical significance of aspartic proteases is exemplified by their extensive utilization throughout history, with notable examples such as chymosin being employed in cheese production for thousands of years. Evidence of early milk processing techniques associated with microbial aspartic proteases dates back to 5500-2000 B.C. in the Libyan Sahara, where archaeological findings revealed pots containing cheese remnants in the tombs of Horus-aha, the second king of Egypt's first dynasty (3000-2800 B.C.). References to cheese production are also found in hieroglyphic texts from ancient Egypt. Furthermore, in China, the use of microbial aspartic proteases in soy sauce production can be traced as far back as the Zhou dynasty (1028-220 B.C.),

aligning with the construction era of the Great Wall. This historical context underscores the enduring and widespread application of microbial aspartic proteases in various culinary and cultural practices.<sup>13</sup>

APs have gained significant attention in the industrial and biotechnological fields due to their diverse and versatile properties.<sup>14</sup> They are widely distributed among bacteria, fungi, and yeasts, and their biosynthesis, characteristics, and applications have been studied extensively.<sup>15-16</sup> Microbial acid proteases exhibit promising potential across various domains of the food and feed industries. These applications encompass the hydrolysis of milk proteins (including casein and whey) to enhance cheese flavors in the dairy sector, as well as improving the texture, flavor, and color of dough in baked goods manufacturing within the baking industry. Furthermore, they play a role in of meat proteins, hydrolyzation of fish proteins, meat tenderization, and augmentation of the digestibility of animal feeds.<sup>13</sup>

The objective was to study the biosynthesis, characteristics, and emerging applications of these enzymes. The review aims to cover the latest developments in the field, including recent research findings, emerging trends, and potential applications. The study seeks to provide an explanation about some factors which may possess some influences on the biosynthesis and characteristics of microbial aspartic proteases, including their molecular structure, substrate specificity, and regulation mechanisms.

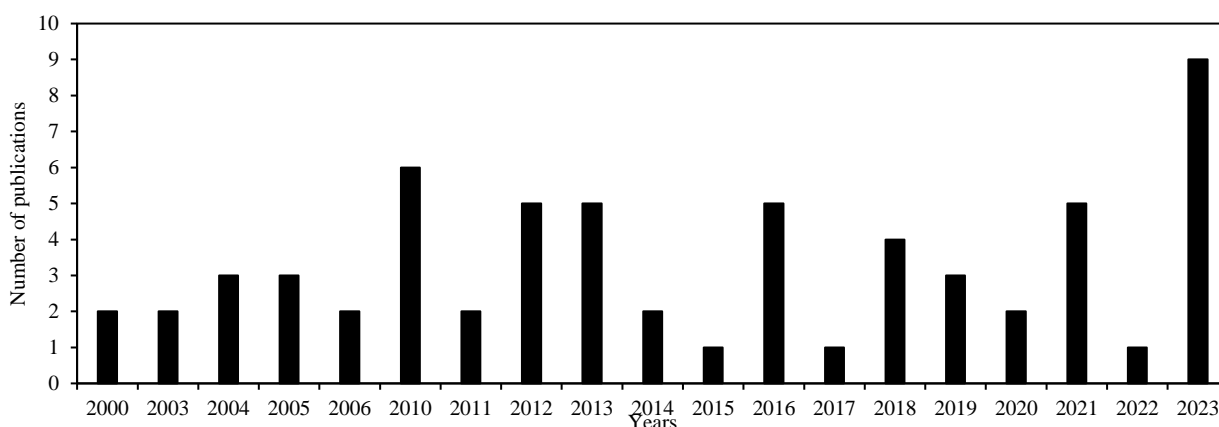
The review also aims to explore the emerging microbial aspartic proteases applications in various industries, including food, pharmaceutical, and biotechnology. This includes discussing the potential use of these enzymes as biocatalysts for the

bioactive peptides production, as well as their use in the production of fermented foods and protein hydrolysates. Overall, the study seeks to offer a comprehensive overview of microbial aspartic proteases, their characteristics, and their potential applications, highlighting their importance in various industries and their potential as a tool for biotechnological applications.

### 1. Review methodology

The investigators meticulously explored two databases, Web of Science and Scopus, situated in London, UK, aiming to compile data on "Microbial

Aspartic Proteases." They specifically concentrated on the "Food" subtopic, as illustrated in Figure 1, opting for Web of Science due to its indexed journals, extensive article collection, and user-friendly interface. Their initial keyword-based search, including terms like "Microbes," "Aspartic protease," "Industrial applications," and "Biosynthesis," yielded 1074 articles. The figure highlights a substantial increase in the field of food industry using Microbial Aspartic Proteases over past 23 years, with a noticeable increase in interest from 2000 to 2023 and a much greater rise in publications, approximately five per year.



**Fig. 1:** Comparing research papers on "microbes; aspartic protease, industrial applications, and biosynthesis" published over time in Web of Science and Scopus databases with focus on keywords

#### 1.1. Biosynthesis of microbial aspartic proteases

The present global enzyme demands are not being met by animal and plant proteases, which has resulted in an increased emphasis on microbial proteases. Microbial proteases are more desirable than animal and plant proteases for biotechnological applications because they possess the desired traits. Microorganisms are an outstanding enzyme source due to their broad range of biochemical variations and ease of genetic modification. Microbial sources contribute about 40% to the total enzyme sales worldwide. Though molds and yeasts contain aspartic protease, it is not commonly found in

bacteria. Since they almost perfectly match the criteria needed for their biotechnological uses, proteases derived from microbial sources are preferable to those from plant and animal sources.<sup>16</sup> However, genomic analysis of two bacterial strains, *Haemophilus influenzae* and *Escherichia coli*, uncovered that the recombinant proteins synthesized from the expression of their respective DNA regions function as active aspartic proteinases. In cheese production, *Bacillus subtilis* has emerged as a safe and genetically recognized acid protease (GRAS), gradually replacing chymosin. The protease from *B. subtilis* var. natto was observed to induce milk clotting. Additionally, a microbial aspartic protease

derived from *Bacillus amyloliquefaciens* found application in crafting miniature cheddar-style cheeses. Chymosin is rapidly being replaced in the production of cheese by the proteases produced by GRAS (genetically regarded as safe)-approved bacteria such *Mucor michei*, *Bacillus subtilis*, and *Endothia parasitica*.<sup>17-19</sup>

Fungi have a more extensive enzyme repertoire than bacteria, with *Aspergillus oryzae* being a notable example as it is capable of producing acid, neutral, and alkaline proteases. *Botrytis cinerea* was reported to produce aspartic acid proteases that have specific industrial uses.<sup>20</sup> Fungi capable of producing milk clotting enzymes are widespread and can be easily isolated from various environments.<sup>21</sup> The biosynthesis of microbial aspartic proteases involves several steps, including genetic regulation, enzyme synthesis, secretion, and post-translational modifications. Both external and intracellular aspartyl proteases are produced by fungi in the yeast or mycelium forms. In *Candida* spp., the secretory aspartyl protease (Sap) family has received substantial research and is regarded as a key virulence component. Yapsins (Yaps), the second class of extracellular aspartyl proteases, have a GPI moiety in their C-terminal region. Through this moiety, Yaps bind to the fungus cell's plasma membrane and appear to be involved in the construction, remodeling, and acquisition of nutrients; as a result, some of them are virulence factors. During nitrogen starvation and the sporulation process, mature vacuolar enzymes are essential for cell survival. Vacuolar aspartyl proteases, designated for the lysosomal vacuole, play a pivotal role, while pseudo vacuolar aspartyl proteases contribute to the activation of carboxypeptidases and other enzymes. Furthermore, these enzymes have the capability to be secreted.<sup>21</sup> Table 1 shows microbial sources for aspartic proteases.

### 1.2. Genetic regulation of aspartic protease production

The production of aspartic proteases is regulated at the genetic level, with several transcription factors and regulatory elements playing a role in the process.<sup>22</sup>

One of the most well-studied aspartic proteases is the human enzyme pepsin, which is produced in the stomach and is involved in the proteins digestion.<sup>23</sup> The pepsin production is regulated by many variables, for example the food presents in the stomach and the hormones activity such as gastrin and secretin. The gene encoding pepsin contains several regulatory elements, including a TATA box and multiple binding sites for transcription factors such as C/EBP and Sp1.<sup>24</sup>

In addition to pepsin, aspartic proteases are also produced by other organisms, such as plants, fungi and bacteria. The production of these enzymes is regulated by a variety of factors, including environmental cues and developmental signals. For example, in the yeast *Saccharomyces cerevisiae*, the production of the aspartic protease yapsin is regulated by the transcription factor Rlm1, which is activated by cell wall stress.<sup>25</sup>

The regulation of aspartic protease production is not limited to transcriptional control. Post-transcriptional and post-translational modifications also play a role in regulating the activity of these enzymes.<sup>26</sup> For example, in the fungus *Aspergillus niger*, the activity of the aspartic protease pepsin-like protease A is regulated by cleavage of the propeptide region, which is mediated by a separate protease.<sup>27</sup> Similarly, in humans, the activity of the aspartic protease cathepsin D is regulated by proteolytic processing and glycosylation.<sup>28</sup>

The regulation of aspartic protease production is a multifaceted and complex process involving biochemical and genetic mechanisms. Despite the

diversity of organisms that produce these enzymes, many of the regulatory elements and transcription factors involved in their production are conserved across different species. More researches are necessary to fully understand the molecular

mechanisms that govern aspartic protease production and activity, as well as their role in physiological processes.

**Table 1:** Microbial sources for aspartic proteases “class: Hydrolases, Acting on peptide bonds (peptidases)”

Aspartic protease name	EC enzyme code	Microbial source
HIV-1 retropepsin	3.4.23.16	HIV
Aspergillopepsin I	3.4.23.18	<i>Aspergillus</i> species (imperfect fungi)
Aspergillopepsin II	3.4.23.19	<i>Aspergillus niger</i> var. macrosporus
Penicillopepsin	3.4.23.20	imperfect fungus <i>Penicillium janthinellum</i>
Rhizopuspepsin	3.4.23.21	zygomycete fungus <i>Rhizopus chinensis</i> , <i>R. niveus</i>
Endothiapepsin	3.4.23.22	Ascomycete <i>Endothia parasitica</i>
Mucorpepsin	3.4.23.23	zygomycete fungi <i>Mucor pusillus</i> and <i>M. miehei</i>
Candidapepsin	3.4.23.24	Imperfect yeast <i>Candida albicans</i>
Saccharopepsin	3.4.23.25	<i>Saccharomyces cerevisiae</i>
Rhodotorulapepsin	3.4.23.26	<i>Rhodotorula glutinis</i> and <i>Cladosporium</i> sp.
Acrocylindropepsin	3.4.23.28	<i>Acrocylindrium</i> sp.
Polyporopepsin	3.4.23.29	Basidiomycete <i>Polyporus tulipiferae</i> (formerly <i>Irpex lacteus</i> )
Pycnoporopepsin	3.4.23.30	Basidiomycete <i>Pycnopus sanguineus</i>
Scytalidopepsin	3.4.23.31	<i>Scytalidium lignicolum</i>
Scytalidopepsin B	3.4.23.32	A second enzyme from <i>Scytalidium lignicolum</i> , <i>Ganoderma lucidum</i> (similar enzyme), <i>Lentinus edodes</i> (similar enzyme)
Cathepsin E	3.4.23.34	-
Barrierpepsin	3.4.23.35	<i>Saccharomyces cerevisiae</i>
Signal peptidase II	3.4.23.36	<i>Staphylococcus aureus</i> , <i>Enterobacter aerogenes</i> , <i>E. coli</i>
Pseudomonapepsin	3.4.23.37	<i>Pseudomonas</i> sp., <i>Xanthomonas</i> sp
Yapsin 1	3.4.23.41	<i>Saccharomyces cerevisiae</i>
Thermopsin	3.4.23.42	<i>Sulfolobus acidocaldarius</i> , a thermophilic archaeon
Prepilin peptidase	3.4.23.43	Many bacterium species have pili.
Nodavirus endopeptidase	3.4.23.44	derived from various insect pathogen nodaviruses
HIV-2 retropepsin	3.4.23.47	HIV-2
Plasminogen activator Pla	3.4.23.48	<i>Yersinia pestis</i> that causes plague
Omptin	3.4.23.49	<i>Escherichia coli</i> ompT gene product
Human endogenous Retrovirus K Endopeptidase	3.4.23.50	HIV-1
HycI peptidase	3.4.23.51	<i>Escherichia coli</i> <i>Thermoascus aurantiacus</i> Pleurotus sojur-caju (white rot fungi)
Aspartic proteases (Milk clotting)	-	<i>Metschnikowia reukaufii</i> <i>Thermomucor indicae-seudaticae</i> N31 <i>Enterococcus faecalis</i>

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*Myxococcus xanthus**Bacillus subtilis**Bacillus licheniformis**Nocardiopsis* sp.

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### **1.3. Mechanisms of aspartic protease secretion**

The mechanisms of microbial aspartic protease secretion involve multiple steps and components, including protein synthesis, folding, targeting, transport, and release.<sup>29</sup>

The first step in aspartic protease secretion is the synthesis of the protein itself.<sup>30</sup> This process is controlled by the genetic information encoded in the microbial DNA, which is transcribed into messenger RNA (mRNA) and then translated by ribosomes into the amino acid sequence of the protein. After synthesis, the protein must be correctly folded, which is facilitated by chaperone proteins that help guide the folding process and prevent misfolding or aggregation.<sup>31</sup> Once the protein is properly folded, it must be targeted to the appropriate location within the cell, a process mediated by specific signal sequences within the protein that are recognized by targeting factors. Regulatory proteins, including transcription factors and other regulatory factors, play a crucial role in controlling the expression of aspartic proteases. These proteins interact with specific DNA sequences within the promoter regions of the corresponding genes, thereby influencing their expression levels. In the context of aspartic proteases, several transcription factors have been identified as important regulators of their expression. Examples include CREB (cAMP response element-binding protein), which binds to cAMP response elements (CREs) in gene promoters, SP1 (Specificity protein 1), which binds to GC-rich motifs known as SP1 binding sites, and NF- $\kappa$ B (Nuclear factor kappa-light-chain-enhancer of activated B cells), which binds to specific DNA

sequences called  $\kappa$ B sites. In addition to transcription factors, specific DNA sequences within the promoter regions of aspartic protease genes act as regulatory elements, modulating gene expression. Promoter proximal elements, located close to the transcription start site, such as TATA boxes, GC-rich regions, and CAAT boxes, serve as binding sites for transcription factors like SP1 and contribute to the regulation of aspartic protease gene expression. Enhancers and silencers, which can be located at various distances from the transcription start site, interact with transcription factors and other regulatory proteins to influence gene expression. Response elements, such as cAMP response elements (CREs), can interact with specific transcription factors (e.g., CREB) in response to elevated intracellular cAMP levels, activating or repressing gene expression. The interaction of transcription factors and regulatory elements within the promoter regions of aspartic protease genes forms a complex regulatory network that determines their expression levels. The binding of specific transcription factors to their cognate binding sites and the modulation of their activity by signaling pathways contribute to the fine-tuned control of aspartic protease expression in various biological contexts.<sup>30-31</sup>

After the aspartic protease has been targeted to the cell membrane, it must be transported across the membrane to be secreted into the extracellular environment.<sup>32</sup> The transport process is facilitated by a dedicated transport system that recognizes the aspartic protease and moves it across the membrane. The dedicated transport system responsible for moving the aspartic protease across the membrane

as the endoplasmic reticulum-associated protein degradation (ERAD) pathway which involves a complex machinery of proteins that recognize the protease and facilitate its translocation across the ER membrane through a process called retrotranslocation. The proteasome degrades the protease. The cleavage of the propeptide is mediated by specific endoproteases such as furin, cathepsins, and other protease family members. Once the aspartic protease has been transported across the cell membrane, it is released into the extracellular environment, either passively or actively depending on the specific transport system involved. Understanding the complex mechanisms of microbial aspartic protease secretion is essential for

understanding the function of these enzymes in microorganisms and developing new therapeutic strategies to target them.<sup>33</sup>

The mechanisms of microbial aspartic protease secretion are a complex and multi-step process. It involves protein synthesis, folding, targeting, transport, and release. These enzymes are essential for microbial metabolism and pathogenesis, and understanding their mechanisms of secretion can lead to the development of new therapeutic strategies to target them. The study of aspartic protease secretion is an ongoing area of research, and further exploration of these mechanisms will shed light on the complex interactions between microorganisms and their environment.



**Fig. 2:** Microbial aspartic protease secretion steps

#### **1.4. Post-translational modifications of microbial aspartic proteases**

Post-translational modifications (PTMs) of aspartic proteases are modifications that occur after translation of the protein, which can influence the stability, activity and substrate specificity of enzyme.<sup>34</sup>

Glycosylation is the addition of a sugar molecule to the protein's amino acid residues. This modification can affect the enzyme's stability, solubility, and substrate specificity. For example, in the case of the human immunodeficiency virus (HIV) protease, glycosylation has been shown to enhance the enzyme's activity, stability, and resistance to

inhibitors.<sup>35-36</sup> Phosphorylation is the addition of a phosphate group to the protein's amino acid residues. This modification can regulate enzyme activity by altering the enzyme's conformation or by recruiting other proteins to the enzyme's active site. In the case of the *Candida albicans* secreted aspartic protease, phosphorylation has been shown to regulate the enzyme's activity and substrate specificity.<sup>38</sup> Acetylation is another PTM of microbial aspartic proteases.<sup>37,39</sup> Acetylation is the addition of an acetyl group to the protein's amino acid residues. This modification can alter the enzyme's activity by changing its conformation or by affecting its interactions with other proteins. Ubiquitination is the addition of a small protein called ubiquitin to the

protein's amino acid residues. This modification can target the enzyme for degradation by the proteasome or can regulate the enzyme's activity by altering its localization or interactions with other proteins. In the case of the *Saccharomyces cerevisiae* aspartic protease, ubiquitination has been shown to regulate the enzyme's turnover rate and substrate specificity.<sup>40-41</sup> Post-translational modifications of microbial aspartic proteases can significantly affect the enzyme's activity, stability, and substrate specificity. Glycosylation, phosphorylation, acetylation, and ubiquitination are some of the common PTMs observed in these enzymes. Understanding the effects of these modifications on microbial aspartic proteases can provide valuable insights into the biological processes these enzymes are involved in and can aid in the development of new therapeutic agents targeting these enzymes.

## 2. Applications of microbial aspartic proteases in industry

The industrial applications of microbial aspartic proteases have been studied extensively, and they are used in various industries, including food, pharmaceutical, and biotechnology.<sup>42</sup> Figure 3 illustrates the diverse applications of these industries, which will be better described in the following sections.

### 2.1. Application in Food industry

The primary reason for the high demand for acid proteases in the food industry lies in their exceptional ability to coagulate proteins, particularly milk proteins. This capability is particularly crucial as it facilitates the production of cheese, which represents the primary application of acid proteases in this sector. The coagulation of milk proteins results in the formation of solid masses, or curds, from which cheese can be produced by separating the liquid whey.

In comparison, the quality ranking of curd generated by *Rhizopus*-derived proteinase placed it in the second position. *B. subtilis* enzymes exhibited the highest proteolytic activity, while commercial rennet demonstrated the most potent milk-clotting enzymes.<sup>18</sup> Analysis through a scanning electron microscope (SEM) revealed that the microstructure of the curd formed by commercial rennet was denser, firmer, and smoother compared to the curds produced by the other two enzymes. Furthermore, fresh cheese produced with extracellular acid protease from *Aspergillus niger* FFB1 and reconstituted cow milk showcased similar attributes, including a pH of 4.5, an acidic taste, and a white color, when compared to cheeses made with calf rennet.<sup>43</sup> Additionally, the concentration of free amino acids (FAA) and physiochemical characteristics in Turkish white brined cheese produced using calf rennet and microbial rennet from *Rhizomucor miehei* were identified to be comparable.

Furthermore, fungal aspartic proteases have found extensive application in the realm of food development, particularly in enhancing flavors and optimizing protein-rich foods like bread. Following a 48-hour fermentation process of liquid dough derived from wheat flour, employing a blend of enterococci and *Rhizopus oryzae* proteases (referred to as dough B), it was observed that the concentration of water-soluble peptides in the dough was three times higher than in the control, which consisted of chemically acidified dough (CAD). Additionally, dough B exhibited a heightened concentration of free amino acids. Although gliadins in dough B were nearly completely degraded, the albumin and glutenin fractions remained incompletely hydrolyzed, as confirmed through SDS-PAGE analysis. In a distinct investigation, gluten treated with pepsin displayed a band of less than 10 kDa, whereas gluten treated with pronase,



chymotrypsin, and papain revealed two bands corresponding to 40 and 10 kDa in HPLC analysis. These findings suggest that gluten treated with pepsin contains fewer protease-resistant peptides compared to gluten treated with pronase, chymotrypsin, and papain.<sup>16</sup> Application of the acid protease from *Aspergillus usarii* to wheat gluten under optimal conditions resulted in the hydrolysis of wheat gluten, leading to a significant increase in its solubility. This enzymatic process also induced a notable enhancement in wheat gluten's emulsifying activity index (EAI), as well as its ability to retain water and oil. Analysis of the distribution of molecular weights indicated that the majority of peptides larger than 10 kDa were fragmented into smaller peptides. Furthermore, the functional characteristics of wheat gluten were improved following this hydrolysis process.<sup>44</sup>

To prevent haziness or turbidity in commercially produced fruit-based beverages, a common practice involves the clarification process. This aims to eliminate proteins responsible for the haze. In the production of fruit juices and certain alcoholic drinks, acid proteases derived from *A. saitoi*, especially aspergillopepsin I, are employed. Researchers have explored alternatives by introducing acid proteinase from *Saccharomyces fibuligera* 1570 and *Torulopsis magnoliae* 1536, along with brewer's yeast, in bench-scale fermentations at 200°C. Their observations indicate that the resulting beer displayed resistance to haze formation, with only a slight decrease in ethanol concentration.<sup>45</sup> Furthermore, the introduction of proteinase A at a concentration of 0.5 mU/ml into sweet wort and its incubation for 144 hours at 250 °C led to a notable reduction in the hydrophobic nature of the wort. Proteinase A demonstrated a capacity to decrease the hydrophobic character of high gravity sweet wort by approximately 47%, indicating a modification in hydrophobic attributes

rather than the molecular size of the wort polypeptide. Likewise, the application of commercial protease enzymes derived from *Bacillus subtilis* resulted in elevated levels of total soluble nitrogen,  $\alpha$ -amino nitrogen, wort color, and extract recovery during mashing with 100% raw barley malt. However, as the protease concentration increased, the efficiency of the enzyme diminished.<sup>46</sup>

Pinelo et al.<sup>47</sup> observed that adding a commercial protease (ENZECO fungal acid protease) from *A. niger* to cherry juice significantly reduced the immediate cloudiness of the juice. However, during cold storage, the impact on clarification was minimal. This finding paralleled results in the production of black currant juice, where commercially available acid proteases from *A. niger* and *Mucor miehei* were applied.<sup>48</sup> Byarugaba-Bazirake et al.<sup>49</sup> also observed similar outcomes when producing banana wine with the addition of commercially available proteases (Zumizyme). They found that the wines made from protease-treated juices had significantly lower turbidity compared to the control group. A longer incubation period resulted in greater reduction in turbidity, and protease addition also had a significant effect in reducing protein haze. The clarity of white wine is an important factor for consumers, making it crucial to maintain the wine's stability before bottling. A stable white wine is one that remains clear and free from any precipitates from the time of bottling to consumption. Hazy wine with precipitates is the result of microbial instability, tartrate instability, and protein heat instability. To achieve microbial stability before bottling, Sulfur dioxide is introduced, and filtration is conducted. Tartrate stability is attained through three approaches: cold stabilization, ion exchange resins, and electro dialysis, as detailed in reference.<sup>20</sup> Marangon et al.<sup>50</sup> attribute wine turbidity to the presence of

heat-unstable grape proteins. Under specific conditions, grape proteins can aggregate, forming particles that scatter light and induce haze. Pathogenesis-related (PR) proteins, including thaumatin-like proteins (TLPs) and chitinases, found in grapes play a significant role in wine haze formation. Although  $\beta$ -glucanases have been linked to haze formation, they are less prevalent in wine compared to TLPs and chitinases. However, the extent of their involvement in wine haze formation is not extensively explored. The subsequent Table (2) illustrates the potential application of Aspartic Protease in various food and feed industries.

### 2.2. Dairy Industry

Aspartic proteases are essential for cheese production, as they hydrolyze peptide bonds and coagulate milk proteins to create curds. They are also used in lactose reduction and flavour modification. Fungi such as *Aspergillus*, *Mucor*, *Entothia*, *Rhizopus*, *Penicillium*, and *Fusarium* are the primary producers of aspartic proteases. Solid-state fermentation (SSF) increases extracellular enzyme recovery and lowers process capital costs.<sup>26,51-55</sup>

### 2.3. Beer Industry

In this context, acid proteases are used to remove proteins from malt barley, facilitating the production of brewing wort. The challenge of hazes arising from proteinaceous compounds has grown more complex. Therefore, the utilization of the aspartic protease enzyme is essential to prevent the accumulation of insoluble complexes. Beer consumers highly prioritize characteristics like clarity, color, and froth, making haze formation a significant concern in beer manufacturing. The use of acidic protease effectively hinders the production of cold haze in bottled beer without compromising the desired qualities of beer foam.<sup>54-57</sup>

### 2.4. Wine Industry

Ensuring the stability of wine before bottling is crucial to meet customer expectations. However, the presence of heat-sensitive grape proteins, particularly pathogenesis-related (PR) proteins like thaumatin-like proteins (TLPs) and chitinases, can lead to haziness in the final wine products.<sup>20,58</sup>

### 2.5. Bakery Industry

Endo and exo-proteinases from *Aspergillus oryzae*, as well as fungal aspartic proteases, were used to enhance wheat gluten via restricted proteolysis. Gluten-free pasta, created through the fermentation of gluten-free sourdough with lactic acid bacteria and fungal proteases, demonstrated superior performance compared to durum wheat pasta. This was evident in chemical scores, biological value, essential amino acid profile, and nutritional index. Enzymatic hydrolysis of wheat gluten resulted in a notable increase in emulsifying activity index (EAI), molecular weight determination, as well as water and oil-holding capacity. The use of Prolyl endopeptidase from *Aspergillus niger* (AN-PEP) effectively eradicated all 9 immunogenic epitopes found in the 26-mer and 33-mer gliadin fragments within the stomach's pH range, and at a significantly lower dosage compared to digestive enzyme supplements.<sup>59-62</sup>

### 2.6. Soy protein degradation

The aspartic protease (Apa1) (molecular weight 50 kDA) demonstrated a robust activity level of 1500 U/mL. It exhibited optimal pH and temperature conditions and displayed stability within the temperature range of 30-60 °C and pH between 2.0-5.0. Successful expression in *P. pastoris* was achieved, resulting in the highest rate of protein hydrolysis observed for the degradation of soybean isolate protein. This discovery presents a novel

protease option well-suited for applications in the feed industry.<sup>61</sup>

### 2.7. Soy sauce production

In this research, the focus was on examining the essential enzymes and flavors produced by *Aspergillus oryzae* throughout the fermentation process of soy sauce. The findings revealed a synergistic relationship between protease and amylolytic enzymes in generating a variety of flavors, including alcohols, acids, esters, aldehydes, furans, and pyrazine.<sup>62</sup>

### 2.8. Meat production

*Rhizomucor miehei* CAU432 (a thermophilic fungus) was used to clone a new aspartic protease gene (RmproA), which was expressed in *Pichia pastoris*. The extracellular protease RmproA was effectively expressed in *P. pastoris*. High cell-density fermentation produced a high protease

activity of 3480.4 U/mL. The two-step methods resulted in the homogeneous purification of the protease. By using SDS-PAGE and gel filtration, it was determined that the RmproA's molecular mass was 50.6 kDa and 52.4 kDa, respectively. At pH 5.5 and 55 °C, respectively, the pure enzyme was most active. The enzyme's substrate selectivity was quite varied. At a relatively low concentration, pig muscle treated with RmproA had a lower shear force than a sample treated with papain, indicating that RmproA is efficient at rendering meat softer. Additionally, turtle hydrolysis by RmproA produced several short peptides with strong ACE-inhibitory properties. RmproA might therefore be a contender for a number of industrial uses. Inactive for 7 and 14 days at 4°C, *Aspergillus oryzae* aspartic protease is stored post-mortem under vacuum.<sup>63-64</sup> Finally Table 2 shows the Aspartic Protease application of in various feed and food industries.

**Table 2.** Application of Aspartic Protease in various food and feed industries

Food type	Effect or role	Source
Dairy Industry	Aspartic proteases are essential for cheese production, as they hydrolyze peptide bonds and coagulate milk proteins to create curds. They are also used in lactose reduction and flavour modification. Fungi such as <i>Aspergillus</i> , <i>Mucor</i> , <i>Entothia</i> , <i>Rhizopus</i> , <i>Penicillium</i> , and <i>Fusarium</i> are the primary producers of aspartic proteases. Solid-state fermentation (SSF) increases extracellular enzyme recovery and lowers process capital costs.	[26, 51, 52,53]
Beer Industry	In the brewing sector, acid proteases play a crucial role in eliminating proteins from malt barley to create brewing wort. The challenge of hazes has intensified due to proteinaceous compounds, prompting the use of the aspartic protease enzyme to prevent the formation of insoluble complexes. Consumers highly prize beer attributes such as clarity, color, and froth, making haze formation a significant concern in beer production. The application of acidic protease effectively hinders the occurrence of cold haze in bottled beer without compromising the desired characteristics of ideal beer foam.	[52-55]
Wine Industry	Ensuring the stability of wine before bottling is crucial for meeting customer expectations. However, the presence of grape proteins sensitive to heat, particularly	[20,56]

	pathogenesis-related (PR) proteins like thaumatin-like proteins (TLPs) and chitinases, can result in haziness in the final wine products.	
Bakery Industry	Endo and exo proteinases from <i>Aspergillus oryzae</i> , as well as fungal aspartic proteases, were used to enhance wheat gluten via restricted proteolysis. Gluten-free pasta created through the fermentation of gluten-free sourdough with lactic acid bacteria and fungal proteases, denoted as E-GFp, exhibited superior performance compared to durum wheat pasta (CDWp) across various parameters such as chemical scores, biological value, essential amino acid profile, and nutritional index. Enzymatic hydrolysis of wheat gluten resulted in a noteworthy increase in emulsifying activity index (EAI), molecular weight determination, as well as water and oil-holding capacity.	[52-57]
Soy protein degradation	Aspartic protease (Apa1) was found to have a high activity level of 1500 U/mL, 50 kDa molecular weight, optimal pH and temperature, and stable at pH 2.0–5.0 and 30–60 °C. It was successfully expressed in <i>P. pastoris</i> and achieved the highest protein hydrolysis rate to soybean isolate protein degradation. This provides a new protease suitable for the feed industry.	[61]
Soy sauce production	In this research, the focus was on exploring the primary enzymes and flavors produced by <i>Aspergillus oryzae</i> throughout the fermentation process of soy sauce. The findings revealed that protease and amylolytic enzymes work in tandem to contribute to the creation of flavors, including alcohols, acids, esters, aldehydes, furans, and pyrazines.	[62]
Meat production	At a relatively low concentration, pig muscle treated with aspartic protease gene (RmproA) had a lower shear force than a sample treated with papain, indicating that RmproA is efficient at rendering meat softer. Additionally, turtle hydrolysis by RmproA produced several short peptides with strong ACE-inhibitory properties. RmproA might therefore be a contender for a number of industrial uses. Inactive for 7 and 14 days at 4 °C, <i>Aspergillus oryzae</i> aspartic protease is stored post-mortem under vacuum.	[63,64]

### 3. Characteristics of microbial aspartic proteases

Aspartic proteases are a diverse class of enzymes with unique structural and functional properties, including substrate specificity, enzyme kinetics, and optimal pH and temperature. They play crucial roles in numerous biological processes and have significant implications for the design and the development of drug.<sup>65</sup> The active site is usually

covered by a flap or loop that controls substrate access, and it is located at the interface between the two  $\beta$ -barrels. The catalytic dyad is highly conserved among aspartic proteases, and any mutations to these residues usually result in the loss of enzyme activity. Aspartic proteases exhibit a pH-dependent activity profile, with maximal activity typically observed at acidic pH values.<sup>66</sup> The mechanism of catalysis involves the formation of a covalent intermediate between the enzyme and the substrate, followed by

its hydrolysis to release the products. Aspartic proteases show a high degree of substrate specificity and selectivity, with different enzymes recognizing specific peptide sequences or structures.<sup>67</sup> Some aspartic proteases, such as pepsin, have a preference for hydrophobic residues, while others, such as renin, prefer hydrophilic residues. The substrate specificity of aspartic proteases is affected by temperature, pH and inhibitors or activators.<sup>68</sup>

The activity of aspartic proteases is highly dependent on pH and temperature. The optimal pH for activity varies among different aspartic proteases, with some enzymes having a pH optimum as low as 1.5 (e.g., pepsin)<sup>69</sup>, while others have a pH optimum closer to neutral (e.g., cathepsin D). Similarly, the optimal temperature for activity varies, with some enzymes exhibiting maximal activity at relatively low temperatures (e.g., HIV-1 protease), while others have a higher temperature optimum (e.g., plasmepsin II). The activity of aspartic proteases can also be influenced by factors such as ionic strength and the presence of cofactors or inhibitors.<sup>70-72</sup>

#### **4. Conclusions, challenges and future directions**

In conclusion, aspartic proteases are one of the most significant subgroups of protease enzymes that can accelerate a wide range of metabolic processes in an acidic environment. Animals, plants, and microbes all frequently synthesize aspartic protease. Microorganisms are the ideal candidates for aspartic protease synthesis for a number of reasons. Aspartic proteases are employed throughout a variety of sectors, but they are most frequently found in the pharmaceutical, food, and beverage industries. Additionally, a variety of environmental and physiological conditions affect the aspartic protease's ability to produce itself at its highest level. As a result of more research, aspartic proteases have

a bright future in a variety of industrial applications. Future directions in aspartic protease research involve improving enzyme production techniques, identifying new enzyme sources, optimizing engineering techniques for enhanced catalytic properties, and developing sustainable and eco-friendly methods for production and purification. Combining aspartic proteases with other enzymes or bio-molecules is also a promising area that requires further exploration. In general, future trends in the study of microbial aspartic proteases may include: (a) Advanced genetic engineering and protein engineering techniques to optimize the biosynthesis and characteristics of microbial aspartic proteases for specific industrial applications. (b) Exploration of novel post-translational modifications and their effects on the activity, stability, and substrate specificity of aspartic proteases, opening new avenues for enzyme optimization and industrial use. (c) Integration of microbial aspartic proteases in emerging fields such as personalized nutrition, functional foods, and precision medicine, leveraging their ability to modify protein structures and enhance nutritional properties. (d) Utilization of microbial aspartic proteases in waste valorization and bioremediation processes, aiming to develop sustainable solutions for environmental and industrial challenges. (e) Collaborative research efforts focusing on the development of standardized protocols for enzyme characterization and industrial-scale production, ensuring reproducibility and scalability across diverse applications.

In essence, the study of microbial aspartic proteases holds significant promise for driving innovations in various industries while contributing to the sustainable and efficient use of natural resources. Further exploration and application of the knowledge encompassed in this review are poised to unlock novel opportunities and solutions in biotechnology and industrial processing.

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## پروتئاز آسپارتیک میکروبی: بیوسنتز، ویژگی ها و کاربردهای در حال ظهور: یک بررسی

صادق فنجان حسناوی<sup>۱</sup>، علاء جبار عبد المنهل<sup>۱</sup>، زینة کاظم الیونس<sup>۱</sup>، عمار بدران رمضان التمیمی<sup>۱\*</sup>، طارق جمال عبدالمقصود<sup>۲</sup>،

محمدعلی حصارى نژاد<sup>۴\*</sup>

۱- گروه علوم و صنایع غذایی، دانشکده کشاورزی، دانشگاه بصره، بصره، عراق

۲- گروه تولیدات دامی، دانشکده کشاورزی، دانشگاه میسان، میسان، عراق

۳- گروه علوم و صنایع غذایی، دانشکده کشاورزی، دانشگاه قاهره، جیزه، مصر

۴- گروه فرآوری مواد غذایی، موسسه پژوهشی علوم و صنایع غذایی، مشهد، ایران

### چکیده

### اطلاعات مقاله

پروتئازهای آسپارتیک نقش حیاتی در فرآیندهای بیولوژیکی متنوع ایفا می‌کنند و انواع میکروبی توجه زیادی را به دلیل پتانسیل تجاری خود در صنایع مختلف به خود جلب می‌کنند. این بررسی جامع به جنبه‌های پیچیده پروتئازهای آسپارتیک میکروبی می‌پردازد که شامل بیوسنتز، تنظیم ژنتیکی، مکانیسم‌های ترشح، تغییرات پس از ترجمه، ویژگی‌ها و کاربردهای در حال ظهور می‌شود. بیوسنتز این پروتئازها شامل ترجمه ژن‌های مربوطه به پروتئین‌های پیش‌ساز است که تحت یک سری مراحل پردازش قرار می‌گیرند که منجر به تشکیل آنزیم‌های بالغ می‌شود. کارایی تولید و تجاری سازی پروتئاز آسپارتیک به شدت به درک مکانیسم‌های حاکم بر ترشح آن‌ها بستگی دارد. قابل توجه است که تغییرات پس از ترجمه، مانند گلیکوزیلاسیون و فسفوریلاسیون، نقش مهمی در تأثیرگذاری بر فعالیت و پایداری این آنزیم‌ها دارند. این بررسی بیشتر به بررسی ویژگی سوبسترا و خواص کاتالیزوری پروتئازهای آسپارتیک میکروبی، همراه با کاربردهای بالقوه آنها در صنایع غذایی، داروسازی و بخش‌های بیوتکنولوژی می‌پردازد. در صنایع غذایی، این پروتئازها در افزایش طعم، بافت و ارزش غذایی مفید هستند. در حوزه داروسازی، آنها به کشف و توسعه دارو کمک می‌کنند. علاوه بر این، در بیوتکنولوژی، پروتئازهای آسپارتیک میکروبی تطبیق پذیری در کاربردهایی مانند مهندسی پروتئین، سنتز پپتید و زیست پالایی از خود نشان می‌دهند. این بررسی جامع، بینش‌های ارزشمندی را در مورد ماهیت چند وجهی این آنزیم‌ها ارائه می‌کند، بیوسنتز، تنظیم ژنتیکی، مکانیسم‌های ترشح، تغییرات پس از ترجمه، ویژگی‌ها و کاربردهای تجاری امیدوارکننده را روشن می‌کند.

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### کلمات کلیدی:

پروتئاز آسپارتیک؛

بیوسنتز؛

کاربردهای صنعتی؛

بیوتکنولوژی.

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\* مسئول مکاتبات:

ammam.ramddan@uobasrah.edu.iq;  
ma.hesarinejad@rifst.ac.ir