Review Article

The Role of Microbial Aspartic Protease Enzyme in Food and Beverage Industries

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Proteases represent one of the three largest groups of industrial enzymes and account for about 60% of the total global enzymes sale. According to the Nomenclature Committee of the International Union of Biochemistry and Molecular Biology, proteases are classified in enzymes of class 3, the hydrolases, and the subclass 3.4, the peptide hydrolases or peptidase. Proteases are generally grouped into two main classes based on their site of action, that is, exopeptidases and endopeptidases. Protease has also been grouped into four classes based on their catalytic action: aspartic, cysteine, metallo, and serine proteases. However, lately, three new systems have been defined: the threonine-based proteasome system, the glutamate-glutamine system of eqolisin, and the serine-glutamate-aspartate system of sedolisin. Aspartic proteases (EC 3.4.23) are peptidases that display various activities and specificities. It has two aspartic acid residues (Asp32 and Asp215) within their active site which are useful for their catalytic activity. Most of the aspartic proteases display best enzyme activity at low pH (pH 3 to 4) and have isoelectric points in the pH range of 3 to 4.5. They are inhibited by pepstatin. The failure of the plant and animal proteases to meet the present global enzyme demand has directed to an increasing interest in microbial proteases. Microbial proteases are preferred over plant protease because they have most of the characteristics required for their biotechnological applications. Aspartic proteases are found in molds and yeasts but rarely in bacteria. Aspartic protease enzymes from microbial sources are mainly categorized into two groups: (i) the pepsin-like enzymes produced by *Aspergillus*, *Penicillium*, *Rhizopus*, and *Neurospora* and (ii) the rennin-like enzymes produced by *Endothia* and *Mucor* spp., such as *Mucor miehei*, *M. pusillus*, and *Endothia parasitica*. Aspartic proteases of microbial origin have a wide range of application in food and beverage industries. These include as milk-clotting enzyme for cheese manufacturing, degradation of protein turbidity complex in fruit juices and alcoholic liquors, and modifying wheat gluten in bread by proteolysis.

1. Introduction

Enzymes are proteins produced by living organisms which catalyze the chemical reaction in greatly efficient ways and are environment friendly. They have substantial advantages over chemical catalysts, in its specificity, high catalytic activity, its capability to work at moderate temperatures, and the ability to be produced in large amounts [1]. The present high demand for better use of renewable resources and the burden on industry to work within an environment-friendly process encouraged the production of new enzyme-catalyst [1].

Proteases represent one of the three major groups of industrial enzymes and occupy 60% of the total global enzyme

sale [1]. They have a wide range of application in various industries to make a change in product taste, texture, and appearance and in waste recovery. Besides this, they have extensive applications in food industry, laundry detergents, leather treatment, bioremediation processes, and pharmaceutical industry. Their depolymerization activity also plays a major role in nutrition. The Novo industry of Denmark is among the major protease producers of the world, which occupies 40% of the market share of proteases. It produces three types of proteases, such as Aquaderm, NUE, and Pyrase, which are used for soaking, dehairing, and bating, respectively [1].

All cells, tissues, and organisms require proteolysis for growth and metabolism. Even a virus, the smallest nucleic

acid-based self-replicating organism, typically requires either host cell proteolysis or enzymes coded by its own genetic material to provide processing of initial viral gene products. Microorganisms can be used as an excellent source of protease. The incapability of plant and animal proteases to meet the current global enzyme demand increased the interest for the microbial protease. Microbial protease is preferred than other sources because they possess almost all the characteristics desired for their biotechnological applications [1].

According to the Nomenclature Committee of the International Union of Biochemistry and Molecular Biology, proteases are classified in enzymes of class 3, the hydrolases, and the subclass 3.4, the peptide hydrolases or peptidases. The term “peptidase” is recommended by the Nomenclature Committee of International Union of Biochemistry and Molecular Biology to be used as synonymous with “peptide hydrolase” for any of the enzyme that hydrolyzes peptide bonds [1, 3].

However, proteases do not act in accordance with the universal enzyme nomenclature system due to their high structural diversity and specificity. At present, proteases are classified depending on three major criteria: (i) type of reaction catalyzed, (ii) chemical nature of the catalytic site, and (iii) evolutionary relationship with reference to structure [1]. Currently, the term “peptidase” is also used equivalently with “protease” and “proteinase.” Peptidase was restricted to the enzymes included in subclasses EC 3.4.11–19, the exopeptidases in the Enzyme Nomenclature (1984), while the term “proteinases” was previously used for the enzymes included in subclasses EC 3.4.21–99 having the same meaning as “endopeptidase.” However, the terms “protease” and “proteinase” are still preferred by many scientists [3].

1.1. Classification of Protease. Proteases are generally categorized into two major groups based on their site of action, that is, exopeptidases and endopeptidases. Exopeptidases are those proteases that cleave the peptide bond proximal to the amino or carboxy termini of the substrate (cleave N- or C-terminal peptide bonds of a polypeptide chain), whereas endopeptidases cleave peptide bonds distant from the termini of the substrate (cleave internal peptide bonds) [3, 4]. Proteases are also classified into acid, alkaline, and neutral proteases based on the pH at which they are active [1].

On the basis of their catalytic action, protease has been also grouped into four categories as aspartic, cysteine, metallo, and serine proteases. However, recently, three new systems have been defined: the threonine-based proteasome system, the glutamate-glutamine system of eqolisin, and the serine-glutamate-aspartate system of sedolisin [2].

1.1.1. Serine Proteases. Serine proteases (EC 3.4.21) are described by having a serine group in their active site. They are abundant and common among viruses, bacteria, and eukaryotes, indicating that they are useful for the organisms. Serine proteases are found in the exopeptidase, endopeptidase, oligopeptidase, and omegapeptidase groups [4, 5]. Most of the neutral and alkaline protease that are commercial serine proteases are produced from bacteria of the genus *Bacillus*. Similar serine enzymes can also be produced

from other bacteria, such as *Thermus caldophilus* and *Desulfurococcus mucosus*, *Streptomyces*, *Aeromonas*, and *Escherichia*. Fungi species like *Aspergillus oryzae* similarly produce several serine proteases [1] (Table 1).

1.1.2. Cysteine/Thiol Proteases. Cysteine proteases (EC 3.4.22) occur in both prokaryotes and eukaryotes [4, 5]. There are about 20 families of cysteine proteases. The activity of all cysteine proteases was determined on a catalytic dyad consisting of cysteine and histidine. The order of Cys and His (Cys-His or His-Cys) residues varies among the families. Generally, cysteine proteases are active only in the presence of reducing agents, such as HCN or cysteine. Cysteine proteases are broadly categorized into four groups based on the specificity of their side chain: (i) papain like, (ii) trypsin like with preference for cleavage at the arginine residue, (iii) specific to glutamic acid, and (iv) others. Papain cysteine proteases have optimum activity at neutral pH, while a few of them like lysosomal proteases have maximum activity at acidic pH. They are sensitive to sulfhydryl agents such as PCMB but are not affected by DFP and metal-chelating agents [4]. Cysteine proteases are not so broadly distributed as was seen with serine and aspartic proteinases [1] (Table 1).

1.1.3. Aspartic Proteases. Aspartic proteinases (EC 3.4.23) or aspartyl proteinases are endopeptidases having two aspartic acid residues (Asp32 and Asp215, pepsin numbering) within their active site that are vital for their catalytic activity [7]. It is commonly known as acidic proteases [4].

Acidic proteases have been categorized into three families, that is, pepsin (A1), retropepsin (A2), and enzymes from pararetroviruses (A3). They have been placed in clan AA. Most of the aspartic proteases (Aps) show the best activity at low pH (pH 3 to 4) and have isoelectric points in the pH range of 3 to 4.5 [4]. They are inhibited by a hexapeptide from *Streptomyces* that contains two statin residues called pepstatin. Aspartic proteases are also sensitive to diazoacetyl-DL-norleucine methyl ester (DAN) and 1, 2-epoxy-3-(*p*-nitrophenoxy) propane (EPNP) in the presence of copper ions. Microbial acid proteases exhibit specificity against aromatic or bulky amino acid residues on both sides of the peptide bond, which is similar to pepsin, but their action is less stringent than that of pepsin [4, 7].

Acid proteases represent an essential group of enzymes, extensively used in food, beverage, and pharmaceutical industries. For most of these applications, the crude enzymes should at least partially purified and free from substances that could alter the characteristics of the product [8].

Aspartic protease enzymes from microbial sources are mainly categorized into two groups: (i) the pepsin-like enzymes produced by *Aspergillus*, *Penicillium*, *Rhizopus*, and *Neurospora* and (ii) the rennin-like enzymes produced by *Endothia* and *Mucor* spp., such as *Mucor miehei*, *M. pusillus*, and *Endothia parasitica* [4, 6] (Tables 1 and 2).

1.1.4. Metalloproteases. Metalloproteases (EC 3.4.24) are highly diversified types of proteases. They contain enzymes

TABLE 1: The characteristics of four types of protease [6].

Properties	EC number	Molar mass range (kDa)	pH optimum	Temperature optimum	Metal requirement (s)	Active amino acid(s)	Major inhibitor(s)	Major sources
Aspartyl or carboxyl protease	3.4.23	30–45	3–5	40–55	Ca ²⁺	Aspartate or cysteine	Pepstatin	<i>Aspergillus</i> , <i>Mucor</i> , <i>Endothia</i> , <i>Rhizopus</i> , <i>Penicillium</i> , <i>Neurospora</i> , animal tissue (stomach)
Cysteine or thiol protease	3.4.22	34–35	2–3	40–55	—	Aspartate or cysteine	Iodoacetamide, <i>p</i> -CMB	<i>Aspergillus</i> , stem of pineapple (<i>Ananas comosus</i>), latex of fig tree (<i>Ficus</i> sp), papaya (<i>Carica papaya</i>), <i>Streptococcus</i> , <i>Clostridium</i>
Metalloprotease	3.4.24	19–37	5–7	65–85	Zn ²⁺ , Ca ²⁺	Phenylalanine or leucine	Chelating agents such as EDTA and EGTA	<i>Bacillus</i> , <i>Aspergillus</i> , <i>Penicillium</i> , <i>Pseudomonas</i> , <i>Streptomyces</i>
Serine protease	3.4.21	18–35	6–11	50–70	Ca ²⁺	Serine, histidine, and aspartate	PMSF, DIFP, EDTA, soybean trypsin inhibitor, phosphate buffers, indole, phenol, and triamino acetic acid	<i>Bacillus</i> , <i>Aspergillus</i> , animal tissue (gut), <i>Tritirachium album</i> (thermostable)

from different origins, such as collagenases from higher organisms, hemorrhagic toxins from snake venoms, and thermolysin from bacteria. For their actions, they require divalent metal ion. A total of about 30 families of metalloproteases have been documented, out of which 17 contain only endopeptidases, 12 contain only exopeptidases, and 1 (M3) contains both endo- and exopeptidases [4, 5] (Table 1).

1.2. Mechanism of Action of Aspartic Protease. Aspartic proteases (EC 3.4.23) are peptidases that exhibit various activities and specificities. They are found in animals, plants, fungi, and viruses. Aspartic proteases (Aps) have been connected to a wide range of physiological functions, including mammalian digestion of nutrients (e.g., chymosin and pepsin A), defense against pathogens, yeast virulence (e.g., candidapepsins), metastasis of breast cancer (e.g., cathepsin D), pollen-pistil interactions (e.g., cardosin A), control of blood pressure (e.g., renin), degradation of hemoglobin by parasites (e.g., plasmepsins), and maturation of HIV proteins (retropepsin) [11].

Aspartic proteases (Aps) belong to the A1 pepsin family structurally. They are synthesized as proenzymes similar to other pepsin enzymes. The proenzyme is secreted and autocatalytically activated after cleavage of the signal peptide. Commonly, the active enzymes contain a single peptide chain of about 320–360 amino acid residues with a molecular mass of 32–36 kDa. Aps mostly have a β -strand secondary structure arranged in a bilobal conformation as confirmed by X-ray crystallographic analyses [11]. The two lobes are homologous

to each other and have evolved by gene duplication. The catalytic center is located between the two lobes and contains a pair of aspartate residues, one in each lobe, that are essential for the catalytic activity [11]. The retropepsin molecule has only one lobe, which consists of only one aspartic residue, and the activity requires the formation of a noncovalent homodimer [4]. Generally, aspartic endopeptidases depend on its aspartic acid residues for their catalytic activity [4].

In pepsin family enzymes, the catalytic Asp residues are mostly contained in an Asp-Thr-X motif, where X is Ser or Thr. The role of these Asp residues is to activate a water molecule that facilitates the nucleophilic attack on peptide bond of the substrate and also to find another water molecule that is used in substrate binding by the formation of hydrogen bond. The catalytic center is enough to accommodate as a minimum of seven residues of the polypeptide substrate. A flexible structure (flap) found at the entry of the catalytic site controls the specificity of the enzyme [11]. APs are mostly active at acidic pH. The optimum pH of aspartic protease is determined by the electrostatic potential at the active site, which in turn is determined by the position and orientation of all residues near the active site [11] (Table 2).

2. Microbial Source of Aspartic Protease

The failure of the plant and animal proteases to meet the present world demands of the enzyme has directed to an increased interest in microbial proteases. The presence of desired characteristics for biotechnological applications in microbial protease enzymes helps it to be preferred over

TABLE 2: Characteristics of various microbial aspartic proteases (EC 3.4.23) [9, 10].

Number	Enzyme number	Accepted name	Other names	Source	Substrate	Optimum pH	Optimum temp	Comment
1	EC 3.4.23.8	Yeast proteinase A	Saccharopepsin	—	—	—	—	Now EC 3.4.23.25
2	EC 3.4.23.9	<i>Rhizopus</i> acid proteinase	Rhizopuspepsin	—	—	—	—	Now EC 3.4.23.21
3	EC 3.4.23.10	<i>Endothia</i> acid proteinase	Endothiapepsin	—	—	—	—	Now EC 3.4.23.22
4	EC 3.4.23.16	Retropepsin	HIV aspartyl protease, HIV proteinase, retroproteinase, HIV-1 protease, HIV-2 protease	HIV	Viral gag and gag-pol protein precursor	5.5–6.0	30 (37°C)	
5	EC 3.4.23.18	Aspergillopepsin I	Proteinase, <i>Aspergillus</i> acid protease, <i>Aspergillus</i> acid proteinase, <i>Aspergillus</i> aspartic proteinase, <i>Aspergillus</i> carboxyl proteinase, denapsin, proctase B, P, proteinase B, sumizyme AP, trypsinogen kinase, pepsin-type aspartic proteinase, carboxyl proteinase	Found in a variety of <i>Aspergillus</i> species (imperfect fungi)	Hydrolysis of proteins with broad specificity	Differs from substrate to substrate but is in the range of 1.6–6.5	Differs but in the range of 30–50	Formerly included in EC 3.4.23.6
6	EC 3.4.23.19	Aspergillopepsin II	Proteinase A, proctase A, <i>Aspergillus niger</i> var. <i>macrosporus</i> aspartic proteinase, nonpepsin-type acid proteinase	Isolated from <i>Aspergillus niger</i> var. <i>macrosporus</i>	Preferential cleavage in B chain of insulin	—	30°C	Formerly included in EC 3.4.23.6
7	EC 3.4.23.20	Penicillopepsin	Peptidase A, <i>Penicillium janthinellum</i> aspartic proteinase, acid protease A, <i>Penicillium</i> X acid proteinase	From the imperfect fungus <i>Penicillium janthinellum</i>	Hydrolysis of proteins with broad specificity similar to that of pepsin A, preferring hydrophobic residues	Differs but in the range of 2.5–3.6	Differs but in the range of 50–75	Formerly EC 3.4.23.7; formerly included in EC 3.4.23.6

TABLE 2: Continued.

Number	Enzyme number	Accepted name	Other names	Source	Substrate	Optimum pH	Optimum temp	Comment
8	EC 3.4.23.21	Rhizopuspepsin	<i>Rhizopus</i> aspartic proteinase, neurase, proteinase, <i>Rhizopus</i> acid protease, <i>Rhizopus</i> acid proteinase	From the zygomycete fungus <i>Rhizopus chinensis</i> , <i>R. niveus</i> activates trypsinogen	Hydrolysis of proteins with broad specificity, prefers hydrophobic residues, clots milk, and activates trypsinogen	3–4 (trypsinogen); 3.5–4.0 (casein)	Differs from substrate to substrate but in the range of 25 and 50	EC 3.4.23.9 (formerly), EC 3.4.23.6 (formerly included in)
9	EC 3.4.23.22	Endothiapepsin	<i>Endothia</i> aspartic proteinase, <i>Endothia</i> acid proteinase, <i>Endothia parasitica</i> acid proteinase, <i>Endothia parasitica</i> aspartic proteinase	From the ascomycete <i>Endothia parasitica</i>	Hydrolysis of proteins with specificity similar to that of pepsin A, prefers hydrophobic residues	—	30°C	EC 3.4.23.10 (formerly), EC 3.4.23.6 (formerly included in)
10	EC 3.4.23.23	Mucorpepsin	Proteinase, <i>Mucor</i> acid proteinase, <i>Mucor</i> acid protease, <i>Mucor</i> rennin, <i>Mucor</i> aspartic proteinase, <i>Mucor</i> pusillus emporase fromase 100, <i>Mucor</i> pusillus rennin fromase 46TL, <i>Mucor miehei</i> rennin	Isolated from the zygomycete fungi <i>Mucor pusillus</i> and <i>M. miehei</i>	Hydrolysis of proteins, favouring hydrophobic residues, clots milk	Differs but in the range of 3.5–5.6	Differs but in the range of 40–63	EC 3.4.23.6 (formerly included in)
11	EC 3.4.23.24	Candidapepsin	<i>Candida albicans</i> aspartic proteinase, <i>Candida albicans</i> carboxyl proteinase, <i>Candida albicans</i> secretory acid proteinase, <i>Candida olea</i> acid proteinase, <i>Candida</i> aspartic proteinase, <i>Candida olea</i> aspartic proteinase	Imperfect yeast <i>Candida albicans</i>	Hydrolyzed protein (preferential cleavage at the carboxyl of hydrophobic aa, activates trypsinogen, and degrades keratin)	Varies between 2.5 and 5.5	42°C (denatured hemoglobin); 45 (at pH 3.0)	EC 3.4.23.6 (formerly included in)
12	EC 3.4.23.25	Saccharopepsin	Yeast endopeptidase A, <i>Saccharomyces</i> aspartic proteinase, aspartic proteinase yscA, proteinase A, proteinase yscA, yeast proteinase A, <i>Saccharomyces cerevisiae</i> aspartic proteinase A, yeast proteinase A, PRA	<i>Saccharomyces cerevisiae</i>	Hydrolysis of proteins with broad specificity for peptide bonds	4–6.5	25°C	EC 3.4.23.8 (formerly), EC 3.4.23.6 (formerly included in)

TABLE 2: Continued.

Number	Enzyme number	Accepted name	Other names	Source	Substrate	Optimum pH	Optimum temp	Comment
13	EC 3.4.23.26	Rhodotorulapepsin	Rhodotorula aspartic proteinase, <i>Cladosporium</i> acid protease, <i>Paecilomyces</i> proteinase, <i>Rhodotorula glutinis</i> aspartic Proteinase, <i>Rhodotorula glutinis</i> aspartic, <i>Rhodotorula glutinis</i> acid proteinase	<i>Rhodotorula glutinis</i> and <i>Cladosporium</i> sp.	Cleaves benzyloxycarbonyl-Lys- + -Ala-Ala-Ala and activates trypsinogen	2.0–2.5 (casein), 2.5–2.7 (casein, hemoglobin), 2.5–3.0 (acid-denatured hemoglobin)	Differs between 55 and 60°C	Formerly included in EC 3.4.99.15
14	EC 3.4.23.28	Acrocylindropepsin	Acrocylindricum proteinase, Acrocylindrium acid proteinase	<i>Acrocylindrium</i> sp.	Preference for hydrophobic residues at P1 and P10 and action on the B chain of insulin	2.0 (casein)	—	EC 3.4.99.1 (formerly), EC 3.4.23.6 (formerly included in)
15	EC 3.4.23.29	Polyporopepsin	Polyporus aspartic proteinase, <i>Irpex lacteus</i> aspartic proteinase, <i>Irpex lacteus</i> carboxyl proteinase B	Basidiomycete <i>Polyporus tulipiferae</i> (formerly <i>Irpex lacteus</i>)	Milk-clotting activity, broad specificity, but fails to cleave Leu15-Tyr or Tyr16-Leu of insulin B chain	2.8 (hemoglobin), 4.0 (Phe-Leu-Ala-Ala)	—	—
16	EC 3.4.23.30 synonyms	Pycnoporopepsin	Proteinase ia, <i>Pycnopus coccineus</i> aspartic proteinase, trametes acid proteinase	Basidiomycete <i>Pycnopus sanguineus</i> , formerly known as <i>P. coccineus</i> and <i>Trametes sanguinea</i>	Cleaving only three bonds in the B chain of insulin: Ala ¹⁴ + Leu, Tyr ¹⁶ + Leu, and Phe ²⁴ + Phe	—	—	EC 3.4.99.25 (formerly), EC 3.4.23.6 (formerly included in)
17	EC 3.4.23.31	Scytalidopepsin	<i>Scytalidium</i> aspartic proteinase A, <i>Scytalidium lignicolum</i> aspartic proteinase, <i>Scytalidium lignicolum</i> carboxyl proteinase, <i>Scytalidium lignicolum</i> acid proteinase	<i>Scytalidium lignicolum</i>	Hydrolysis of proteins with specificity similar to that of pepsin A but also cleaves Cys (SO ₃ H) ⁷ Gly and Leu17 Val in the B chain of insulin	2–3.5 (casein), 3.6 (benzyloxycarbonyl-Phe-Glu-Ala-Ala)	50 and 55	—

TABLE 2: Continued.

Number	Enzyme number	Accepted name	Other names	Source	Substrate	Optimum pH	Optimum temp	Comment
18	EC 3.4.23.32	Scytalidopepsin B	<p><i>Scytalidium</i> aspartic proteinase B, <i>Ganoderma lucidum</i> carboxyl proteinase, <i>Ganoderma lucidum</i> aspartic proteinase, <i>Scytalidium lignicolum</i> aspartic proteinase B, SLB</p>	<p>A 2nd enzyme from <i>Scytalidium lignicolum</i>, <i>Lentinus edodes</i> (similar enzyme), <i>Ganoderma lucidum</i> (similar enzyme)</p>	<p>Hydrolysis of proteins with broad specificity, cleaving Phe24 Phe, but not Leu15-Tyr and Phe25-Tyr in the B chain of insulin</p>	2.0–2.7 (casein), 2.9–3.2 (hemoglobin)	50, 52, and 65	—
19	EC 3.4.23.33	Xanthomonapepsin	<p><i>Xanthomonas</i> aspartic proteinase <i>Xanthomonas</i> aspartic PCP, pseudomonas carboxyl proteinase</p>	<p><i>Pseudomonas</i> sp., expression in <i>E. coli</i>, <i>Xanthomonas</i> sp</p>	—	2.7 (casein, acid-denatured hemoglobin), 3 (acid-denatured hemoglobin, casein)	50 and 55	Now transferred to EC 3.4.21.101, xanthomonalisin
20	EC 3.4.23.35	Barrierpepsin	Barrier proteinase, bar proteinase	<i>Saccharomyces cerevisiae</i>	Selective cleavage of -Leu ⁶ + Lys- bond in the mating pheromone a-factor	5–5.3	—	—
21	EC 3.4.23.36	Signal peptidase II	<p>Premurein-leader peptidase, prolipoprotein signal peptidase, leader peptidase II, leader peptidase II</p>	<p><i>E. coli</i>, <i>Enterobacter aerogenes</i>, <i>Staphylococcus aureus</i></p>	<p>Hydrolyzes -Xaa-Yaa-Zaa+ (S, diacylglyceryl)Cys-, in which Xaa is hydrophobic (preferably Leu) and Yaa (Ala or Ser) and Zaa (Gly or Ala) have Small, neutral side chains</p>	6	37	—
22	EC 3.4.23.37	Pseudomonapepsin	<p><i>Pseudomonas</i> sp. pepstatin-insensitive carboxyl proteinase, pepstatin-insensitive carboxyl proteinase</p>	<p><i>Pseudomonas</i> sp., <i>Xanthomonas</i> sp.</p>	<p>Hydrolysis of the B-chain of insulin at Glu13-Ala-, Leu15-Tyr-, Phe25-Tyr-, and angiotensin I at Tyr4-Ile. A good synthetic substrate is Lys-Pro-Ile-Glu-Phe-(4-nitro) Phe-Arg-Leu)</p>	3 (acid-denatured hemoglobin, casein)	50	Now EC 3.4.21.100, pseudomonalisin
23	EC 3.4.23.41	Yapsin 1	Yeast aspartic protease 3, Yap3	<i>Saccharomyces cerevisiae</i>	Hydrolyzes various precursor proteins with Arg or Lys in P1, and commonly Arg or Lys also in P2	—	—	—

TABLE 2: Continued.

Number	Enzyme number	Accepted name	Other names	Source	Substrate	Optimum pH	Optimum temp	Comment
24	EC 3.4.23.42	Thermopsin	—	Thermophilic archaeon <i>Sulfolobus acidocaldarius</i>	Similar in specificity to pepsin A preferring bulky hydrophobic amino acids in P1 and P10	—	—	—
25	EC 3.4.23.43	Prepilin peptidase	—	Many species of bacteria carry pili	Typically cleaves a -Gly+ Phe-	—	—	—
26	EC 3.4.23.44	Nodavirus endopeptidase	Black Beetle virus endopeptidase, flock house virus endopeptidase	From several nodaviruses that are pathogens of insects	Hydrolysis of an asparaginyl bond, typically -Asn+ Ala- or -Asn+ Phe-	—	—	—
27	EC 3.4.23.47	HIV-2 retropepsin	—	HIV-2	—	—	—	—
28	EC 3.4.23.48	Plasminogen activator Pla	—	<i>Yersinia pestis</i> that causes plague	Converts human Glu-plasminogen to plasmin by cleaving the Arg560 + Val peptide bond, also cleaves arginyl bonds in other proteins	—	—	—
29	EC 3.4.23.49	Omptin	Protease VII, protease A, ompT protease, protein a, protease VII, OmpT	A product of the ompT gene of <i>Escherichia coli</i>	Has a virtual requirement for Arg in the P1 position	—	—	—
30	EC 3.4.23.50	Human endogenous retrovirus K endopeptidase	Human endogenous retrovirus K10 endopeptidase, endogenous retrovirus HERV-K10 putative protease, human endogenous retrovirus K retropepsin	HIV-1	Cleavage of the -SQNY+ PIVQ- cleavage site	—	—	—
31	EC 3.4.23.51	HycI peptidase	HycI, HycE processing protein	<i>Escherichia coli</i>	Removes a 32-aa acid peptide from the C-terminus of the precursor of the hydrogenase 3	—	—	—

TABLE 3: Microbial sources of milk-clotting aspartic proteases [13].

Microorganisms	Properties
<i>Pleurotus sojur-caju</i> (white rot fungi)	Clotting activity under cheese-making conditions
<i>Mucor bacilliformis</i>	High structural similarity to bovine chymosin lower thermostability than <i>Rhizomucor miehei</i> protease
<i>Thermoascus aurantiacus</i>	Enzymatic hydrolysis of bovine casein differed largely from proteolysis patterns generated by bovine chymosin
<i>Thermomucor indicae-seudaticae</i> N31	Crude enzymatic extract showed high milk-clotting and low proteolytic activity and low thermostability
<i>Metschnikowia reukaufii</i>	Milk-clotting activity, successfully cloned into <i>Escherichia coli</i>
<i>Myxococcus xanthus</i>	Molecular mass: 40 kDa, highest clotting activity at pH 6 and 37°C, acceptable yield and properties of the curd in cheese-making experiments, successfully cloned into <i>Escherichia coli</i>
<i>Enterococcus faecalis</i>	Similar electrophoretic patterns of hydrolyzed k-casein as <i>Rhizomucor miehei</i> , effectively applied for camembert cheese manufacture
<i>Nocardiopsis</i> sp.	Milk-clotting ability of extracellular extracts, optimization of enzyme yield by fermentation conditions
<i>Bacillus subtilis</i>	Ratio of milk clotting to proteolytic activity is comparable with commercial fungal protease but has high thermostability
<i>Bacillus licheniformis</i>	Shows typical milk-clotting kinetics

plant and animal proteases. Microorganisms represent an excellent source of enzymes owing to their wide biochemical diversity and their susceptibility to genetic manipulation. About 40% of the total global enzyme sales are from microbial sources [4]. Aspartic protease is found in molds and yeasts, but rarely in bacteria [6].

2.1. Fungal Aspartic Proteases. Fungi produce a wider range of enzymes than do bacteria. For instance, *Aspergillus oryzae* produces all types of proteases such as acid, neutral, and alkaline proteases. The pH ranges (pH 4 to 11) of fungi protease are wide, and this shows their broad substrate specificity. However, they have a lower reaction rate and low heat tolerance than do the bacterial enzymes. Fungal enzymes can be conveniently produced in a solid-state fermentation process [4].

Due to the global scarcity of calf chymosin, fungal aspartic proteases (Aps) have been used as milk-clotting enzymes in the dairy industry for about 30 years. The aspartic protease enzymes produced from *Mucor miehei*, *Mucor pusillus*, and *Cryphonectria (Endothia) parasitica* and marketed under the trade names Rennilase®, Fromase®, Novoren®, Marzyme®, Hannilase®, Marzyme®, and Suparen® are usually used for the production of various types of cheeses [11]. The production of aspartic acid proteases that have a certain industrial application was also reported from *Botrytis cinerea* [12]. An alternative for milk clotting enzyme for cheese production was also reported from more than 100 fungal sources. Fungi that produce milk clotting enzyme are universal and easily isolated from various environments [13].

Most of these extracellular fungal aspartic proteases produced from *Aspergillus* species. These include *Aspergillus oryzae*, *Aspergillus fumigatus*, *Aspergillus saitoi*, *Aspergillus*

awamori, and *Aspergillus niger* [5]. Aspartic proteases from *Candida albicans* have been intensively studied due to its role in various forms of candidiasis. The presence of secreted aspartic protease enzyme in *C. albicans* contributes to its virulence factor. The major proteases secreted in vitro by *C. albicans*, *C. parapsilosis*, and *C. tropicalis* have been termed as Sap2, Sapp1, and Sapt1, respectively [5].

A different study has revealed that *C. albicans* have at least eight secreted aspartic protease genes (SAP genes). Out of these, SAP2 gene is the leading form expressed in a number of strains. SAP2 encodes 398-residue pre-protein that is processed to a 342-residue mature enzyme, a typical aspartic proteinase having an optimum pH between 3 and 4 and inhibited by pepstatin A [14]. The optimum pH for fungal aspartic proteases is between 4 and 4.5; however, they are stable at a pH range between 2.5 and 6.0. Fungal aspartic protease enzymes are specifically useful in the cheese-making industry due to their narrow pH and temperature specificities [4] (Tables 2 and 3).

2.2. Bacteria. It has been widely assumed that bacteria do not produce clotting enzymes due to only a few research works conducted on bacteria [15]. But the study conducted on the genomes of two bacteria, *Escherichia coli* and *Haemophilus influenzae*, showed that the recombinant proteins resulting from the expression of each of these DNA regions are active aspartic proteinases [15].

A novel retropepsin-like enzyme (APRc) has been also found in two pathogenic species of *Rickettsia* such as *R. conorii* and *R. rickettsia*. This APRc enzyme is particularly inhibited by drugs clinically used to treat HIV infections, and therefore, this enzyme can be used as a target for

therapeutic intervention. This implies that a retropepsin type of aspartic protease enzymes is found in prokaryotes, signifying that these enzymes may represent an ancestral form of these proteases [16]. Acid protease produced by *Bacillus subtilis*, which is GRAS (genetically regarded as safe), is gradually replacing chymosin in cheese making, and protease produced from *B. subtilis* var. *natto* has showed milk clotting [4, 17]. Microbial aspartic protease produced from *Bacillus amyloliquefaciens* was also used for the production of miniature cheddar-type cheeses [18] (Tables 2 and 3).

2.3. Viruses. The concern to viral proteases has been started as a result of its involvement in the cause of certain fatal diseases, such as AIDS and cancer. Several types of viruses have serine, aspartic, and cysteine peptidases. All of the virus-encoded peptidases are endopeptidases, but there are no metallopeptidases in viruses [4]. The crystal structures of aspartic proteases from retroviruses such as HIV and Rous sarcoma have been widely studied and determined since 1989 [5]. The aspartyl proteases of retroviruses have a great role in viral assembly and replication. The aspartyl proteases of retroviruses are homodimers and expressed as a part of the polyprotein precursor. The mature protease is released by autolysis of the precursor [4].

Microbes serve as an ideal source of protease enzymes, even though they are widespread in nature. The rapid growth, the lesser space required for cultivation, and the ease for genetic manipulation to generate new enzymes with improved properties make microbes desirable for protease enzyme production [4] (Table 2).

3. Application of Microbial Aspartic Protease

The proteases have been considered as one of the most essential groups of enzymes in enzyme industry and have various applications in different industries, such as detergents, foods, pharmaceuticals, and leather [19].

Microbial acid proteases have an application mostly in three industries: food, beverage, and pharmaceuticals [5]. However, there is limited evidence available on the application of aspartic proteinases other than cheese industry that has been the preferred area of application to date [7].

3.1. Application in Dairy Industry. The major application of acid proteases is for cheese production in dairy industry. The microbial milk-coagulating proteases belong to a class of acid aspartate proteases and have molecular weights between 30,000 and 40,000 [4]. The major role of acid proteases in cheese production is to hydrolyze the specific peptide bond (the Phe105-Met106 bond) to generate para-K-casein and macropeptides. Chymosin is preferred due to its high specificity for casein, which accounts for its exceptional performance in cheese production. The aspartic proteases produced by microbes such as *Mucor miehei*, *B. subtilis*, and *Endothia parasitica*, which is GRAS (genetically regarded as safe), are gradually replacing chymosin in cheese making [4].

The enzymatic coagulation of milk is a two-phase process; any variation in the chemical environment can affect

the two phases of reaction separately [7]. In the first phase, calf rennet (CAR) and most microbial proteases clot milk with the cleavage of K-casein at the Phenylalanine105-Methionine 106 bond, which liberates hydrophilic glycopeptide (residues 106–169) that enters into the whey and para-K-casein (hydrophobic) [7, 20]. Proteinase from *Cryphonectria parasitica* cleaves the S104-F105 bond. Rennin can also hydrolyze other milk proteins (α s1-, α s2-, and β -caseins and α -lactalbumin) at a lower rate. Fungal proteases cause extensive nonspecific hydrolysis of both K-casein and para-K-casein as compared to rennin in which its activity is limited to the hydrolysis of K-casein with the formation of only macropeptide and para-K-casein [20].

The second phase is nonenzymatic in which para-K-casein and other caseins aggregate under the influence of Ca^{2+} , which eventually leads to gel formation. These two steps of milk-clotting activity overlap with each other where the aggregation of micelles starts before the end of the enzymatic process [20].

The production of UF (ultrafiltrated) white soft cheese using fungal rennin (1 ml fungal rennin/100 ml milk) from *Rhizomucor miehei* NRRL 2034 in the laboratory showed very close properties to calf rennet cheese used as a control. The cheese produced with fungal rennet revealed better values of soluble nitrogen (SN), total volatile fatty acids (TVFAs), tyrosine, and tryptophan than control cheese. Moreover, the sensorial examination conducted on cheese produced with fungal rennet showed that the experimental cheese had a soft body, smooth texture, and desirable taste during cold storage for 2 months [21, 22].

The production of miniature cheddar-type cheeses using microbial rennet from *Bacillus amyloliquefaciens* (clotting enzyme (MCE)) and calf rennet (CAR) did not have significant differences in gross composition with the exception of pH. The level of α s1-casein and β -casein hydrolysis determined by urea-PAGE was equivalent for both cheese samples. The concentration of peptides in 2 cheese samples was increased during the course of ripening. However, the ratio of hydrophobic to hydrophilic peptides was higher in CAR-C than in MCE-C. The MCE-C was softer than CAR-C as a result of higher protein hydrolysis. Microbial rennet from *B. amyloliquefaciens* contributed to higher photolytic rates that reduced ripening time [18].

In the study that compares the milk-clotting activity of proteinase produced by *B. subtilis* var. *natto*, *Rhizopus oligosporus*, and commercial rennet, the curd formed by the commercial rennet had the highest viscosity and curd tension and the shortest clotting time followed by the curd produced by proteinase from *Rhizopus* among the three enzymes. The highest proteolytic activity was recorded by the enzymes from *B. subtilis*, while the highest milk-clotting enzymes were noticed in commercial rennet. Observations of microstructures by scanning electron microscope (SEM) showed that the three-dimensional network of curd formed by commercial rennet was denser, firmer, and smoother than others [17]. Fresh cheese produced using an ochratoxin-free extracellular acid protease from *Aspergillus niger* FFB1 and reconstituted cow milk as a substrate showed similar basic characteristics (pH 4.5, acid taste, and white

color) as cheeses produced with calf rennet [23]. The concentration of free amino acid (FAA) and physiochemical characteristics were similar in the Turkish, white, brined cheese produced using calf rennet and microbial rennet from *Rhizomucor miehei* [24].

The experimental study conducted on cheese produced by calf rennet and microbial rennet from *Rhizomucor miehei* after 90 days of ripening period revealed that the physico-chemical characteristics and the contents of the total bitter tasting amino acids (Phe, Leu-Ile, Val, and Pro) were similar for both types of cheeses. Phe, Leu-Ile, Gln, Val, Pro, and Ala were the principal free amino acids (FAAs) in the white cheeses at all stages of ripening [25].

The study during 2 months under pickling conditions of domiati cheese produced using the *M. mucedo* KP736529 enzyme (E-cheese) and commercial calf rennet (C-cheese) as control showed that the yield and chemical properties of E-cheese were better than those of C-cheese. The fungal enzyme (MCE) showed higher proteolytic activity than calf rennet with the absence of bitter taste defects. The organoleptic scores of E-cheese were also higher than C-cheese [26].

In the study conducted at Hyderabad, the milk-clotting enzyme produced from *Rhizomucor miehei* was subjected to ammonium sulfate and acetone precipitation and then used for cheese production. The result indicates that higher quantity of cheese (280 g/500 ml milk) was obtained by ammonium sulfate precipitation than with acetone precipitation (220 g/500 ml milk). But the milk curdling time was found to be rapid with the enzyme obtained by acetone precipitation than that obtained by saturated ammonium sulfate precipitation [27].

3.2. Application in the Bakery Industry. Wheat flour is a major component of baking processes. It comprises an insoluble protein called “gluten” that regulates the dough property. Endo and exoproteases from *Aspergillus oryzae* have been applied to improve the wheat gluten by limited proteolysis [4]. Moreover, fungal aspartic proteases have also been broadly used in the production of food seasonings and the improvement of protein-rich foods such as bread and related foodstuffs [5].

The fermentation of liquid dough made from wheat flour with the combination of enterococci and *Rhizopus oryzae* proteases (dough B) for 48 h revealed that it had threefold higher concentration of water-soluble peptides than the chemically acidified dough (CAD) used as the control. The concentration of free amino acids was also being higher in dough B. The SDS-PAGE analysis showed that gliadins were almost fully degraded in dough B, while albumin and glutenin fractions were incompletely hydrolyzed [28]. In another study, gluten treated with pepsin had shown a band less than 10 kDa, while gluten treated with pronase, chymotrypsin, and papain showed two bands corresponding to 40 and 10 kDa by HPLC analysis. These results suggest that less protease-resistant peptides exist in gluten treated with pepsin as compared to gluten treated with pronase, chymotrypsin, and papain [29].

The experimental gluten-free pasta (E-GFp) produced from gluten-free sourdough after fermentation by lactic acid

bacteria and fungal proteases showed higher chemical scores, essential amino acid profile, biological value, and nutritional index than those of durum wheat pasta (C-DWp). This might be due to proteolysis during sourdough fermentation. The in vitro protein digestibility of E-GFp has also resulted in the highest value. The sensory characteristic of E-GFp was acceptable as shown by sensory analysis [30].

The hydrolysis of wheat gluten by the acid protease from *Aspergillus usamii* under optimized conditions has greatly increased the solubility of wheat gluten. Enzymatic hydrolysis of wheat gluten resulted in a radical increase in emulsifying activity index (EAI), water, and oil-holding capacity. The molecular weight determination study also showed that most of the peptides above 10 kDa have been hydrolyzed into smaller peptides. Furthermore, the functional properties of wheat gluten improved after hydrolysis [31].

The treatment of nine immunogenic epitopes of the 26-mer and 33-mer gliadin fragments by prolyl endopeptidase from *Aspergillus niger* (AN-PEP) successfully degraded all the nine epitopes in the stomach pH range at considerable lesser dosage than the digestive enzyme supplements. The digestive enzyme supplements showed comparable proteolytic activities with near neutral pH optima and modest gluten detoxification properties as determined by ELISA [32].

3.3. Application in the Beer Industry. The visual aspects of beer such as clarity, color, and foam are vital for consumers. Foam affects the consumers' views, flavor, and mouth feel about the beer. Brewers desire the presence of sufficient, stable, white, and finely textured foam to satisfy consumers' concern [33].

Haze formation is a major problem in beer production, as it affects the qualities of the end product [34]. During the production of beer, proteins and polyphenols extracted from the plant tissue may interact and form haze [35]. Beer haze comprises numerous components: the most common organic parts are proteins (40–75%), polyphenols (in combination with proteins), and, to a smaller percentage, carbohydrate (2–15%) [34]. The group of proteins that contributes to haze formation is called “cystine-rich proteoses” (albumins and barley hordeins) [36].

There are two types of haze: the cold break (chill haze) and the age-related haze. The cold break haze is formed at 0°C and disappears at higher temperatures. If cold break haze does not dissolve, age-related haze, which is irreversible, develops. Chill haze is produced after polypeptides and polyphenols bound noncovalently. Permanent haze is produced in a similar way primarily, but covalent bonds are soon formed and insoluble complexes are created that will not dissolve when heated [34]. Both chill haze and age-related haze formation are not desired by the consumers, as they show the oldness and staleness of beer and alter the physical stability of beer [36, 37].

The production of undesirable chill haze in the final beer product can be removed by several ways. These include hydrolyzing the undesirable proteins in finishing the operation, adsorbing proteins using silica adsorbents or silica

hydrogels, and/or using polyvinylpyrrolidone to remove polyphenols that contribute to protein condensation reactions [36]. The application of proline-specific endoproteinases (i.e., Brewers Clarex®, DSM, France) that target on the degradation of haze-active proteins (i.e., hordeins) reduces the formation of storage haze in final beer product. These hydrolyzed proteins are unable to condense with polyphenols and hence do not form haze [37]. The addition of a brewer's yeast that secretes a proteinase enzyme that can degrade haze-forming proteins into brewer's wort during wort fermentation is also a possible alternative to remove chill-proofing beer [36].

The addition of acid proteinase from *Saccharomycopsis fibuligera* 1570 and *Torulopsis magnoliae* 1536 along with brewer's yeast into brewer's wort in bench-scale fermentations conducted at 200°C showed that the final bottled beer was resistant to haze formation with a slight reduction in the final ethanol concentration [36]. The addition of proteinase A (0.5 mU/ml concentration) to the sweet wort and incubating for 144 h at 250°C also showed a significant decrease in hydrophobic character of wort. Moreover, the activity of proteinase A contributed for about 47% reduction in hydrophobic nature of high gravity sweet wort. This result implies that proteinase A alters the hydrophobic character rather than the molecular size of the wort polypeptide [34]. Similarly, the use of commercial protease enzyme from *Bacillus subtilis* correspondingly increased the level of total soluble nitrogen, levels of α -amino nitrogen, wort color, and extract recovery levels in wort when mashing with 100% raw barley malt. However, as the level of protease increased, the efficiency of the protease decreased [38].

The incubation of beer wort with acidic proline-specific protease from *Aspergillus niger* in small-scale brewing experiment extensively hydrolyzed the proline-rich proteins and produced a peptide fraction unable to form a haze. Subsequent pilot plant trials also verified that the addition of this acidic enzyme even at low levels during wort fermentation efficiently inhibits chill haze formation in bottled beer. Results from the determination of beer foam stability indicated that the enzyme treatment did not affect the beer foam [35].

The addition of protease enzyme during beer fermentation also has a significant benefit beyond haze removal. The expression of aspartyl protease in recombinant yeast used for fuel ethanol production improves the capability of the yeast to metabolize soluble proteins and leads to significant increase in ethanol production. Furthermore, the recombinant yeast strains exhibited advanced growth rate, viability, and lower yields of by-products such as glycerol and pyruvic acid [39]. Likewise, the use of the multicomponent protease enzyme (Flavourzyme) with brewer's yeast strain *Weihenstephan* 34/70 for beer fermentation showed a significant increase in nitrogen availability during the course of beer fermentation [40].

3.4. Application in Wine Industry. The production of clear wine, particularly for white wines, is one of the very important parameters from the consumer's perspective. Therefore, maintaining the stability of wine before bottling is a challenging and crucial step in the winemaking process.

A stable white wine is a clear wine that is free from any precipitates at the time of bottling till to consumption. Hazy wine with precipitate is formed as a result of microbial instability, tartrate instability, and protein heat instability [41]. Microbial stability is attained prior to bottling by the addition of sulfur dioxide and filtration, whereas tartrate stability is accomplished by three techniques, such as cold stabilization, ion exchange resins, and/or electrodialysis [41].

Heat unstable grape protein could remain and cause hazy appearance in final wine products. Grape proteins in wines that exist as unstable under certain conditions can aggregate into light-dispersing particles and cause wines to appear turbid. Specifically, grape pathogenesis-related (PR) proteins, such as thaumatin-like proteins (TLPs) and chitinases, contribute for wine haze formation [42]. Other proteins like β -glucanases have also been associated with haze formation, while they are much less abundant than chitinases and TLPs in wine. But the role of β -glucanases in wine haze formation is not widely studied [41].

The mechanism of haze formation is started by the unfolding and aggregation of grape-derived wine proteins. The experimental study proved that wine protein unfolding and aggregation are the two discrete events occurred in wine processing. Heat experiment has shown that proteins can unfold as soon as the wine is heated, while haze is formed after wine cooling [41].

In commercial winemaking, the stability of proteins is accomplished by the addition of bentonite. Bentonite is a clay cation exchanger that binds with proteins and eliminates them from wine through precipitation, and it has been commonly used in oenology as a fining agent since the 1930s [41, 42]. Proteins that bound with bentonite settle to the bottom of wine tanks as lees, and it covers 3–10% of the total wine volume. Wine is recovered from bentonite lees using rotary drum vacuum filtration, specialized lees filtration equipment, or centrifugation processes [41]. Bentonite fining has some negative characteristics such as dilution of the wine by the bentonite slurry, elimination of wine flavor, high labor expenses, trouble in bentonite spent disposal, and loss of wine quality [42].

For the abovementioned reasons, alternative methods for stabilizing white wine have been extensively studied [41, 42]. Several alternatives have been suggested, like the use of other adsorbents, ultrafiltration, and flash pasteurization, but none of them has proven suitably effective to replace bentonite. One ideal solution to this issue would be the use of proteolytic enzymes capable to degrade the heat unstable proteins [42].

Acid protease enzyme is suitable for the degradation of the turbidity complex produced from proteins in fruit juices and alcoholic liquors [6]. Some fungal aspartic proteases have been used to hydrolyze proteins that cause turbidity in juices and wine. These are the protease BcAP8 from *Botrytis cinerea* and the aspergillopepsin I from *Aspergillus saitoi* (commercially marketed as Molsin F by Kikkoman Corp., Japan), both used in the winery as they successfully remove haze-forming proteins and hence reducing bentonite requirements [19]. The addition of AGP (a combination of Aspergillopepsin I (EC 3.4.23.18) and Aspergillopepsin II

(EC 3.4.23.19)) into two clarified grape juices (Chardonnay and Sauvignon blanc juices) with heat treatment (at 750°C, 1 min) and without heat treatments prior to fermentation exhibited about 20% total protein reduction as compared to the control wine. However, it showed the best activity when the enzymes were combined with juice heating ($\approx 90\%$ total protein reduction). But the more heat-stable grape proteins (i.e., those do not contribute to wine hazing) were not affected by the treatments and hence account for the remaining 10% of proteins still found in the solution after the treatments. The major physicochemical parameters and sensorial characteristics of wines produced with AGP were comparable with the control [42].

The application of Aspergilloglutamic peptidase (AGP) (commercially known as Proctase and formerly known as Aspergillopepsin II) to clarified grape juice prior to flash pasteurization and fermentation resulted in heat-stable wines that were completely free from haze-forming proteins. The results of chemical and sensory analysis also indicated that there were no significant changes in physicochemical parameters of wine preference. This combined use of protease with flash pasteurization has been shown to be effective at industrial scale, and consequently, the use of AGP in wine has been recently accepted in Australian winemaking. The overall cost of AGP application is comparable to bentonite treatment, and this makes AGP a potentially cost-effective and commercially viable alternative for bentonite [41].

The clarification of blackcurrant juice with acid protease (Enzeco and Novozyme 89L) after precentrifugation and cold storage showed a significant reduction in haze development. The addition of Enzeco protease (conc. 0.025 g/L) and gallic acid (conc. 0.050 g/L) into blackcurrant juice and allowing it to react in the juice for 90 min at 50°C showed the lowest levels of haze formation after 28 days of storage at 20°C [43]. In other studies, the treatment of cherry juice with protease (Enzeco, enzyme preparations derived from *Aspergillus* spp.) resulted in a considerable reduction in immediate turbidity but had low clarification impact during the subsequent cold storage [44]. The combined treatment of wines with heat (90°C for 1 minute) and enzymes (Trenolin blank, a mixed pectolytic and proteolytic enzyme solution and porcine pepsin) also reduced 40%–80% of the protein level in wines [45].

The treatment of banana juice extracted from the banana pulp by two selected commercial protease enzymes (zumi-zyne and papaine) during banana wine making indicated that the wine turbidity was significantly reduced as compared to the control. These proteases had also shown a substantial effect on the protein haze removal after periods of one week and four weeks. Moreover, a longer incubation period leads to a higher decrease in turbidity. Based on the sensorial analysis, the overall organoleptic quality of the wine did not show significant variation than the control ($p > 0.05$) [46].

The application of BcAP8 (*Botrytis* aspartic protease) from the grape fungal pathogen *Botrytis cinerea*, into Australian Semillon and Sauvignon blanc juices, noticeably degraded chitinase, a major class of haze-forming proteins without heat denaturation. Therefore, BcAP8 could potentially benefit winemakers by removing haze-forming

proteins under normal winemaking conditions [12]. The extracellular acid protease secreted by *Saccharomyces cerevisiae* PIR1 during alcoholic fermentation was also found to be active against grape proteins (molecular mass ≈ 25 kDa) at 38°C and pH 3.5 [47].

In another study, the wines treated with protease enzyme have been shown to have a higher amino acid contents than the nonenzyme-treated wines except for arginine and histidine. These results indicated that protease treatment could enhance the concentration of assimilable nitrogen, which was one of the important nutrients for yeast in wine fermentation [48].

4. Conclusions

Aspartic protease enzyme has a wide range of application in food and beverage industries, such as cheese industry, bakery industry, and beer and wine industry. The microbial aspartic protease enzyme produced from bacteria and fungi were used for the production of curd in cheese manufacturing, for haze removal in brewery and winery, and for modification of bread in the bakery.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

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