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Marine Enzymes Biotechnology: Production and Industrial Applications, Part III - Application of Marine Enzymes

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Se-Kwon Kim and Fidel Toldrá

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Marine Enzymes Biotechnology: Production and Industrial Applications, Part III - Application of Marine Enzymes

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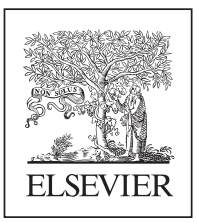

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CONTENTS

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PREFACE

In the last decades, progress in the knowledge of marine enzymes has advanced exponentially. The growing interest on marine enzymes is related to their relevant properties that make them quite attractive because somehow they are different from the well-known terrestrial enzymes. Marine organisms may have to face extreme environmental conditions, and this makes that most of their enzymes be active and stable under extreme conditions like very high or very low temperatures, high pressure, tolerance to high salt concentration, stability to acid or basic pH, and easy adaptation to cold conditions. All these properties make marine enzymes very attractive for new catalytic reactions and, of course, new applications in food and nutrition. In view of this increased interest, *Advances in Food and Nutrition Research* publishes three consecutive volumes focused on the topic *Marine Enzymes Biotechnology: Production and Industrial Application*. Parts I and II were published in 2016 and the third part is published in 2017. Volume 78 corresponded to Part I that was mainly dealing with the production of enzymes from marine sources, volume 79 corresponded to Part II dealing with the marine organisms producing enzymes, and this volume 80 corresponds to Part III dealing with the applications of marine enzymes.

This volume brings 10 chapters reporting the applications of enzymes produced by marine organisms. So, this includes the potential use of enzymes derived from marine sources as therapeutic agents for cancer therapy or other potential biological applications relevant to human health as well as the biomedical applications of enzymes from marine actinobacteria, the biotechnological applications of marine enzymes from algae, bacteria, fungi, and sponges, the use of enzymes from *Bacillus subtilis* to facilitate the nutrients assimilation from unconventional and economic plant resources in aquaculture marine animals, the use of marine probiotic enzymes to improve host digestion and cleave molecular signals involved in quorum sensing in pathogens to control disease in aquaculture, the economic production of actinobacterial enzymes from agricultural wastes as a better alternative for utilization of biomass, the action of marine enzymes for bioremediation of industrial wastes and for the development of efficient processes for bioethanol production, and the use of marine enzymes in

fermented fish products. This volume presents the combined effort of 30 professionals with diverse expertise and background. The Guest Editors wish to thank the publisher production staff and all the contributors for sharing their experience and for making this book possible.

S.-K. Kim and F. Toldrá
Guest Editors

1. INTRODUCTION

Fermented fish products are very popular in Southeast Asian countries; however, the products are actually also found in other parts of the world. Fermentation of fish is an ancient technology that has already been employed by our ancestors a long time ago. The processing is traditionally used to overcome the perishable nature of fish. Fermented fish is an old staple food in European cuisines; for instance, the ancient Greeks and Romans made a famous fermented fish product called "garum." The product has pasta form and very strong smell. Garum is made through a fermentation process of entrails and blood of mackerel (Ching, Mauguin, & Mescle, 1992; Gildberg, Simpson, & Haard, 2000; Skåra, Lars Axelsson, Stefansson, & Ekstran, 2015).

Fermented fish products usually have special consumers because of their ability to provide a certain unique characteristic, especially in terms of aroma, flavor, and texture. This is due to a transformation of organic materials into compounds which are simpler by the activity of microorganisms or enzymes that are encountered in fish muscle tissue during the fermentation process (Beddows, 1998).

The interest of consumers for fermented fish products is primarily due to the specific flavor generated which can induce appetite. In the case of Indonesia, a variety of flavors produced by fermented fish products can actually satisfy the tastes of consumers outside the area of origin of the product. Unfortunately most of fermented fish products are still local and not so easily found nationwide. Only some types of fermented fish products have been widely known, such as fish sauce and shrimp paste (Irianto, 2012).

The ones contributing the most in flavor formation and the changes in texture in fermented fish products are enzymes. Besides enzymes, microorganisms that contribute to the fermentation process also assist in the formation of aroma and flavor (Beddows, 1998). Many researchers from around the world have already explored the enzymes in fermented fish products and pay attention to uncover their role in the fermentation process.

2. ENZYMES IN FISH

Naturally, fish contains enzymes which are distributed in the whole body of the fish. The blood, certain tissues, muscles, and glands, such as kidney, contain very active enzymes (Marsh & Flick, 2012). Enzyme action

often causes significant deteriorative changes prior to noticeable spoilage of bacterial origin (Ghaly, Dave, Budge, & Brooks, 2010). Autolytic enzymes are present at much higher concentration in the viscera and head than in other tissues (Owens & Mendoza, 1985). During rigor mortis, the acidity of the tissues decidedly increases and this change of the hydrogen ion concentration causes an acceleration of autolytic decomposition. Enzymes are most active in dilute solution and do not act in the absence of water. Most of them are destroyed or rendered inactive by concentrated salt solution (Gildberg et al., 2000; Ponce de Leon Valdivia, 1994).

The proteolytic activity is mainly caused by tissue proteases and, to a lesser degree, by gut proteases (Lindgren & Pleje, 1983). Based on the mode of catalysis, proteolytic enzymes from the fish may be classified into: (a) aspartic proteases (pepsin and cathepsin D); (b) serine proteases (trypsin and chymotrypsin); (c) cysteine proteases (calpain, m-calpain, and cathepsins B, H, L); and (d) metalloprotease (Sriket, 2014). Aspartic and alkaline proteases have been found from the viscera and stomach of sardine (Bougatef, Souissi, Fakhfakh, Ellouz-Triki, & Nasri, 2007; Khaled et al., 2011; Salazar-Leyva et al., 2013).

Klomklao (2008) proposed proteases to be classified on the basis of their similarity to well-characterized proteases, such as trypsin-like, chymotrypsin-like, chymosin-like, or cathepsin-like. Trypsins play major roles in biological processes including digestion, activation of zymogens of chymotrypsin, and other enzymes (Cao, Osatomi, Hara, & Ishihara, 2000). Serine collagenases or trypsin-like proteinase were found in the intestines of Atlantic cod, *Gadus morhua* (Hernandez-Herrero, Duflos, Malle, & Bouquelet, 2003), and king crab, *Paralithodes camtschaticus* (Rudenskaya, Kislitsin, & Rebrikov, 2004). Chymotrypsin was isolated from the hepatopancreas of Chinese shrimp, *Fenneropenaeus chinensis* (Shi, Zhao, & Wang, 2008). Chymosin has been isolated and identified from carp and harp seal stomach (Shahidi & Kamil, 2001). Cathepsins also known as lysosomal cysteine proteases, playing an important role in many physiological processes including protein degradation, are mostly active at weakly acid pH values (pH 5). Cathepsins B, C, H, L, and S have been extracted from fish and shellfish muscles to be the major proteases involved in intracellular protein breakdown (Aoki, Ahsan, & Watabe, 2004; Pangkey et al., 2000). Cathepsin D shows some activity in the lowest pH range, predominating postmortem in some fish. The activity of cathepsin D was detected in red or white fish muscle among 24 species, and no difference was found between red- and white-flesh fish, or freshwater fish (Aoki, Yamashita, & Ueno, 2000).

A study on digestive enzymes conducted by Langeland, Lindberg, and Lundh (2013) proved that the high lipase and protease activity and low carbohydrase activity in Eurasian perch (*Perca fluviatilis*) and Arctic charr (*Salvelinus alpinus*) can be linked to their feeding habits. Total carbohydrase activity was higher in Eurasian perch than in Arctic charr, which had a higher total chymotrypsin activity and lipase activity in the mid-intestine. The study suggests that feed formulation should be different for Eurasian perch and Arctic charr in order to match their inherent digestive enzyme activities. This relates specifically to the carbohydrate content and composition of the feed. Based on the observed α-amylase and carbohydrase activities, the carbohydrate content, particularly starch, can be higher in feed for Eurasian perch than in feed for Arctic charr.

In fish, the levels of digestive enzymes may be influenced by type of feeding (Hofer & Schiemer, 1981), the age of the fish (Il'ina & Turetskiy, 1988), season and/or temperature of acclimatization (Kuz'mina, 1991), and so on. Additionally, heavily feeding fish will generally deteriorate rapidly because the enzyme concentration is often higher in the digestive tract of the fish during feeding. The effect of season on enzyme activity varies with the spawning cycle, water temperature, feeding cycle, and other variables (Wheaton & Lawson, 1985). The activity and thermal stability of fish enzymes vary from one species to another. For example, the activity and thermal stability of tryptic enzymes from horse mackerel (*Trachurus mediterraneus ponticus*) are greater than those from sprat (*Sprattus nostamus*). The pepsin from plaice (*Pleuronectes platessa*) is 10 times more active than that from horse mackerel. Generally, white fish have less proteolytic activity than do pelagic species (Mackie, Hardy, & Hobbs, 1971).

The presence of active proteases in muscle and digestive organ, particularly proteolytic and collagenolytic enzymes, makes the flesh fish and shellfish prone to degrade especially during storage in ice, since the digestive organ is not practically removed prior to storage. That occurrence leads to the muscle softening or mushiness of fish and shellfish during storage or distribution. During storage of fish and shellfish, the intensive hydrolysis of myofibrillar and collagenous proteins by proteases was noted. To lower the muscle degradation, different pretreatment methods as well as protease inhibitors have been applied in the stored fish and shellfish. The use of natural serine or trypsin inhibitors is a better way to retard such a textural problem of fish species (Sriket, 2014).

Biochemical properties of trypsin purified from the digestive system of carp *Catla catla* had a similarity with trypsin from other fishes. Stability at high pH and low temperature indicates the potential application of this

protease in detergent and in the food industry. Enzymes extracted from the fish viscera may be used in the food processing industry, thus making beneficial and productive use of the fish processing wastes (Khangembam, Sharma, & Chakrabarti, 2012).

Each type of fish protease extracted from the visceral waste of different fish species had a distinct optimum alkaline pH, temperature, and molecular weight. The crude enzyme from the visceral waste of the Red snapper and Great barracuda can be used to remove the blood stains effectively within 20 min, without the usage of any detergents. It was also observed that the enzyme dehaired the goat hide after 22 h of incubation, without the addition of sodium sulfide. The direct applicability of the crude extract without downstream processing would make its use acceptable as a substitute for the commercial ones (Sabtecha, Jayapriya, & Tamilselvi, 2014).

Atlantic cod as a poikilothermal organism lives in relatively frigid water; thus, it can be predicted that its enzymes are classified as cold active enzymes. Several of these enzymes have been demonstrated to be cold active, including elastase, collagenase, and chymotrypsin. The enzyme activity at low temperatures is helpful in various food processing applications where proteolysis must be performed at low temperature, such as in caviar production and carotenoprotein extraction (Vilhelmsson, 1991).

Fish also contains transglutaminase, playing an important function in surimi production. The role of transglutaminase in surimi production is in the formation of ε-(γ-glutamyl) lysine bonds in the fish protein to produce a high-quality gel. The activity of endogenous fish transglutaminase decreases rapidly after catch, and is almost completely destroyed by freezing (Vilhelmsson, 1991).

3. ENZYMES IN FERMENTED FISH

Proteases play an important role in the production of fermented fish product, particularly during fermentation process to obtain an acceptable product quality. Chadong, Yunchalard, and Piyatheerawong (2015) demonstrated fish protein degradation and peptide formation throughout fermentation process of *Plaa-som*, a traditional fermented fish product from Thailand, due to proteolysis. Proteolysis of Plaa-som occurred under fermentation at 30°C because protein concentration was continually reduced to 8.52 mg/g after 120 h of fermentation. This fact was also supported by the increase of peptide content to 1.95 μmol/g during fermentation.

Fermentation process of fish relies both on naturally occurring enzymes (in the muscle or the intestinal tract) as well as those enzymes from bacteria. In general, the former is most considerable with respect to changing texture as well as producing some of the flavor, and the latter contributes to the development of aroma and flavor (Skåra et al., 2015). In some cases, commercial enzymes are also supplemented for specific purposes, such as quality improvement and process acceleration.

3.1 Endogenous Fish Enzymes

As discussed above, various proteolytic enzymes are found in viscera, digestive tract, and muscle tissue of fish.

Major endogenous proteinases in anchovies were trypsin-like proteinase, pepsin, chymotrypsin, elastase, and aminopeptidase (Martinez & Serra, 1989; Siringan, Raksakulthai, & Yongsawatdigul, 2006). Digestive enzymes of trypsin, chymotrypsin, and pepsin are considered as the three of more important enzymes compared to others (de la Parra, Rosas, Lazo, & Viana, 2007). Pepsin is usually found in the stomach of fish and is a main enzyme of the digestive juices (de la Parra et al., 2007). Trypsin is present in viscera, pyloric caeca, and spleen (Kishimura, Hayashi, Miyashita, & Nonami, 2005, 2006; Kishimura et al., 2007; Klomklao et al., 2006). Hepatopancreas of fish and shellfish digestive organs contains both peptidase and proteinase activities such as aminopeptidase, gelatinolytic proteases, trypsin and chymotrypsin, and collagenolytic proteases (Sriket, 2014).

It was found that most of lipolysis and proteolysis activities in *peda* processing were recorded in the gut, especially at the beginning of the fermentation process; however, the activities fell rapidly during the process (Irianto, 1990). In view that enzymes are found in viscera and digestive tract, evisceration plays an important role in determining the rate and type of enzymatic degradation occurring. Fermented fish products processed using the whole fish will have different characteristic than those manufactured from headed and gutted fish (Wheaton & Lawson, 1985). The enzymatic activity of most visceral and digestive tract enzymes from fish had the greatest activity at near neutral pH values (Bougatef et al., 2007; Munilla-Moran & Saborido-Rey, 1996).

Castillo-Yañez, Pacheco-Aguilar, Garcia-Carreño, and Toro (2004) isolated an acid proteolytic enzyme, which belongs to the aspartic protease class from the viscera of sardines. The enzyme is similar to pepsin II from other fish species and is stable at pH 3–6 and 45°C.

The pyloric caeca represent the organs which are the major source of alkaline proteinases. A trypsin-like enzyme obtained from the pyloric caeca of cod (*G. morhua*) had an isoelectric point of 5.30 and 5.89 and was very similar in amino acid composition to bovine trypsin, but differed in having a higher relative amount of acidic amino acids and a lower amount of basic amino acids. The enzyme also hydrolyzed fish protein substrates (Beirão, Mackie, Teixeira, & Damian, 2001).

Three alkaline proteinases and two acid proteinases were isolated from sardine. Each of the alkaline proteinases hydrolyzed casein more rapidly than other proteins. A major alkaline proteinase (III) hydrolyzed sarcoplasmic proteins from sardine five times faster than other alkaline proteinases. Each of two acid proteinases hydrolyzed hemoglobin and myoglobin more rapidly than the other proteins. After preincubation with 25% NaCl, an alkaline proteinase (III) and an acid proteinase (II) were stable although the other proteinases became unstable. The two proteinases, alkaline proteinase III and acid proteinase II, were also stable for 3 months after the beginning of fish sauce production. The proteolytic activity of each of alkaline and the acid proteinases was strongly inhibited by more than 15% NaCl; however, minimum inhibition was observed when sardine muscle proteins were used as substrate (Noda, Van, Kusakabe, & Murakami, 1982).

Two aminopeptidases (I and II) were extracted from defatted internal organs of sardine and purified using DEAE-cellulose chromatography, gel filtration on Sephadex G-200, and isoelectric focusing. The final preparations of enzymes I and II were judged nearly homogenous by polyacrylamide gel electrophoresis. The molecular weights of enzymes I and II were determined by gel filtration to be 370,000 and 320,000, respectively. The isoelectric points were 4.1 (I) and 4.8 (II), respectively. Both enzymes were inhibited by EDTA and activated by Co^{++}. Bestatin could inhibit enzyme I but not enzyme II. Enzymes I and II rapidly hydrolyzed not only synthetic substrates containing alanine or leucine but also di-, tri-, and tetra-alanine. Based on all of these characteristics, sardine aminopeptidases resemble human alanine aminopeptidase. Enzyme I retained more than 70% of its original activity in 15% NaCl, suggesting the enzyme participates in hydrolyzing fish proteins and peptides during fish sauce production (Vo Van, Kusakabe, & Murakami, 1983).

Activities of alkaline and acid proteinases were compared with bovine trypsin and pepsin and showed that like bovine trypsin the alkaline proteinase from sardines pyloric caeca hydrolyzed casein more effectively than other protein substrates (Noda et al., 1982).

Muscle tissue enzymes, particularly cathepsins, peptidases, transaminases, amidases, amino acid decarboxylases, glutamic dehydrogenases, and related enzymes, are all found in fish muscle tissue (Chaveesuk, 1991), and these enzymes, particularly trypsin, chymotrypsin, and cathepsin, involve in the protein hydrolysis during fish sauce fermentation (Fernandes, 2016). Muscle tissue enzymes are mostly located in the cells. On the other hand, digestive enzymes are exocellular secretion. Even though some studies showed that muscle tissue enzymes have an optimum activity at neutral pH, most reports inform that low pH values accelerate muscle tissue enzyme activities. Most fermented fish products are processed at pH above 4, except for fish silage and some fermented fish products. Accordingly, most muscle tissue enzymes are actually not at optimum pH condition (Mackie et al., 1971).

Partial characterization of cathepsins B from the muscle of horse mackerel indicated similar characteristics with other cathepsin BS. The optimum pH of the cathepsin was 5 with optimum temperature of 50°C. The activity was inhibited by E-64, CA-074, and chymostatin (Yoshida et al., 2015).

Maximum enzyme activities can be achieved by using whole fish including heads and viscera. On the contrary, minimum enzyme activity will occur when deheaded and gutted fish are used to produce fermented fish products. Meanwhile, intermediate enzyme activities can be obtained by removing the guts anytime after the fish are caught to allow some diffusion of visceral enzymes into tissues (Owens & Mendoza, 1985).

In salted fish, the ripening is described by three hypotheses. These are (1) microbiological theory, (2) autolytic theory, and (3) enzymes theory. In microbiological theory, the microorganisms produce the essential active enzymes, and these enzymes penetrate into the flesh and contribute to ripening process. The autolytic theory describes that ripening is a result of the activity of enzymes of the muscles or other tissues, or of the gastrointestinal tract. Finally, the enzyme theory explains the ripening of salted fish as taking place under the influence of certain enzymes, namely, those contained in the muscle tissue, those in the intestinal body organs of the fish, together with those produced by microorganisms (Mackie et al., 1971).

In the maturing of anchovies, maximum autolytic activity of Indian anchovy (*Stolephorus indicus*) was found at 60°C. Autolytic activity decreased with increased NaCl concentration. Crude extract exhibited an optimum pH at 8.5–9.5. Trypsin-like proteinases were the predominant proteinases in the crude extract. Proteinases from Indian anchovy could participate in protein hydrolysis during fish sauce fermentation. Therefore, incubation of Indian anchovy at 60°C and in 10% NaCl for a period of time before full

salting at 25% NaCl could be an effective way to accelerate the fish sauce fermentation process (Siringan et al., 2006).

3.2 Microbial Enzymes

Fermentation of fish is brought about by autolytic enzymes from fish and microorganisms in the presence of salt. The use of salt in fresh fish preservation is as selective microbial agent (Anihouvi, Kindossi, & Hounhouigan, 2012; Majumdar & Basu, 2010).

There are two categories of fermented fish products. They are (1) product preservation primarily by water activity reduction (fish/salt formulation) and (2) product preservation by combined water activity reduction and lactic acid generation (fish/salt/carbohydrate formulation) (Adam, Cooke, & Rattapol, 1985). During salting, genus *Micrococcus* is predominant and its proportion increases gradually from 40% to approximately 90% of the total numbers. Simultaneously an appreciable reduction is observed in the other genera which are named in the following order of importance: *Flavobacterium*, *Achromobacter*, *Pseudomonas*, *Bacillus*, and *Sarcina* (Graikoski, 1973). The salting treatment reduces the water activity of fish from about 0.98 to about 0.70–0.75. At this water activity range, the only possibility is the growth of halophilic bacteria, xerophilic molds, and osmophilic yeast (Grant, 2004; Smith, 1989; Stevenson et al., 2015). Microbial load in fish sauce made from gambusia (*Affinis affinis*) during processing decreased with increased fermentation period, possibly caused by high concentration of salt (Ibrahim, 2010). While similar phenomenon was noted, the growth of halophilic bacteria, lipolytic bacteria, proteolytic bacteria, and lactic acid bacteria in *peda* processing decreased during the first and second fermentation. The bacteria probably contributed significantly only at the beginning of the fermentation, since the number tended to decrease (Irianto, 1990). The role of microorganisms in the fermentation process of fish is different from that in fermented vegetable products. The high salt content of these products leaves only salt-tolerant microorganisms to survive (Rose, 1982).

Microorganisms excrete proteolytic enzymes capable of degrading proteins. Many types of microorganisms excrete proteolytic enzymes, including the fungi *Aspergillus oryzae*, *A. orizae*, and *Rhizopus* sp.; the bacteria *Bacillus subtilis*; the actinomycetes *Streptomyces griseus*; and the yeast *Saccharomyces* spp. and *Candida* sp. (Gupta, Beg, Khan, & Chauhan, 2002; Mackie et al., 1971; Oyeleke, Egwim, Oyewole, & John, 2012). Therefore, careful selection by seeding or controlling the growth environment within the fermentation

container enables the desired microbes to flourish and produce significant quantities of proteolytic enzymes which help to hydrolyze the fish protein (Wheaton & Lawson, 1985).

3.3 Enzymes Added to the Fermentation Process

The fermentation process in the production of traditional fermented fish products is often found running slow, inconsistent product quality, and frequently not as expected. Microorganisms producing enzymes require a long adaptation period, particularly adjustment to environmental conditions with high salt content. However, the process acceleration, consistent product quality guarantee, and product quality improvement can be performed by using enzymes addition during processing. The enzymes may be produced from plant, animal, and microorganisms.

Several enzymes are extracted from plant, and those have been well recognized for their ability to tenderize meats. Commercial plant proteases such as bioprase, pronase, molsin, protease AJ, papain, bromelain, and ficin have all been investigated as enzyme supplements to speed up the rate of fish sauce fermentation (Chaveesuk, 1991; Le et al., 2015; Ooshiro, Ok, Une, Hayashi, & Itakura, 1981; Yongsawatdigul, Rodtong, & Raksakulthai, 2007). Bromelain found in pineapple juice, papain from papaya latex, and ficin from figs are already well known (Wheaton & Lawson, 1985). Those enzymes are quite heat stable and work optimally at near neutral pH (Mackie et al., 1971).

Proteases which have been investigated to be used in processing fermented fish products are extracted from *A. oryzae* (Man & Tuyet, 2006), moderately halophilic marine bacterium *Pseudomonas* sp. (Nakano, Watanabe, Hata, Qua, & Miura, 1986), and others. The most common commercial microbial proteases are Alcalase®, Neutrase®, Protamex®, Flavourzyme®, and Kojizyme® (Aristotelis, Himonides, Anthony, Taylor, & Morris, 2011; Nilsang, Lertsiri, Suphantharika, & Assavanig, 2005; Yongsawatdigul et al., 2007).

Animal-derived enzymes are trypsin, pepsin, and pancreatin. Trypsin has been extracted from digestive system of carp *C. catla* (Khangembam et al., 2012). Fish pepsins have been purified and characterized in various types of fishes including Arctic capelin, rainbow trout, Atlantic cod, bolti fish, Antarctic rock cod, sea bream, African coelacanth, Mandarin fish, smooth hound, orange-spotted grouper, albacore tuna, and European eel (Zhao, Budge, Ghaly, Brooks, & Dave, 2011).

4. ENZYMES IN FISH SAUCE PROCESSING

A fish sauce has attracted research scientists of all over the world to explore the secrets behind its fermentation process. Fish sauce is not only familiar for the people in Southeast Asia but also for those living in other parts of the globe. Fish sauce is known as "kecap ikan" in Indonesia, "nam pla" in Thailand, "patis" in Philippines, "shottsuru" in Japan, "nuöc mâm" in Vietnam, "budu" in Malaysia, "ngapi" in Myanmar, "pissala" in France, "garos" in Greece, "colombo-cure" in Pakistan and India, "yeesu" in China, and "aekjeot" in Korea. Fish sauce is manufactured through fermentation process for 3–12 months, in which fish and salt are previously mixed thoroughly at a ratio of 1:3. After 4–6-month period, a liquid containing fish extract is obtained in fermentation tanks. That liquid is actually fish sauce.

During fermentation process, fish tissue is gradually hydrolyzed, indicating the activity of proteolytic enzymes. The proteolytic enzymes responsible for the protein degradation are either endogenous fish enzymes coming from viscera or enzymes from microorganisms which may previously exist on or in the fish prior to the salting period. Endogenous proteolytic enzymes of fish originate from the digestive tract, internal organs, or muscle tissue (Chaveesuk, 1991; Chayovan, Rao, Liuzzo, & Khan, 1983). However, Orejana and Liston (1981) claimed that endogenous fish enzymes are the major and perhaps sole agents responsible for digestion in the fish sauce process. Fen, Sali, Ahmad, Tze, and Abdullah (2011) revealed similar finding that the endogenous fish enzymes, especially from fish viscera, were the main contributors of protease action during the initial days of fermentation. In addition, bacterial enzymes may be involved in the later stage of fermentation.

Digestive enzymes have a significant role in the fermentation of capelin (*Mallotus villosus*) sauce, in view of the fact that the rate of protein hydrolysis of whole fish was considerably higher than that of eviscerated fish. Intracellular enzyme of cathepsin C was believed to contribute to proteolysis in fish sauce and the formation of the delicious fish sauce taste (Raksakulthai, 1987).

Quality improvement and fermentation process acceleration of fish sauce can be carried out enzymatically through the use of papain (Anon, 1983; Chuapoehuk & Raksakulthai, 1992), bromelain (Chuapoehuk & Raksakulthai, 1992; Handayani, Ratnadewi, & Santoso, 2007; Subroto,

Hutuely, Haerudin, & Purnomo, 1985), pepsin (Kumalaningsih, 1986), trypsin and chymotrypsin (Chaveesuk, 1991), as well as trypsin and pepsin (Kristianawati, Ibrahim, & Rianingsih, 2014).

Subroto et al. (1985) utilized pineapple juice as the source of bromelain in the processing of fish sauce using by-catch fish as raw material. Good quality fish sauce can be acquired by the use of pineapple extract as much as 8% (v/w) with 10-h incubation period. Sangjindavong, Mookdasanit, Wilaipun, Chuapoehuk, and Akkanvanitch (2009) used pineapple core and pineapple peel for producing fish sauce from surimi waste. Handayani et al. (2007) suggested using 15% NaCl to produce sardine (*Sardinella lemuru*) sauce with the addition of crude protease extracted from pineapple.

Use of crude papain has successfully improved quality and accelerated fermentation process of fish sauce (Lopetcharat, Choi, Park, & Daeschel, 2001; Setyahadi, 2013). A better quality of fish sauce made from *Sardinella* sp. as raw material was obtained by a combination of 12.5% salt and 1.5% papain, producing a nitrogen conversion of 13.63%. The more the salt addition, the lower the protein degradation rate will be. Increasing papain amount will induce higher nitrogen conversion rate and water-soluble protein degradation level in the liquid. High salt addition level seems to inhibit enzyme activity. On the contrary, the reduction of salt addition level will encourage the growth of microorganisms and generate undesirable odor in the fish sauce. Increasing added papain amount promotes the formation of nitrogen compounds, but results in a viscous material due to connective tissue degradation (Anon, 1983).

Chuapoehuk and Raksakulthai (1992) prepared oyster sauce by hydrolyzing minced oyster meat using papain or bromelain supplemented with 20% sodium chloride. It was found that 0.7% papain or 0.3% bromelain produced the highest soluble nitrogen in the hydrolysates and showed no significant differences in proximate composition, pH, consistency, and sensory evaluation scores.

Pepsin can be used for fish sauce processing in one condition that the pH of fish is brought down into optimum pH for pepsin activity, i.e., pH 2. Salt amount of 15% is considered suitable for generating an optimum condition in preventing the growth of putrefactive bacteria (Kumalaningsih, 1986).

The use of trypsin and chymotrypsin to accelerate the rate of fish sauce fermentation processed from herring (*Clupea harengus*) increased significantly the rate of proteolysis and the amounts of total nitrogen, formol nitrogen, and free amino acids in the fish sauce product. Fermentation period was

also reduced from 6–12 to 2 months. A significant increase in total nitrogen and free amino acid contents in the end products was observed when enzyme concentration was increased from 0.3% to 0.6%. Supplementation with 0.6% of 25:75 trypsin:chymotrypsin showed the most satisfactory results in terms of total nitrogen, formol nitrogen, and free amino acid contents. The lighter color of herring sauce produced with 0.6% enzyme supplement was preferred to the darker color of the first grade commercially produced fish sauce. There was no significant difference in the preference for aroma and flavor among enzyme-supplemented sauces and the first-grade commercially produced fish sauce (Chaveesuk, 1991).

Acceleration of fish sauce fermentation process was carried out by Kristianawati et al. (2014) by employing proteolytic enzyme addition and salt level reduction with marine catfish viscera as raw material. The addition of trypsin and pepsin with the concentration of 0.3% produced fish sauce with significantly higher yield and enzyme activity values, as well as better sensory performance. The most organoleptically acceptable fish sauce was obtained through processing with 0.3% trypsin, 20% salt addition, and 45-day fermentation period.

Ooshiro et al. (1981) examining papain, bromelain, and trypsin for the production of fish sauce from sardines revealed that papain was the most effective for proteolysis. Fermentation with 0.3% papain at 37°C without adjusting initial pH was the best condition for maximum proteolysis.

Manufacturing fish sauce from anchovy fish using purified protease from *A. oryzae* was investigated by Man and Tuyet (2006). The application of that protease with a suitable procedure of salt addition in fish sauce processing accelerated fish proteolysis and increased the free amino nitrogen content. It should be taken into consideration that high salt content (25%) in the fish–salt mixture decreased the enzyme activity.

5. CONCLUDING REMARKS

Fermented fish products play an important role in daily life of many countries. These products are consumed in small amount, but can give a taste sensation-promoting appetite for eating. Endogenous fish proteases, particularly the ones coming from digestive tracts, are suspected as the main enzymes contributing in degrading protein during fermentation process. Eviscerating by removing the gut is not recommended if faster fermentation to be achieved. Microorganisms through excreting proteases seem to take part in the processing of fermented fish mainly either at the beginning of

the process or prior to the fish receiving salting treatment. In order to obtain optimum involvement of microorganisms in the production of fermented fish, manipulation of the environment to facilitate the most suitable conditions for the growth of microorganisms should be performed.

The fermentation process with correct proteolysis is needed to guarantee a good quality product, but this usually requires a long fermentation period, even up to a year. Economically, the shorter fermentation process is more profitable. Therefore, the accelerated processing by enzymes addition has a good prospect as long as the quality of the product is comparable with that obtained without employing enzymes. Intensive studies have been carried out to accelerate fermentation process of fish sauce using the addition of various enzymes manufactured from plants, animals, and microorganisms. Processing acceleration for other fermented products than fish sauce by involving enzyme supplementation is encouraged to be done. Manipulation of fermentation environment to achieve optimum enzyme activities and microorganism growths for speeding up the processing rate of fermented fish products is recommended.

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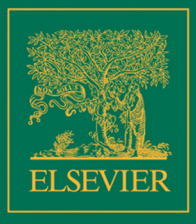

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