J. Fish. Sci. Tech. 2(1), 36~43, 1999

JOURNAL OF
FISHERIES SCIENCE
AND TECHNOLOGY

A Study on the Rapid Hydrolysis of Fish Using Proteolytic Bacteria Isolated from Anchovy *Jeotkal*

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(Received December 1998, Accepted May 1999)

A study on the hydrolysis of anchovy using proteolytic bacteria isolated from anchovy *jeotkal* (a salt-fermented fish) was carried out to develop a rapid process of liquefied anchovy *jeotkal*. Five kinds of proteolytic bacteria, such as *Staphylococcus* sp.-1, *Photobacterium* sp., *Volcaniella* sp., *Staphylococcus* sp.-2 and *Bacillus* sp., were isolated from the anchovy *jeotkal* that fermented with 20% NaCl at room temperature for 2 months. Those grew well at 40℃, pH 7.0 on TPY broth with 2.0% NaCl. The optimal hydrolysis temperature, pH, time and proteolytic bacteria densities for hydrolysis of minced anchovy were 40℃, 7.0, 6 hours and 1.8×10^8 cells/g raw anchovy, respectively.

Key words: rapid hydrolysis, liquefied anchovy *jeotkal*, proteolytic bacteria

Introduction

A Jeotkal is the most well known traditional fermented seafood in Korea, and it is fermented and matured by using reaction of autolysis enzyme and enzyme produced from microorganism which is related to the fermentation of *jeotkal*. It is reported that *jeotkals* contained some peptides, and they show some characteristics of ACE inhibition, mutagenicity inhibitor, and oxidation inhibitor (Kim et al., 1993-a,-b). Since the high concentration of salt was added to the meat, viscera, gonad of fish-shell for prevention of spoilage during fermentation, it would be induce some kinds of adult diseases (Lee et al., 1983). And the long time fermentation of *jeotkal* resulted to decrease amount of high unsaturated fatty acids such as EPA and DHA, whereas to increase amount of TMA which could be induced undesirable odor (Han, 1997). The undesirable odors in *jeotkals* have might be limit the utilization of it except for seasonings in *Kimchi*, and became one of the main reason why most young people, who were familiar to the Western food, disliked it. Therefore, it is important to develop various type products containing original functional characteristics of *jeotkals* with low salt contents and better flavor. In this study proteolytic bacteria isolated from the anchovy *jeotkal*, possibility of development on the rapid process of liquefied anchovy food was investigated by using proteolytic bacteria isolated from the anchovy *jeotkal*.

Materials and Methods

Sample : Anchovy, *Engraulis japonica*, were purchased at Daepyun harbor, GiJang Gun, Pusan, in March, 1997. The size of those were 17~24 cm in length, and 60~80 g in weight. The anchovy were packaged double with polyethylene film (25×30 cm) and kept at −30℃ until analyzed.

Isolation and Identification of proteolytic bacteria : The anchovy with 20% NaCl was fermented at room temperature for 2 months. Proteolytic bacteria were isolated from anchovy *jeotkal* by the methods of Lee and Choi (1974) and Cha et al. (1988). The isolated proteolytic bacteria were identified by the methods of MacFaddin (1984) and ATB system (automated test for

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bacteriology system, Biomerieux Co., France). In ATB system, proteolytic bacteria were inoculated on the sheep blood agar plate and nutrient agar, thioglycollate broth containing 5% NaCl, and classified with the shape after incubation on that medium at 25~30℃ for 24~72 hrs (Logan and Berkeley, 1983; Goodfellow and Minnikin, 1985). The classified bacteria were cultivated purely on the sheep blood agar plate again, and the purely cultivated bacteria were deciphered with ATB system reader which used a rapid ID 32E and a ID 32GN (Biomerieux Co., France), a kinds of commercial kit.

Characteristics of proteolytic bacteria during culture : In order to determine the optimal culture temperature, time for the growth of proteolytic bacteria, proteolytic bacteria were inoculated on TPY broth (tryptone 0.5%, peptone 0.5%, yeast extract 0.3%) and were cultivated on shaking incubator (Han Baek Scientific Co., HB-201SF, Korea) at 25~55℃, 200 rpm. The cell density was determined by measuring of absorbance at optical density (O.D. : 660 nm). The growth of bacteria with changes of pH, NaCl contents in TPY broth were determined by measuring of cell density after culture at 40℃, for 20 hrs. The pH and NaCl contents in TPY broth were adjusted from 2 to 12 with 0.1 N NaOH or 0.1 N HCl, and from 0 to 20%, respectively.

Hydrolysis : In order to investigate conditions for the hydrolysis of minced anchovy, proteolytic bacteria, which were cultivated at optimal conditions for 20 hrs, were added to minced anchovy which was minced the mixture of water and raw anchovy mixed with 2 : 1 ratio. And it was hydrolyzed on shaking incubator with various conditions such as cell density, hydrolysis time, pH and temperature. The hydrolysis ratio was calculated by the method of Han et al. (1990).

Chemical analysis : Contents of moisture, crude protein, crude lipid, ash and carbohydrate were determined using A.O.A.C. methods (1990) and pH was determined using the pH meter (Suntex model: sp-7). Contents of pure protein, amino nitrogen (NH_2-N), histamine and volatile basic nitrogen (VBN) were determined using the method of Barnstein (Kohara et al., 1982), A.O.A.C. (1990), Park et al. (1980) and micro diffusion (Miwa and Iida, 1973), respectively.

Results and Discussion

Proximate composition and characteristic values of raw anchovy : Proximate compositions and characteristic values of raw anchovy are shown in Table 1. The composition of crude protein, histamine and VBN were 19.69%, 9.70 mg/100 g and 18.6 mg/100 g, respectively. Considering these results, it was considered that the raw anchovy might be kept in fresh state.

Isolation and identification of proteolytic bacteria : In order to isolate proteolytic bacteria from anchovy *jeotkal* fermented for 2 months, 30 kinds of bacteria were isolated and among these five kinds of proteolytic bacteria were collected by the method of Cha et al. (1988), and then cultivated on TPY broth. The cultural and biochemical characteristics of the selected proteolytic bacteria are shown in Table 2. According to Table 2, proteolytic bacteria were identified as follows, comparising with Bergey's manual (1984, 1986) ; Strain A was gram positive, non-spore forming, cocci, and produced acid by using glucose, fructose, ribose, sucrose, arabinose, maltose, and not utilized urea, arginine, ornithine, cellobiose. Therefore, strain A was identified as *Staphylococcus* sp.-1.

Strain B was gram negative, non-spore forming, rod, and produced acid by using arabinose, glucose, maltose, ribose, and could utilized cellobiose, but not utilized galactose, mannitol, rhamnose, arginine, ornitine, urea. Therefore, strain B was identified as *Photobacterium* sp.

Strain C was gram negative, non spore forming, rod, and produced acid by using arabinose, maltose, and could utilized acetate, cellobiose, mannose, rhamnose, but not utilize galactose, mannitol, ornitine. Therefore, strain C was identified as *Volcaniella* sp.

Strain D was gram positive, non spore forming, cocci. Comparing strain A, it was considered as similar species with strain A, but was shown some differences in utilization of sucrose, lactose,

Table 1. Proximate compositions and some characteristic values of minced anchovy

Components	Contents	Components	Contents
Moisture	73.15%	VBN	18.60 mg/100 g
Crude protein	19.69%	NH_2-N	107.10 mg/100 g
Crude lipid	4.83%	Pure protein	1,742 mg/100 g
Crude ash	3.70%	Histamine	9.70 mg/100 g
Carbohydrate	4.47%	pH	6.3

Table 2. Characteristics of the proteolytic bacteria isolated from anchovy *jeotkal*

Test \ Strain	A	B	C	D	E
Form	cocci	rod	rod	cocci	rod
Spore	−	−	−	−	+
Gram stain	+	−	−	+	+
Indole		−	−		−
Voges-Proskauer test	−	−	−	−	−
Catalase	+	+	+	+	−
Oxidase	−	−	−	−	−
Nitrate reduction	+				+
Tetrathionate reduction			+	−	+
Bacitracin (sensitive)	−			+	
Novobiocin (sensitive)	+				
Acid production from					
Arabinose	+	+	+		
Fructose	+			+	+
α-galactose		−	−		
D-arabinose				−	−
D-tagatose					+
D-turanose					+
D-xylose					+
L-arabinose					+
Glucose	+	+		+	+
Maltose	+	+	+	+	
Ribose	+	+	−		
Raffinose	−		−	−	−
Sucrose	+	−	−	−	+
Utilization of					
Acetate			+		
Adonitol		−	−		−
Amygdaline		+			
Cellobiose	−	+	+		+
Galactose		−	−		−
Glycerol					+
Glycogen					+
Inuline					−
Lactose					+
L-xylose					−
Melibiose			+	+	−
Mannitol	−	−	−	−	+
Mannose	−		+	+	+
N-acetyl glucosamine	+				+
Rhamnose		−	+		+
Sorbitol			−		+
Trehalose	−	−	−	−	+
Turanose	−				
Urea	−	−	−	−	−
Arginine	−			−	+
Esculine	−		−		+
Ornithine	−	−	−		−
Starch		+			+
Hydrolysis of		+			+
Gelatine					+
Production of					
Alkaline phosphatase	−			+	
β-galactosidase	−				+
Phenylalanine deaminase		−	−		
15% NaCl tolerance	+	+	+	+	+

+ : Positive reaction, − : Negative reaction.

mannose, melibiose and production of alkaline phosphatase. Therefore, strain D was identified as *Staphylococcus* sp.-2 that unliked with strain A.

Strain E was gram positive, spore forming, rod, and produced acid by using glucose, fructose, sucrose, D-arabinose, D-xylose, arabinose, and utilized cellobiose, glycogen, lactose, mannitol, mannose acetate, but could not utilize galactose, L-xylose. Therefore, strain E was identified as *Bacillus* sp.

Optimal conditions for proteolytic bacteria culture : To determine optimal growth temperature for *Staphylococcus* sp.-1, *Photobacterium* sp., *Volcaniella* sp., *Staphylococcus* sp.-2 and *Bacillus* sp., these proteolytic bacteria were inoculated on TPY broth, pH 7.0. The maximum specific growth rates (μ_{max}) of proteolytic bacteria at 25~45℃ are shown in Fig. 1. The μ_{max} of all kinds of proteolytic bacteria were the highest at 40℃ culture. Two μ_{max} of *Volcaniella* sp. and *Staphylococcus* sp.-2 were shown higher than the others, whereas that of *Bacillus* sp. was shown lower relatively. The cell densities were actually ca. 6×10^7~1.1×10^8cells/mℓ ranges with kinds of bacteria after 20 hrs culture.

Fig. 2 and 3 show that the growth of proteolytic bacteria with changes of pH and NaCl contents in TPY broth after 20 hrs culture.

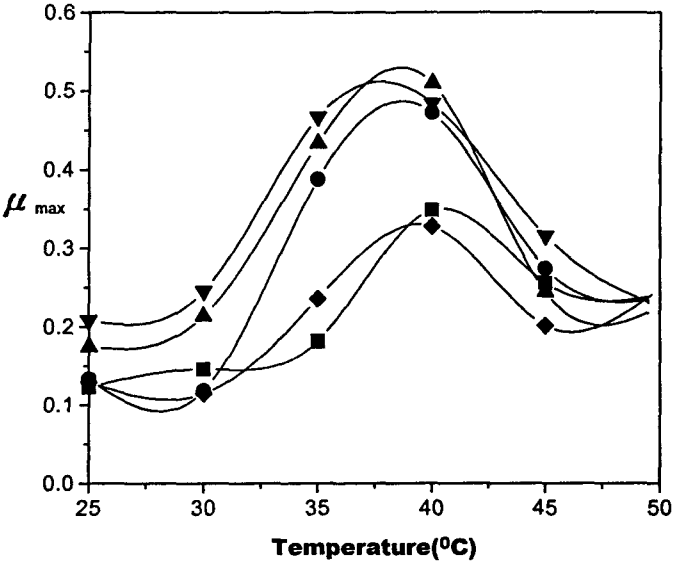

Fig. 1. Maximum specific growth rate of the proteolytic bacteria during shaking culture in TPY broth at different temperature, pH 7.0. −■− : A, −●− : B, −▲− : C, −▼− : D, −◆− : E
A: *Staphylococcus* sp.-1, B: *Photobacterium* sp., C: *Volcaniella* sp., D: *Staphylococcus* sp.-2, E: *Bacillus* sp.

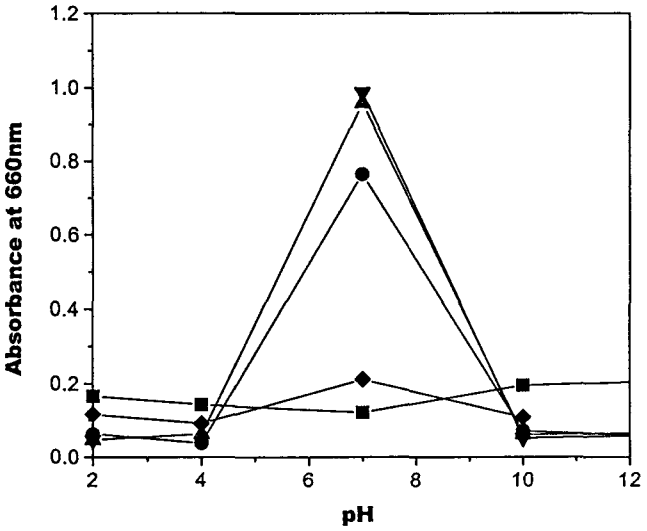

Fig. 2. Influence of pH on the growth of proteolytic bacteria after 20 hrs shaking culture in TPY broth.
—■— : A, —●— : B, —▲— : C, —▼— : D, —◆— : E
*A, B, C, D and E : See in Fig. 1.

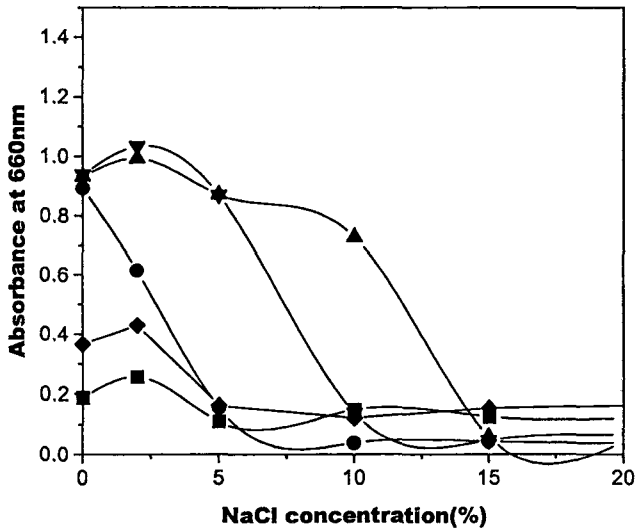

Fig. 3. Influence of NaCl contents on the growth of proteolytic bacteria after 20 hrs shaking culture in TPY broth, at pH 7.0.
—■— : A, —●— : B, —▲— : C, —▼— : D, —◆— : E
*A, B, C, D and E : See in Fig. 1.

Most proteolytic bacteria grew well at pH 7.0 in TPY broth, but unlike with the others, growth of *Staphylococcus* sp.-1 was not affected significantly from changes of pH in TPY broth. When NaCl contents were changed with ranges of 0~20% in TPY broth, proteolytic bacteria grew well at 2.0% except *Photobacterium* sp., and *Volcaniella* sp., *Staphylococcus* sp.-2 grew well even though above 5.0%.

Ryu and Jin (1995) reported that *Bacillus* sp., isolated from ocean, shown high growth and enzyme activity. Cha et al. (1988) and Cha and Lee (1989) reported that the growth rates and produced enzyme activities of proteolytic bacteria were shown the highest on cultivation at optimal conditions. Thus, the pre-culturing conditions of proteolytic bacteria for hydrolysis of minced anchovy were determined as follows : culture temperature and pH, NaCl contents in TPY broth were 40℃, 7.0 and 2.0 %, respectively.

Added proteolytic bacteria concentration : It was reported that the low salt anchovy *jeotkal* fermented with proteolytic bacteria has no significant differences compared with conventional *jeotkal* fermented with traditional method on the view of the flavor containing taste, odor (Cha et al., 1990). To determine the rational added concentration of proteolytic bacteria for the rapid processing of low salt liquefied anchovy *jeotkal*, the proteolytic bacteria, which were cultivated at 40℃ for 20hrs in pH 7.0 TPY broth containing 2% salt, were used in anchovy hydrolysis. Salt contents in minced anchovy, which mixed anchovy and water with 1 : 2, were controlled 2%, and filled in 250 mℓ round flask and then proteolytic bacteria of 2×10^{6}~2×10^{8}cells against to the raw anchovy of 1g were added in minced anchovy. The results after hydrolysis at 40℃ for 5hrs are shown in Fig. 4.

The hydrolysis ratios were increased radically by increasing of the added concentration of proteolytic bacteria against raw anchovy of 1g, until to the 1.8×10^{8}cells, but the increasing of hydrolysis ratio were slow above concentration of that. It was suggested that these results were probably because the amount of substrate were decreased relatively by increasing of the concentration of proteolytic bacteria and they resulted in decreasing the saturated degree of enzyme produced from proteolytic bacteria in substrate (Han et al, 1990; Bae et al., 1990-a, -b). Therefore, the rational added concentration of proteolytic bacteria for the rapid processing of low salt liquefied anchovy *jeotkal* were determined with 1.8×10^{8}cells/g raw anchovy.

Hydrolysis temperature, time and pH : In order to determine the optimal hydrolysis temperature, time and pH on minced anchovy by using 5 kinds of proteolytic bacteria, minced anchovy was hydrolized with proteolytic bacteria.

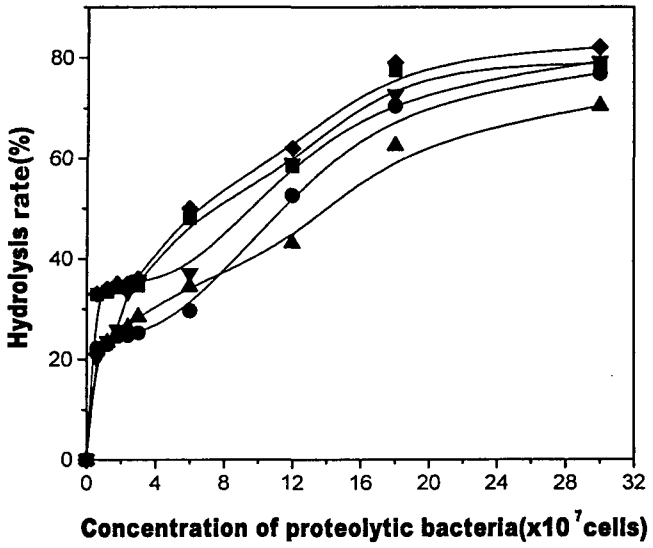

Fig. 4. Comparison of hydrolysis ratio of minced anchovy by the concentration of proteolytic bacteria.
*Hydrolysis ratio was measured after 5 hr shaking incubation at 40℃, and NaCl concentration of minced anchovy was 2.0 %, the mixed ratio of water and raw anchovy was 2 : 1 in minced anchovy.
−■− : A, −●− : B, −▲− : C, −▼− : D, −◆− : E
*A, B, C, D and E : See in Fig. 1.

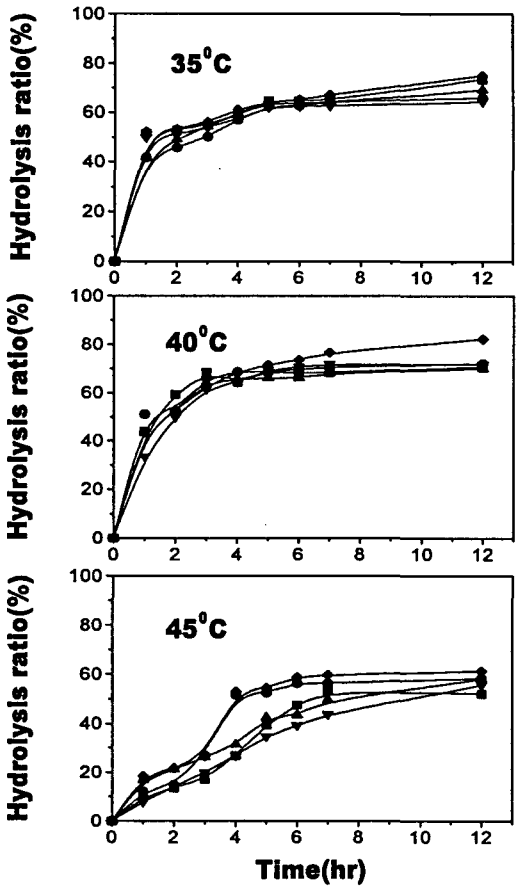

Fig. 5. Comparison of hydrolysis ratio of minced anchovy by hydrolysis time, temperature, and kinds of proteolytic bacteria.
*NaCl concentration of minced anchovy was 2.0% and the mixed ratio of water and raw anchovy was 2 : 1 in minced anchovy, concentrations of proteolytic bacteria were 1.8×10^8cells/g raw anchovy.
−■− : A, −●− : B, −▲− : C, −▼− : D, −◆− : E
*A, B, C, D and E : See in Fig. 1.

Fig 5. shows changes of hydrolysis ratios with hydrolysis temperature, time when the minced anchovy was hydrolyzed with proteolytic bacteria of 1.8×10^8cells/g raw anchovy containing 2% NaCl.

During minced anchovy was hydrolyzed at 35~50℃ for 12hrs, all kinds of proteolytic bacteria were shown relatively higher hydrolysis ratio at 40℃. It was considered that these results were because the activities of proteolytic enzyme produced from proteolytic bacteria were the highest at the optimal growth condition considering the above results, which shown the maximum specific growth rate of proteolytic bacteria was highest at 40℃. As similar results, Cha et al. (1988) also reported that the activity of proteolytic enzyme produced from proteolytic bacteria were the highest at the optimal growth condition.

In order to determine the rational hydrolysis time from the results of Fig. 5, the rational hydrolysis time based on the decrease of apparent hydrolysis activity against increase of hydrolysis time were determined by the method of Bae et al. (1990-a, -b), as shown in Fig. 6. The hydrolysis activities were the highest at initial 1 hr and decreased radically until 6 hr, and decreased slowly after 6 hr. Even though hydrolysis activities were the highest at initial 1hr, the hydrolysis ratios were low because of insufficient hydrolysis time at that time. The whole hydrolysis sections were divided two section with different slop, except initial 1 hr section. Thus, the rational hydrolysis time could be determined 6 hr when two line with different slope were crossed.

Cha et al. (1990) reported that hydrolysis ratio of anchovy was shown about 80% after hydrolysis for 15hrs in the study on the processing of rapid fermented anchovy with low salt contents by proteolytic bacteria. But there were some differences between that results and results in this study. Even though some differences were shown with kinds of proteolytic bacteria, the hydrolysis ratios were

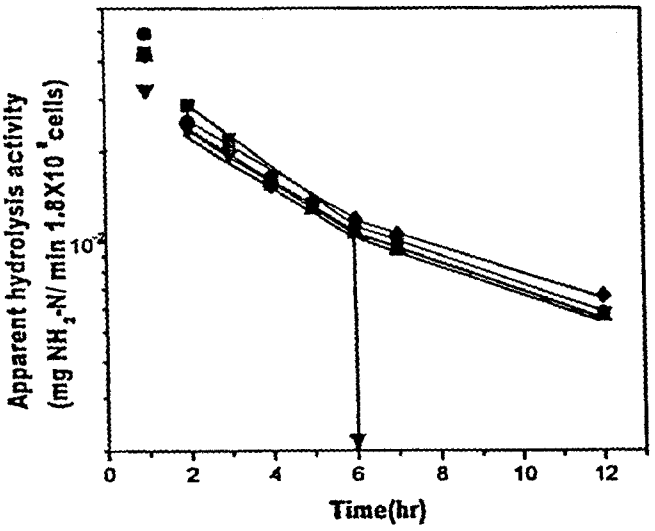

Fig. 6. Influence of hydrolysis time on the apparent hydrolysis activity.
—■— : A, —●— : B, —▲— : C, —▼— : D, —◆— : E
*A, B, C, D and E : See in Fig. 1.

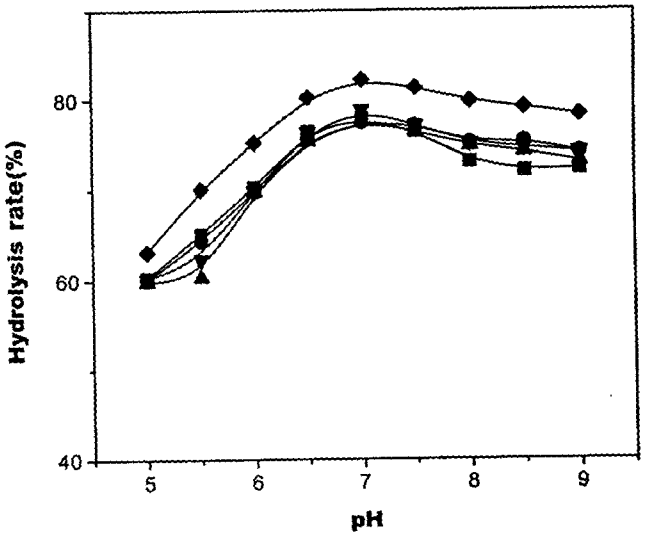

Fig. 7. Comparison of hydrolysis ratio of minced anchovy by pH and kinds of proteolytic bacteria.
*Hydrolysis ratio was measured after 6 hr shaking incubation at 40℃, and NaCl concentration of minced anchovy was 2.0 %, the mixed ratio of water and raw anchovy was 2 : 1 in minced anchovy, concentrations of proteolytic bacteria were 1.8×10^{8}cells/g raw anchovy.
—■— : A, —●— : B, —▲— : C, —▼— : D, —◆— : E
*A, B, C, D and E : See in Fig. 1.

shown almost 80% after only 6hrs hydrolysis. It was considered that these differences were caused by the differences of concentration of proteolytic bacteria added in minced anchovy, i.e., the added concentration of proteolytic bacteria in Cha et al. (1990) were 6×10^{3}cells/g raw anchovy, but the added concentration of that in this study were 1.8×10^{8}cells/g raw anchovy which was more 30,000 times than that of Cha et al. (1990). Otherwise, considering these results, it may be suggested that the proteolytic bacteria cultivated to the high density was more effective on the hydrolysis of minced anchovy with proteolytic bacteria.

In order to investigate resonable pH on hydrolysis of minced anchovy, the pH in minced anchovy containing 2% NaCl were controlled 5~9 and the concentration of added proteolytic bacteria were 1.8×10^{8}cells/g raw anchovy, and the minced anchovy were hydrolyzed at 40℃ for 6hrs. The changes of hydrolysis ratios with pH are shown in Fig. 7.

There were some differences in hydrolysis ratios according to the kinds of proteolytic bacteria, but the hydrolysis ratios were shown the highest when pH in the minced anchovy was 7.0. Comparing with above results that the maximum specific growth rates of proteolytic bacteria were highest at 40℃, pH 7.0, it may be suggested that the activities of enzyme produced from proteolytic bacteria were the highest at the optimal growth conditions for proteolytic bacteria.

As similar these results, it was reported that the activity of enzyme produced from microorganism was the highest when the microorganism was cultivated at the optimal growth pH (Cha et al, 1988; Uchiyama et al., 19 94). In study on the hydrolysis of minced anchovy using commercial enzyme, Kim et al. (1997) reported that controlling optimal pH of added commercial enzyme was more effective than that of autolysis enzyme in view of increasing of hydrolysis ratio of minced anchovy. Ishida et al. (1994) reported that salted anchovy muscle could be solubilized by some kinds of thermostable proteases, these enzymes shown the strongest proteolytic activity at pH 7.4. These proteolytic enzymes were shown the strongest proteolytic activity at different pH range but the hydrolysis ratio was shown the highest at the optimal pH range of added enzyme on the hydrolysis fish using commercial enzyme when commercial enzyme and autolysis enzyme were acted complexively in fish hydrolysis (Bae, 1989).

Conclusion

A study on the rapid hydrolysis of anchovy using proteolytic bacteria was carried out. From the anchovy *jeotkal*, 5 kinds of proteolytic bacteria, such as *Staphylococcus* sp.-1, *Photobacterium* sp., *Volcaniella* sp., *Staphylococcus* sp.-2 and *Bacillus* sp., were isolated and identified. Optimal conditions for bacterial growth were determined with temperature, pH, time, NaCl contents in TPY broth. Those proteolytic bacteria were cultivated at optimal condition, and then used for anchovy hydrolysis. When minced anchovy was hydrolyzed with 5 kinds of proteolytic bacteria at the optimal conditions, the hydrolysis ratio of anchovy was shown the highest.

Acknowledgements

This paper was devoted to the late Prof. Dr. Bong-Ho Han for praising his academic achivement.

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