CHAPTER 3

Isolation and characterization of proteolytic enzymes from salt-tolerant bacteria

Introduction

There has been considerable interest in microbes which exist in extreme environments. Extremophiles are a source of enzymes with extreme stability, and the application of these enzymes as biocatalysts is attractive because they are stable and active under stress conditions. Salt tolerant microorganisms or their enzyme may play potentially significant roles in industrial process that require high salt environment (Moriguchi *et al*., 1994). Hypersaline environments such as salt lakes and salted food products can be used as the source for the isolation of salt tolerant microorganisms. There are many kinds of fermented protein food products in Thailand. For example, fermented fish products, which are high protein such as Nam-pla (fish sauce), Pla-ra (fermented fish), Ka-pi (fish and shrimp paste), and Tai-pla (fermented fish visceral), and fermented soy products such as soy sauce and Thua-nao (popular in northern Thailand), all of which contain salt. Fermented foods have undergone degradative changes through enzymatic or microbiological activity either in the presence or absence of salt (Essuman, 1992). More halophilic extra- and intracellular enzymes have been isolated and characterized from moderate halobacteria. The exoenzymes include several amylases, nucleases, proteases and 5'-nucleotidases from several moderately halophilic bacteria.

The objectives of this study were to isolate and characterize the proteases from salt-tolerant bacteria isolated from various sources in Thailand.

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Materials and Methods

Isolation of salt-tolerant bacteria

The salt tolerant bacteria were isolated from Thai fermented foods such as Nam-pla (fish sauce), Ka-pi (shrimp paste), Pla-ra (fermented fish), Pla-chom (fermented fish), Bu-du (fermented fish in liquid form), Tai-pla (fermented fish liquid entrails), Kaey (fermented small shrimp), Pung-pla(fermented fish visceral mass), Pu-chem (salted crab), Pla-too-chem (salted fish), Tao-chieo (fermented soybean), Phak-kard-dong (pickle mustards), Naw-mai-dong (fermented bamboo shoot), Gra-teim-dong (fermented garlic), and Hua-phak-kard-chem (turnip with salt), soil from salt pan, salt, sea sand, sea water and sea shell solids wastes. The various samples were collected at different locations in Thailand from Chiang Mai, Sukhothai, Ratchaburi, Khonkan, Cholburi, Rayong, Samutprakarn, Samutsakorn, Samutsongkram and Trang. One gram of each sample was dissolved in 9 ml of sterilized water and make serial dilution. The 0.1 ml of sample solution was spread on nutrient agar (NA) containing (w/v) 0.3 % beef extract, 0.5 % peptone, 1.5 % agar and added 1.5 % NaCl (pH 7.0) incubated at ambient temperature for 48 h. The bacteria on the medium were purified by streak plated technique for the individual colonies on NA medium. Pure cultures of new isolated strains were maintained in NA agar in the presence of 1.5 % NaCl and kept at 4 °C for further studies.

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The study of the characteristics of isolated bacteria

All of isolated bacteria were studied the morphological characteristic after incubation of the culture on NA slant at 37 °C for 24 h by Gram’s staining (Appendix A). The cultural characteristic of isolated bacteria were observed on nutrient agar.

The ability of growth of isolated bacteria on medium containing various concentrations of sodium chloride

The ability of growth of isolated bacteria was studied by using a sterile toothpick method inoculated on nutrient agar containing 5, 10, 15, 20 and 25 % NaCl and incubated at 37 °C.

The ability of growth of isolated bacteria at various temperatures

All of isolated bacteria were studied on the ability of growth at various temperatures by using a sterile toothpick method on nutrient agar containing 1.5 % NaCl incubated at 35, 40, 45, 50, 55, and 60 °C for 48 h.

Productivity of proteases by isolated bacteria on skimmed milk agar

The productivity of proteases by isolated bacteria were examined by using a sterile tooth pick method on skimmed milk agar (SMA) containing (w/v) 0.3 % beef extract, 0.5 % peptone, 10 % skimmed milk and 1.5 % agar in the presence of 5, 10, 15, 20, and 25 % NaCl (pH 7.0) at 37 °C. The productivity of proteases was calculated by measuring the diameter of the clear zone formed after bacterial growth.

Productivity of proteases by isolated bacteria in PY medium

The isolated bacteria were grown in 2 ml of PY medium containing (w/v) 1.0 % peptone, 0.1 % yeast extract and 1.0 % NaCl in test tube on horizontal shaker 150 rpm at ambient temperature for 24 h. After growth, culture medium was centrifuged at 5,000 rpm, 4 °C for 10 min. The supernatant obtained was used for assay of proteases activity.

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Effect of composition of medium on proteases production

The bacteria were grown in the test tube containing 2 ml of various media 1- 9 on horizontal shaker 150 rpm at ambient temperature for 24 h. After growth, culture medium was centrifuged at 5,000 rpm, 4 °C for 10 min and the supernatant was used for assay of proteases activity. The medium 1- medium 9 were applied from the literature reviews. The composition of various medium 1- medium 9 were summarized in Table 3.1.

Time course of four bacteria

Four bacteria, *Bacillus* sp., K52, K53, K61 and KK128 that produced high proteases activity were determined. One to two loops of bacteria were cultivated in 100 ml of medium containing (w/v) 1.0 % glucose, 0.25 % yeast extract, 0.05 % $CaCl_2$ and 1 % NaCl in 250 ml Erlenmeyer flask incubated on rotary shaker at 150 rpm and incubated at 37 °C for 5 days. Five ml samples were withdrawn at 12 h intervals for 5 days. The culture broth was centrifuged at 5,000 rpm, 4 °C for 10 min. Cell growth was measured the optical density at 660 nm and supernatants obtained were used to assay proteases activity.

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Effects of concentrations of carbon and nitrogen sources on proteases production

The concentrations of carbon and nitrogen sources on proteases production of *Bacillus* sp., K52, K53, K61 and KK128 were studied by modified medium 3 containing (w/v) 1.0 % glucose, 0.25 % yeast extract, 0.02 % $CaCl_2.2H_2O$ and 1 % NaCl.

Table 3.1 The composition of medium 1 – medium 9

Composition (% w/v)	Medium								
	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**
Glucose	-	-	1	0.25	1.0	-	1.0	1.0	-
Peptone	1.0	-	-	-	0.5	-	-	0.5	-
Yeast extract	0.1	1.0	0.25	0.25	0.5	-	-	-	0.2
Beef extract	-	-	-	-	-	-	-	0.3	-
Casein	-	-	-	-	-	-	-	-	0.4
NaCl	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0
$CaCl_2$	-	-	0.05	0.002	-		0.15	-	0.08
$MgSO_4$	-	0.05	-	-	0.02		-	-	0.08
$NaNO_3$	-	-	-	-	-		0.05	-	-
$FeSO_4$	-	-	-	-	-		-	-	-
K_2HPO_4	-	0.1	-	-	-		1.0	-	0.1
KH_2PO_4	-	-	-	-	0.02		-	-	0.15
Na_2CO_3	-	-	-		1.0		-	-	
$(NH4)_2SO_4$	-	-	-	-	-		-	-	0.2
Sodium citrate	-	-	-	-	-		-	-	0.15
pH	7.0	7.0	7.0	7.0	7.0	7.0	7.0	7.0	7.0

- = no added

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The composition of each formula of medium 3 were summarized in Table 3.2. One to two loops of bacteria were cultivated in 100 ml of the medium in 500 ml Erlenmeyer flask on rotary shaker at 150 rpm incubated at 37 °C. The culture medium

was taken at 0, 24, 48 and 72 h and then centrifuged at 9,000 rpm at 4 °C for 10 min. The supernatant obtained was dialyzed against 10 mM KPB (pH 6.8) The dialyzed supernatant was used for assay of proteases activity.

Productivity of extracellular proteases from *Bacillus* sp., K52, K53, K61 and KK128 in medium 3 formula 1- formula 7

One to two loops of *Bacillus* sp., K52, K53, K61 and KK128 were cultivated in 100 ml of medium 3 formula 1-formula 7 in Table 3.2 in 500 ml Erlenmeyer flask on rotary shaker at 150 rpm incubated at 37 °C. The culture medium was taken at 0, 24, 48 and 72 h and then centrifuged at 9,000 rpm at 4 °C for 10 min. The supernatant obtained was dialyzed against 10 mM KPB (pH 6.8). The dialyzed supernatant was used for assay of proteases activity.

Table 3.2 The composition of medium 3 formula 1- formula 7

Composition (% w/v)	**Medium 3 Formula**						
	1	**2**	**3**	**4**	**5**	**6**	**7**
Glucose	1.0	1.0	0.25	0.5	1.0	1.0	1.0
Peptone	0.5	1.0	1.0	1.0	1.0	0.25	0.5
Yeast extract	0.25	0.25	-	-	-	0.02	0.02
$CaCl_2.2H_2O$	0.05	0.05	0.05	0.05	0.05	0.05	0.05
KH_2PO_4	-	-	0.1	0.1	0.1	0.01	0.01
K_2HPO_4	-	-	0.1	0.1	0.1	0.1	0.1

- = no added

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Productivity of intracellular proteases from *Bacillus* sp., K52, K53, K61 and KK128 in medium 3 formula 1 and formula 2

One to two loops of *Bacillus* sp., K52, K53, K61 and KK128 were cultivated in 100 ml of medium 3 formula 1 and formula 2. Each medium containing 100 ml in 500 ml Erlenmeyer flask on rotary shaker at 150 rpm incubated at 37 °C. Five ml of culture medium was withdraw at 0, 24, 48 and 72 h and then centrifuged at 9,000 rpm at 4 °C for 20 min. The harvested cells were washed twice with 10 mM KPB (pH 6.8) and subsequently suspended in 10 mM KPB. Cell-free extracts were prepared by sonication. The disrupted cells were collected by centrifugation at 9,000 rpm for 20 min at 4 °C. The cell-free extract was dialyzed against 10 mM KPB and was used for assay of aminopeptidase activity. The supernatant obtained was dialyzed against 10 mM KPB (pH 6.8). The dialyzed supernatant was used for assay of proteases activity.

Productivity of extra- and intracellular aminopeptidases of *Bacillus* sp., K52, K53, K61 and KK128 in medium 3 formula 1 and formula 2

One to two loops of *Bacillus* sp., K52, K53, K61 and KK128 were cultivated in 100 ml of medium 3 formula 1 and formula 2. Each medium containing 100 ml in 500 ml Erlenmeyer flask on rotary shaker 150 rpm incubated at 37 °C. The culture medium was taken at 0, 24, 48 and 72 h and then centrifuged at 9,000 rpm at 4 °C for 20 min. The harvested cells were washed twice with 10 mM KPB (pH 6.8) and subsequently suspended in 10 mM KPB. Cell-free extracts from washed cells were prepared by sonication. The disrupted cells were collected by centrifugation at 9,000 rpm for 20 min at 4 °C. The cell-free extract was dialyzed against 10 mM KPB and was used for assay of aminopeptidase activity.

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Identification of four bacteria, K52, K53, K61 and KK128

The morphological characteristic of bacteria, K52, K53, K61 and KK128 were observed after incubation of the culture on NA slant at 37 ^{o}C for 24 h by Gram's staining. The cultural characteristic was also observe on nutrient agar. The carbon assimilation and fermentation were examined by using test kit API 50CH incubated at 37 °C.

Enzyme assay

Proteases activity was assayed according to modified Anson's method (Anson, 1938). The reaction mixture contained 200 mM sodium phosphate buffer (pH 7.0), 0.25 ml of 1 % casein and 0.25 ml of enzyme in the final volume of 0.5 ml. The reaction was started by addition of enzyme. After 10 min at 40 °C, the reaction was stopped by adding 0.5 ml of 7 % trichloroacetic acid (TCA). After incubation for 30 min at ambient temperature, the mixture was centrifuged at 5,000 rpm at 4 °C for 10 min. The supernatant was mixed with 0.625 ml of 2 % Na_2CO_3 in 0.1 M NaOH and then incubated for 10 min. Further, 0.125 ml of Folin's reagent (2-fold dilution) was added. After incubating at 40 °C for 30 min, the absorbance at 660 nm was measured. Control samples were assayed in the same method as the test samples, except that the substrate was incubated with the denatured enzyme by the addition of TCA. One unit of proteases activity was defined as the amount of enzyme that catalyzes the formation of 1 µmole tyrosine per min under the given conditions.

Protein determination

Protein concentration was determined using Lowry method with egg albumin (BSA) as a standard (Appendix B).

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Effect of pH on enzyme activity

The effect of pH on crude enzyme activity was measured in various 200 mM buffer systems; citrate buffer (pH 5.0), phosphate buffer (pH 6.0, 7.0, 8.0), glycine/NaOH buffer (pH 9.0, 10.0) and Na_2HPO_4/NaOH buffer (pH 11.0). The reaction mixtures were pre-incubated for 30 min at room temperature, and assays started by addition of the enzyme. The proteases activity was determined after incubation at 40 ^{o}C for 10 min, as described above.

Effect of temperature on enzyme activity

The effect of temperature on crude enzyme activity was measured in 200 mM sodium phosphate buffer (pH 7.0) containing 1 % casein from bovine milk as substrate. Reaction mixtures were incubated at room temperature, 30, 35, 40, 45, 50, 55, 60 and 65 ^{o}C for 10 min and proteases activity was determined as described above.

Thermal stability

The crude proteases were pre-incubated in 200 mM sodium phosphate buffer (pH 7.0) h at 40, 45, 50, 55 and 60 ^{o}C. Samples of the enzyme were withdrawn for activity assay at 0, 15 min, 0.5, 1, 2, 4, 12 and 24 h. The reaction was started by addition of the enzyme and incubated at 40 ^{o}C for 10 min. Proteases activities were determined as described above.

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Effect of cations on enzyme activity

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After the crude enzyme was pre-incubated with various 1 mM cations; $BaCl_2$, $CoSO_4$, $CuSO_4$, $CaSO_4$, KCl, $ZnSO_4$, $K_3Fe(CN)_6$, $Fe_2(SO_4)$, $MnSO_4$, $MgSO_4$, $HgCl_2$, and $CaCl_2$ at ambient temperature for 5 min, the activity was assayed at 40 °C for 10 min using casein from bovine milk as substrate.

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Effect of chemical reagent on enzyme activity

The crude enzyme was pre-incubated with EDTA, mercaptoethanol, SDS, DTT, PMSF at a final concentration 5 mM in 200 mM sodium phosphate buffer (pH 7.0) at ambient temperature for 5 min, the activity was assayed at 40 °C for 10 min using casein from bovine milk as substrate.

Effect of sodium chloride on enzyme activity

After the enzyme was pre-incubated with 0, 5, 10, 15, 20 and 25 % NaCl at ambient temperature for 5 min in 200mM sodium phosphate buffer (pH 7.0), the reaction was started by addition of casein as substrate, and carried out at 40 °C for 10 min.

Substrate specificity

Casein hamersten, bovine serum albumin (BSA), egg albumin, hoe, gluten, whole egg and gelatin were investigated as substrates for the crude proteases. The enzyme was incubated with the substrates in 200 mM sodium phosphate buffer (pH 7.0) and carried out at 40 °C for 10 min. The enzyme activity was determined as described previously.

Results and Discussion

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Isolation of salt-tolerant bacteria

Salt tolerant baeteria were isolated from Thai fermented foods, soil from salt pan, salt, sea sand, sea water and sea shell solid wastes. One hundred and eighty two strains were obtained. It was found that 48, 51, 36, 3, 1, 2, 2, 3, 2, 1, 10, 1, 5, 1, 2, 10, 1, 1, 1, 1 strains were isolated from Nam-pla, Ka-pi, Pla-ra, Pla-chom, Bu-du, Tai-pla,

Kaey, Pung-pla, Pu-chem, Pla-too-chem, Tao-chieo, Phak-kard-dong, Naw-mai-dong, Gra-teim-dong, Hua-phak-kard-chem, soil from salt pan, salt, sea sand, sea water, and sea shell solid wastes, respectively (Table 3.3). The result showed that the number of bacteria from Nam-pla, Ka-pi and Pla-ra were more than the other sources. Because of these sources had more samples and we selected all of colony form that could grow on the medium. On the other hand, in the other sample source we selected some of colony form. Because they appeared the same types of colonies on the medium. In this study we found that most of microorganisms in Thai fermented foods were bacteria. Thongthai and Siriwongpairat (1978) reported that most of microorganisms from fish sauce have been identified as largely bacterial species while rarely as yeast and fungi were rarely found.

The study of characteristics of isolated bacteria

All of isolated bacteria were studied on the morphological characteristics by Gram's staining. It was found that 129, 38 and 15 strains were Gram positive; rods, Gram negative; rods and Gram positive; cocci, respectively (Table 3.4). Salt tolerant bacteria are usually found to occur in protein rich salt brines. Thai fermented foods, such as fermented fish products; Nam-pla (fish sauce), Pla-ra (fermented fish product), fermented soy product; soy sauce could be the sources of salt tolerant bacteria. Tanasupawat and Komagata (1995) reported that *Lactobacillus pentosus, L. plantarum* and *Pediococcus pentosaceus* were dominant in Thai fermented foods Som-fak (fish cake), Sai-krog-prieo (sour pork sausage) whereas *P. acidophilus* strains were present in fish sauce, Bu-du, Tai-pla. Liptasiri (1975) isolated bacteria from Thai fish sauce and classified into 7 genera which were *Micrococcus, Staphylococcus, Bacillus, Streptococcus, Pseudomonas, Sarcina* and *Lactobacillus*. The optimal growth

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Table 3.3 Summary the number of bacteria isolated from various sources

Sources	Number of isolate
Nam-pla (fish sauce)	48
Ka-pi (shrimp paste)	51
Pla-ra (fermented fish)	36
Pla-chom (fermented fish)	3
Bu-du (fermented fish in liquid form)	1
Tai-pla (fermented fish liquid entrails)	2
Kaey (fermented small shrimp)	2
Pung-pla (fermented fish visceral mass)	3
Pu- chem (salted crab)	2
Pla-chem (salted fish)	1
Tao-chieo (fermented soybean)	10
Phak-kard-dong (pickled mustards)	1
Naw–mai-dong (fermented bamboo shoot)	5
Gra-tiem-dong (fermented vegetable)	1
Hua-phak-kard-chem (Turnip with salt)	2
Soil from salt pan	10
Salt	1
Sea sand	1
Sea water	1
Sea shell solid wastes	1
Total	**182**

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Table 3.4 Summary of morphological characteristic of isolated bacteria

Characteristics	Number of isolated bacteria
Gram positive, Rods	129
Gram negative, Rods	28
Gram positive, Cocci	15
Total	**182**

conditions of *Pediococcus halophilus* isolated from Thai fish sauce were at 30-32 °C, pH 6.5 and salt requirement 2-5 % NaCl. Some properties of bacteria isolated from Thai fish sauce made from fresh water and marine fishes were different. *Pediococcus halophilus* was predominant in both samples of Thai fish sauce. It could grow slightly in acid medium at high temperature (43 °C) and in 20 % NaCl (Jayanandana, 1979).

The ability of growth of isolated bacteria on various concentrations of sodium chloride

The isolated bacteria were examined the ability of growth on nutrient agar containing various concentrations of sodium chloride. It was found that all of isolated bacteria showed excellent growth on media in the presence of 0, 5 and 10 % NaCl after cultivation for 24, 24 and 72 h, respectively. One hundred and seventy nine isolates grew on medium containing 15 % NaCl after cultivation for 5 days. After cultivation for 21 days, thirty five and twenty eight bacteria grew on media containing 20 and 25 % NaCl, respectively (Table 3.5). Irwin and Baird (2004) reported that moderate halophiles could grew in up to 15-20 % NaCl. This study showed that most of bacteria

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Table 3.5 Summary of the growth number of bacteria grew on nutrient agar containing various concentrations of sodium chloride

NA containing various concentations of NaCl (%w/v)	Number of isolate	Period of cultivation (days)
0	182	1
5	182	1
10	182	3
15	179	5
20	35	21
25	28	21

might be classified to halophilic bacteria. The colony of isolated bacteria was formed on NA containing 15, 20 and 25 % NaCl after incubation for 5, 21 and 21 days, respectively. This results indicated that high salt concentration environment had the effect on growth of bacteria. Because of increasing of salt concentration to 15, 20 and 25 % NaCl , some of bacteria grew slowly or could not grew. And some of them were not salt tolerant bacteria.

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The ability of growth of bacteria on various temperatures

Temperature profile of isolated bacteria were culture on the nutrient agar containing 1.5 % NaCl and incubated at 35, 40, 45, 50, 55 and 60 °C. It was found that all of isolated bacteria were excellent growth at 35, 40,45 °C, respectively and no growth at 60 °C (Table 3.6). However, only thirty-three and sixteen strains were growth at 50 and 55 °C. Most of the isolated bacteria grew at maximum 45 °C and the result

indicated that most of isolated bacteria were classified to moderately thermophilic bacteria. Aroonpairoj (1997) reported that most of bacteria which isolated from Thai fish sauce had optimal growth at 37 ^{o}C.

Table 3.6 Summary of the number of ability of growth of isolated bacteria at various temperatures

Temperature (^{o}C)	**Number of isolate**
35	182
40	182
45	182
50	33
55	16
60	0

Proteases production on skimmed milk agar (SMA)

The proteases production of isolated bacteria were determined on skimmed milk agar (SMA) containing 0, 5, 10, 15, 20 and 25 % NaCl were studied. It was found that 155 and 143 strains gave clear zone on SMA in the absence of NaCl after cultivation for 10 h. All of them could not gave clear zone on SMA containing 10, 15, 20 and 25 % NaCl (Table 3.7). These results showed that we could not obtain the salt tolerant proteases from these bacteria. It is possible that we isolated the bacteria that not be the dominate group during the stage of fermentation. Because of the author purchased the fermented products from local markets and isolated the bacteria from a final stage of fermentation process.

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Table 3.7 Summary of the number of isolated bacteria giving clear zone on SMA containing various concentrations of sodium chloride

SMA containing various concentrations of NaCl (% w/v)	**The number of isolates gave clear zone**	**Period of cultivation (days)**
0	155	10 h
5	143	2
10	0	21
15	0	21
20	0	21
25	0	21

These fermented products were highly contaminated with various microorganisms. So we could not obtain bacterial strains that play an important role in each stage of fermentation processes. On the other hand, the composition of medium and condition for isolation step were not appropriate for these microorganisms. Thongthai *et al.* (1989) reported that protease activities in natural process of fish sauce fermentation were detected maximally on day 1 of fermentation and decreased rapidly to 5.1 % of original on day 8 to 9.5 % of original on day 15. The activities were barely detectable on day 29 and 37. However, high concentration of sodium chloride was found generally to suppress proteases activities. The higher the concentration, the greater the degree of suppression (Thongthai and Siriwongpairat, 1978).

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Proteases production in PY medium

The productions of proteases by isolated bacteria were determined in PY medium. It was found that twelve bacteria, K21, K24, K52, K53, K61, KK12, KK19, KK128, KK137, TN103, RC194 and RC210 had high specific proteases activity (Table 3.8). These bacteria were obtained from fermented foods. They were Gram positive, rods shape and all of them grow on nutrient agar containing 15 % NaCl. These twelve bacteria were selected for further study.

Effect of composition of medium on proteases production

The effect of composition of the medium on proteases production of twelve bacteria were determined in medium 1- medium 9. The composition of each medium as described previously. The results showed that twelve bacteria produced highest specific proteases activity in medium 3. Bacteria, K61, K53, K52 and KK128 showed highest proteases activity in medium 3, 0.127, 0.140, 0.162, 0.111 U/mg, respectively (Table 3.9). The composition of PY medium containing (% w/v) 1.0 % peptone, 0.1 % yeast extract and 1.0 % NaCl whereas medium 3 containing (% w/v) 1 % glucose, 0.25 % yeast extract, 0.05 % $CaCl_2.2H_2O$ and 1 % NaCl. The composition of medium 3 had $CaCl_2$. The presence of calcium was known to modulate the protease activity (Spungin and Blumberg, 1989). Proteases activity was activated by calcium ion found in *B. subtilis* (Yang *et al*., 2000), *B. pumilus* (Yasuda and Aoyama(2000), *B. latersporus* and *Flavobacaterium* sp. (Sharma *et al*., 1996). Medium 3 was selected for proteases production.

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Table 3.8 Proteases production of bacteria in PY medium

Bacterial Code	Sources	Ability of growth on medium containing NaCl (%)	Specific activity (U/mg)
K21	Nam-pla	15	0.088
K24	Ka-pi	15	0.088
K52	Ka-pi	15	0.115
K53	Pla-ra	15	0.109
K61	Pla-ra	15	0.123
KK12	Ka-pi	15	0.096
KK19	Ka-pi	15	0.095
KK128	Ka-pi	15	0.102
KK137	Ka-pi	15	0.090
TN103	Tau-chieo	15	0.091
RC194	Pla-ra	15	0.090
RC210	Pla-ra	15	0.084

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Table 3.9 Proteases production of bacteria in different composition of medium

Bacterial Code	Specific activity (U/mg)								
	Medium								
	1	2	3	4	5	6	7	8	9
K21	0.088	0.030	0.095	0.019	0.065	0.011	0.024	0.040	0.012
K24	0.088	0.023	0.093	0.040	0.047	0.014	0.019	0.020	0.006
K52	0.115	0.070	0.127	0.057	0.050	0.013	0.013	0.103	0.094
K53	0.109	0.094	0.140	0.058	0.055	0.016	0.013	0.099	0.103
K61	0.123	0.077	0.162	0.069	0.056	0.017	0.020	0.107	0.112
KK12	0.096	0.080	0.103	0.042	0.043	0.014	0.018	0.100	0.062
KK19	0.095	0.081	0.107	0.056	0.046	0.013	0.013	0.098	0.062
KK128	0.102	0.090	0.111	0.069	0.046	0.015	0.017	0.107	0.110
KK137	0.090	0.087	0.109	0.052	0.054	0.011	0.023	0.086	0.031
TN103	0.091	0.088	0.109	0.059	0.043	0.014	0.026	0.068	0.066
RC194	0.090	0.092	0.110	0.083	0.052	0.014	0.023	0.023	0.083
RC210	0.084	0.038	0.100	0.049	0.050	0.016	0.021	0.085	0.046

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Time course of four isolated bacteria in medium 3

Relationship between proteases production and growth of *Bacillus* sp. strain K52, K53, K61 and KK128 were studied in medium 3. It was found that *Bacillus* sp., K52, K53, K61 and KK128 produced maximal proteases activities 0.48, 0.39, 0.52 and 0.45 unit/ml at 84, 84, 96 and 84 h, respectively. The maximum of the cell growth was at 72, 60, 72 and 72 h, respectively (Figure 3.1). The proteases production increased with increasing cell growth, resulting in maximum proteases activity at the end of exponential phase in all strains. These bacteria were similar to a *Bacilllus* sp., isolated from fish sauce, which showed the highest proteases activity when grown in a medium containing up to 12 % NaCl at 52(69 h), 132 (63 h) and 88 (69 h) units/ml, respectively (Pornsettakul, 1991). Proteases activity from *Pseudoalteromonas* sp. strain CP76 showed maximal proteases production at the end of exponential growth phase(Porro *et al*., 2003).

Comparing the growth phase and proteases production of *Bacillus* sp., K52, K53, K61 and KK128 in this study with other reports, the timing of proteases production occured late in the growth cycle. It might be the inoculum used was to small or the culture conditions were not optimized. This would be necessary for further work on this system to be carried out effectively.

There are many factors that effect on proteases production by bacteria. High level of extracellular proteases by *Bacillus* sp. were obtained on soybean flour, casein and starch but low levels on carboxymethylcelluloase, pectin polypectate and polygalacturonate (Mahmood *et al*., 2000). Starch and peptone were the best inducers for maximum proteases production by salt-tolerant *Bacillus subtilis* NCIM no. 64 (Kembhavi *et al*., 1993). Maltose or hydrolyzed starch as the carbonn source, monopotassium phosphate as a phosphorus source, ammonium sulphate as a nitrogen sources. The addition of albumin, peptone or casein, methionine, isoleucine and valine stimulated the synthesis of proteasess from *B. subtilis* strain 103 (Kalinuant *et al*., 1979).

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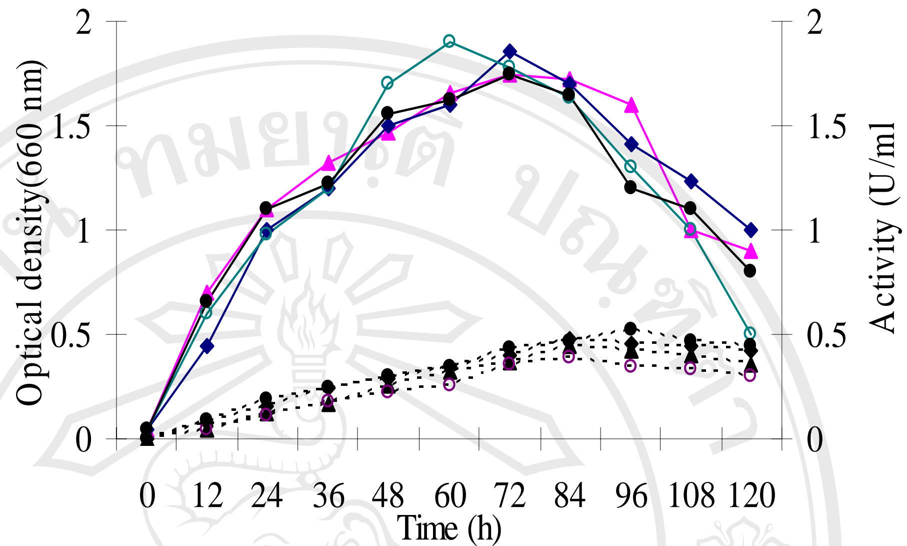

Figure 3.1 Time course of *Bacillus* sp., K52 (—◆—), K53(—○—), K61(—●—) and KK128 (—▲—) and growth, K52 (--◆--), K53 (--○--), K61 (--●--) and KK128 (--▲--) a) of *Bacillus* sp., The strains were cultured at 37 °C for 12 to 120 h in 100 ml of medium containing 1.0 % glucose, 0.25 % yeast extract, 0.05 % $CaCl_2$ and 1 % NaCl in 250 ml Erlenmeyer flask. Enzyme assays were carried out at 12 h intervals.

And 10 g/l of casein was the best nitrogen source for alkaline proteases production by *B. subtilis* (Massucco *et al*., 1980). Starch or its hydrolyzate dextrin, maltose as the carbon source. Ammonium phosphate and casein were to be the optimal nitrogen source for *Bacillus mesentericus* and *B. subtilis.* Complex B vitamins added to the nutrient medium could increase enzyme synthesis 2.5-4 fold (Emtseva, 1975). Fairbairn and Law (1987) found that the medium containing sodium casinate supported good growth and proteases production of *Pseudomonas fluorescens.* Asparagine was the most effective amino acid inducer for proteases production. *Pseudomonas aeruginosa* K-187 could produced higher proteases activity when the initial pH and temperature were at 8.0 and 25 °C, respectively (Oh *et al*., 2000).

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The effects of concentrations of carbon and nitrogen sources on extracellular proteases production

The effects of concentrations of carbon and nitrogen sources on proteases production were studied by modification of medium 3. The protease production of *Bacillus* sp., K52, K53, K61 and KK128 in medium 3 were medium 3 formula 1 and 2 (Figure 3.2 a and b), medium 3 formula 3, 4 and 5 (Figure 3.3 a, b and c) and medium 3 formula 6 and 7 (Figure 3.4 a and b).

The comparison of specific activities of proteases in each medium was showed in Table 3.10. It was found that *Bacillus* sp., K52, K53 and KK128 showed highest proteases specific activity in medium 3 formula 1, 0.14, 0.08, 0.36 and 0.29 at 72, 72, 72 and 72 h, respectively. *Bacillus* sp. K61 showed highest specific protease activity in medium 3 formula 2 0.39 U/mg at 72 h. The result had substantial variation in the proteases activity of different medium. It could have been due to the use of different concentration of glucose, peptone and yeast extract in medium. Metals might increase or decrease the production of proteases as well as their stability (Kohlman *et al*., 1991, Olson *et al*., 1992, Sexton *et al*., 1994 and Stepaniak *et al*., 1982). The addition of 10 mM $MgCl_2$ to a minimal salt medium containing 1 % yeast extract, 1 % K_2HPO_4, 1 % NaH_2PO_4 increased the specific proteases activity two fold. A medium consisting of 1 % yeast extract, 1 % K_2HPO_4, 1 % NaH_2PO_4 with 1 % $MgCl_2$ provided the simplest and cheapest medium for the growth of *Burkholderia* strain 2.2 N and purification of a large amount of proteases. Addition of metal ions to growth media has been shown to not only increase proteases activity, but may also be required for the

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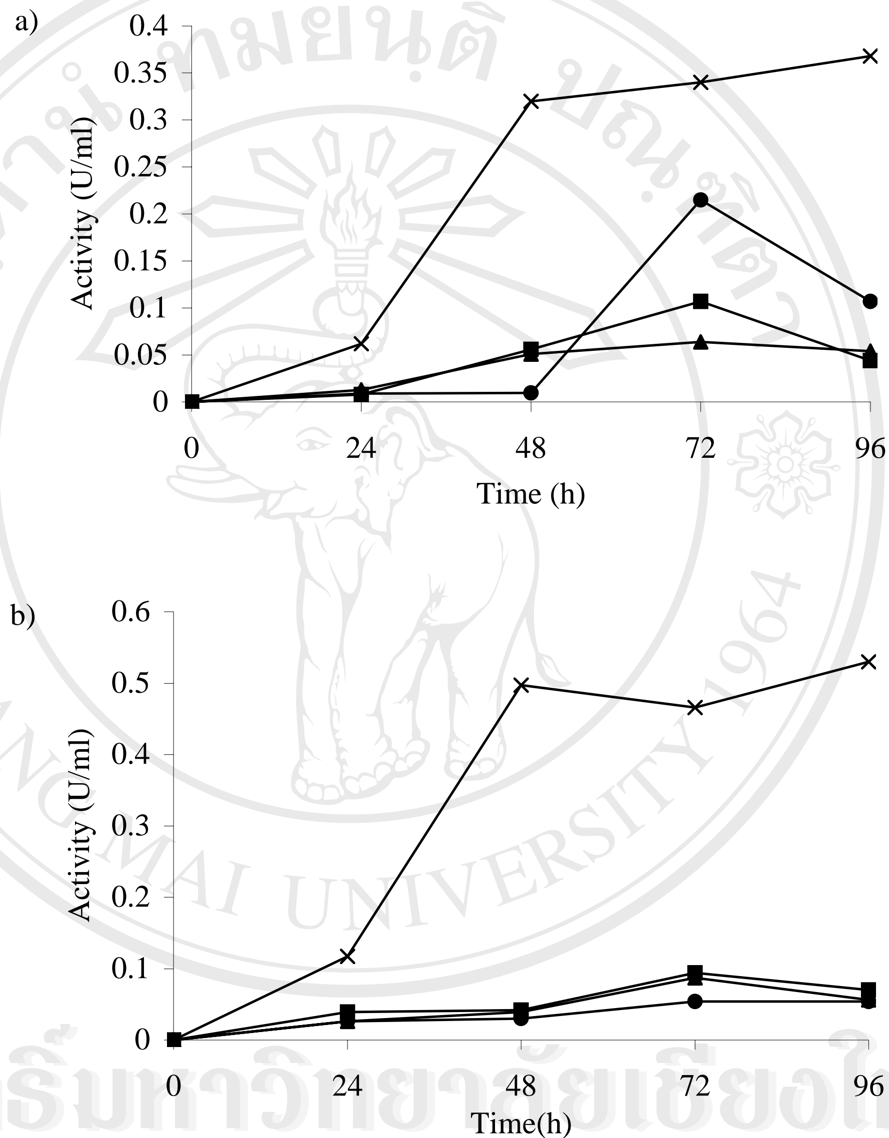

Figure 3.2 Production of extracellular proteases by *Bacillus* sp., K52(■), K53 (▲), K61(✕) and KK128 (●) a) medium 3 formula 1; 1 % glucose, 0.5 % peptone 0.25 % yeast extract, , 0.05 % $CaCl_2.2H_2O$ b) medium 3 formula 2; 1 % glucose, 1 % peptone, 0.5 % yeast extract, 0.02 % $CaCl_2.2H_2O$

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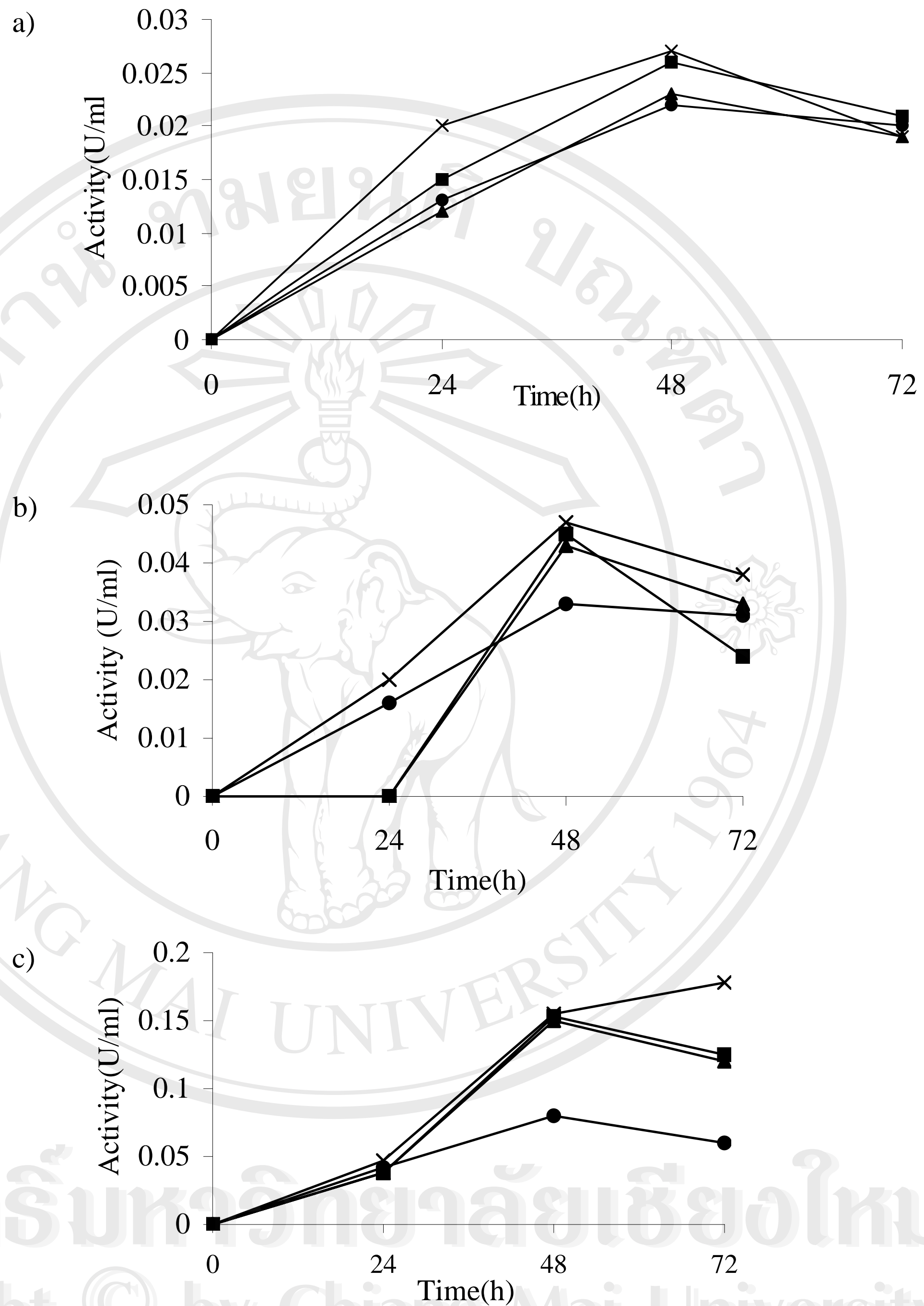

Figure 3.3 Production of extracellular proteases by *Bacillus* sp., K52(■), K53 (▲), K61(✕) and KK128(●) a) medium 3 formula 3; 0.25 % glucose, 1 % peptone, 0.1 % KH_2PO_4, 0.1 % K_2HPO_4, 0.05 % $CaCl_2$, 0.02 % yeast extract pH 7.0, b) medium 3 formula 4; 0.5 % glucose, 1 % peptone, 0.1 % KH_2PO_4, 0.1 % K_2HPO_4, 0.05 % $CaCl_2$, 0.02% yeast extract pH 7.0, c) medium 3 formula 5; 1% glucose, 1 % peptone, 0.1 % KH_2PO_4, 0.1 % K_2HPO_4, 0.05 % $CaCl_2$, 0.02 % yeast extract pH 7.0

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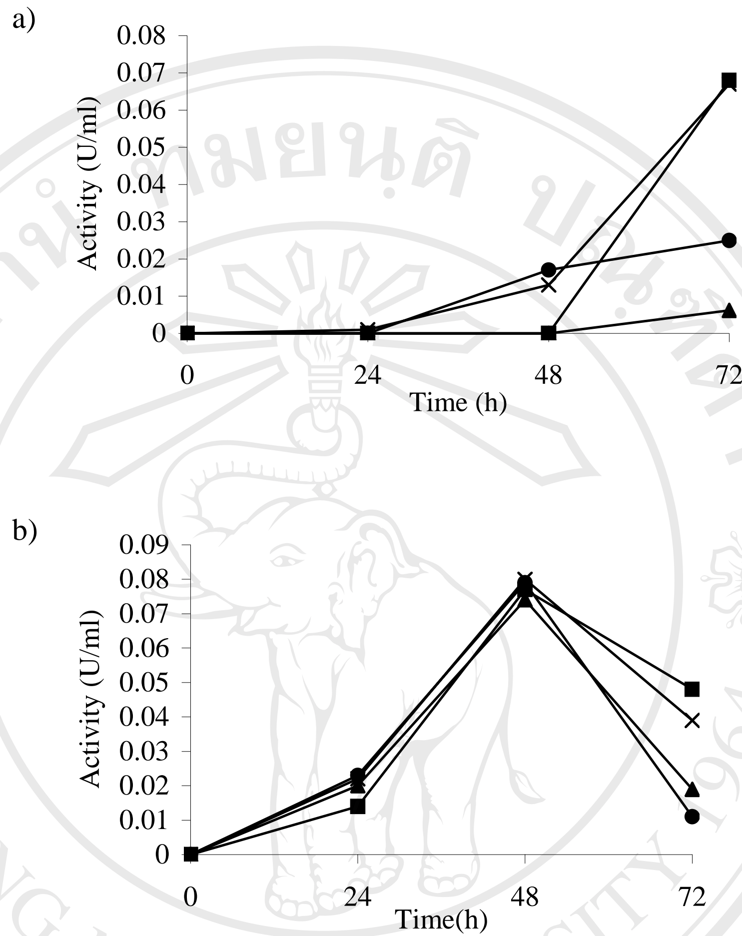

Figure 3.4 Production of extracellular proteases by *Bacillus* sp., K52 (■), K53 (▲), K61 (✕) and KK128 (●). The strains were cultured at 37 ^{o}C for 12 to 72 h in 100 ml of medium 3 formula 6 in 500 ml flask. medium 3 formula 6 a) 1 % glucose, 0.25 % peptone, 0.1 % KH_2PO_4, 0.1 % K_2HPO_4, 0.05 % $CaCl_2$, 0.02 % yeast extract pH 7.0 and medium 3 formula 7 b) 1 % glucose, 0.5 % peptone, 0.1 % KH_2PO_4, 0.1 % K_2HPO_4, 0.05 % $CaCl_2$ and 0.02 % yeast extract, pH 7.0

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production and stability of some proteases (Liao and McCallus, 1997; Kohlman *et al*., 1991; Olson and Ohman, 1992).

In this study the results showed that 1 % glucose and 0.5 % peptone were the optimal concentration of carbon and nitrogen sources for proteases production of *Bacillus* sp., K52, K53 and KK128 and 1 % glucose , 1 % peptone for *Bacillus* sp. K61. *Bacillus* sp. K61 showed highest specific protease activity when compared with the other strain. So strain K61 was selected for further study.

Table 3.10 The specific activity of extracellular protease of *Bacillus* sp., K52, K53, K61 and KK128 in various formula of medium 3

Bacteria	**Specific activity (U/mg)**						
	Medium 3 Formula						
	1	**2**	**3**	**4**	**5**	**6**	**7**
K52							
24 h	0.01	0.01	0	0	0	0	0.01
48 h	0.03	0.01	0.01	0.01	0.02	0	0.03
72 h	0.14	0.06	0	0	0.03	0.01	0.02
K53							
24 h	0.01	0.01	0	0	0	0	0.01
48 h	0.03	0.01	0.01	0.01	0.01	0	0.02
72 h	0.08	0.06	0	0.01	0.02	0.01	0.01
K61							
24 h	0.07	0.06	0	0.01	0	0.02	0.01
48 h	0.22	0.18	0.01	0.01	0.03	0.01	0.02
72 h	0.36	0.39	0	0	0.02	0.06	0.01
KK128							
24 h	0.01	0.01	0	0	0	0	0.01
48 h	0.01	0.01	0.01	0.01	0.01	0.02	0.02
72 h	0.29	0.04	0	0	0.02	0.02	0

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Productivity of intracellular proteases by *Bacillus* sp., K52, K53, K61 and KK128

The intracellular proteases of *Bacillus* sp., K52, K53, K61 and KK128 in medium 3 formula 1 and formula 2 were determined. It was found that all of them showed low specific proteases activity in both formula (Table 3.11). These results indicated that *Bacillus* sp., K52, K53, K61 and KK128 produced extracellular proteases.

Table 3.11 Specific activity of intracellular proteases of *Bacillus* sp. K52, K53, K61 and KK128 in medium 3 formula 1 and formula 2

Time (h)	Specific activity (U/mg)							
	Intracellular proteases							
	Medium 3 formula1				Medium 3 formula2			
	K52	K53	K61	KK128	K52	K53	K61	KK128
24	0	0.03	0.01	0.04	0	0	0.04	0.01
48	0.02	0.04	0.11	0.04	0.04	0.04	0.09	0.04
72	0.03	0.03	0.05	0.05	0.01	0.01	0.06	0.03

Productivity of extra- and intracellular aminopeptidase by *Bacillus* sp., K52, K53, K61 and KK128

The extra- and intracellular aminopeptidase of *Bacillus* sp. strain K52, K53, K61 and KK128 were examined in medium 3 formula 1 and formula 2. The aminopeptidase activity against various substrates, Leu-pNA, Z-Ala-Ala-Leu-pNA and Pyr-Phe-Leu-pNA were determined. It was found that all of them no extra- and intracellular aminopeptidase activity in every substrate. However, these bacteria may be applied for proteases production and applied in other protein foods which contain high salt concentration. Because these bacteria could grow on the medium contained 15 % NaCl.

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Identification of four bacteria

Four bacteria, K52, K53, K61 and KK128 were identified. It was found that *Bacillus* sp., K52, K53, K61 and KK128 were Gram positive, rod shape and central endospore. All of selected bacteria were white colony. The colony form of K52, K53 and KK128 were circular but K61 was irregular. The elevation of the colony of K52 and K53 were raise and K61 and KK128 were flat. The entire edges of colony were observed in strain K52, K53 and KK128 but K61 was located edge of colony. The colony surface of K61 was contoured and K52, K53 were entire and smooth surface of KK128. The consistencies of colony of all selected bacteria were butyrous. The results showed that all of selected bacteria were spore forming bacteria. All of them could motile and catalases were positive. The growth at various tempearures was studied. It was found that all of selected bacteria could grow at 45 and 50 °C. Strain K52, K61 and KK128 grew at 55 °C but K52 could not grow. All of selected bacteria could not grow at 60 °C. The maximum concentration of NaCl capabing of growth of all of selected bacteria were 15 % NaCl. The characteristics of selected bacteria was showed in Table 3.12. Ventosa and Nieto (1995) reported that a moderate halophile could grow optimally in media containing 3-15 % NaCl. So these bacteria could be classified as moderate halophilic bacteria.

The carbon assimilation and fermentation of four bacteria were examined by API50 CH test kit. The four strains had differences in their cultural characteristic and some biochemical characteristics, suggesting that they were different strains. Strains K52, K53, K61 and KK128 showed similarities to *Bacillus* sp., this genus is a ubiquitous component of the bacterial flora of Thai fermented fish, such as Nam-pla, Pla-ra, Ka-pi. *Bacillus* sp. was found in Thai fish sauce (Liptasiri, 1975). Santinanalerts (1979) reported that *Bacillus, Coryformans* and *Halobacterium* were the majority of organisms with proteolytic enzymes in Thai fish sauce which had demonstrable proteases activity against gelatin and casein. For this study *Bacillus* sp. K61 showed proteases activity higher than the other strains and complete to identify. It was identified as *Bacillus licheniformis.*

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Table 3.12 The characteristics of four bacteria

Characteristics	Bacteria			
	K52	**K53**	**K61**	**KK128**
1. Gram	positive	positive	positive	positive
2. Cell Shape	rods	rods	rods	rods
3. Colony color	white	white	white	white
4. Motility	+	+	+	+
5. Spore	central spore	central spore	central spore	central spore
6. Catalase	+	+	+	+
7. Casein hydrolysis	+	+	+	+
8. Citrate	+	+	+	+
9. MR	-	-	-	-
10. VP	+	+	+	+
11. Nitrate	+	+	+	+
12. Starch hydrolysis	+	+	+	+
13. Lecithinase	+	+	-	+

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Table 3.12 (Continued)

Characteristics	Bacteria			
	K52	**K53**	**K61**	**KK128**
14. Growth at				
- 45 °C	+	+	+	+
- 50 °C	+	+	+	+
- 55 °C	+	-	+	+
- 60 °C	-	-	-	-
15. Growth on				
- 0 % NaCl	+	+	+	+
- 5 % NaCl	+	+	+	+
- 10 % NaCl	+	+	+	+
- 15 % NaCl	+	+	+	+
16. Glycerol	+	+	+	+
17. Erythritol	-	+	+	-
18. D-arabinose	-	+	+	-
19. L-arabinose	+	+	+	+
20. Ribose	+	+	+	+
21. D-xylose	-	+	+	-
22. Adonitol	-	+	+	-
23. β–Methyl-xyloside	+	+	+	-
24. Galactose	+	+	+	+
25. D-glucose	+	+	+	+
26. D- fructose	+	+	+	+

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Table 3.12 (Continued)

Characteristics	Bacteria			
	K52	**K53**	**K61**	**KK128**
27. D-mannose	+	+	+	+
28. L-sorbose	-	+	+	-
29. Rhamnose	-	+	+	-
30. Dulcitol	-	+	+	-
31. Inocitol	+	+	+	+
32. Mannitol	+	+	+	+
33. Sorbitol	+	+	+	+
34. α- Methyl-D-mannoside	+	+	+	-
35. α-Methyl-D-Glucoside	+	+	+	+
36. N-Acetyl glucosamine	+	-	-	-
37. Amygdaline	+	+	+	+
38. Arbutine	+	+	+	+
39. Esculine	+	+	+	+
40. Salicine	+	+	+	+
41. Cellobiose	+	+	+	+
42. Maltose	+	+	+	+
43. Lactose	+	+	+	+
44. Melibiose	+	+	+	+
45. Saccharose	+	+	+	+
46. Trehalose	+	+	+	+
47. Inuline	-	-	-	-
48. Melezitose	+	-	-	-

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Table 3.12 (Continued)

Characteristics	Bacteria			
	K52	**K53**	**K61**	**KK128**
49. D-raffinose	+	+	+	+
50. Amindon	+	+	-	+
51. Glycogene	+	+	+	+
52. Xylitol	-	+	+	-
53. β-gentiobiose	-	+	+	+
54. D-turasose	-	-	±	-
55. D-lyxose	-	-	-	-
56. D-tagatose	-	-	-	-
57. D-fucose	-	-	-	-
58. L-fucose	-	-	-	-
59. D-arabitol	-	-	-	-
60. L-arabitol	-	-	-	-
61. Gluconate	-	-	-	-
62. 2-ceto-gluconate	-	-	-	-
63. 5-ceto-gluconate	-	-	-	-

Remark

+ = positive

± = slightly positive

- = negative

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The effect of pH on enzyme activity

Proteases activity was determined over the range pH 5.0-11.0 using buffer systems as described previously. The optimum pH for crude proteases activities of strains K52, K61 and KK128 was at pH 8.0 but at 7.0 for strain K53 (Figure 3.5). Therefore, the crude proteases produced by *Bacillus* sp., K52, K53, K61 and KK128 belong to the neutral proteases since they had optimal pH at 7.0-8.0. These enzyme had a similar optimum pH, at pH 8.0, for enzyme activity as has been reported for *B. licheniformis* (Tanskul and Trirattananukul, 1998) and *Bacillus latersporus* (Sharma *et al*., 1996). The optimal pH and temperature for highest proteases activity produced by other *Bacillus* sp. were 7.0-8.0 and 55 ^{o}C, respectively (Yang *et al*., 2000 , Choorit and Prasertsan, 1992).

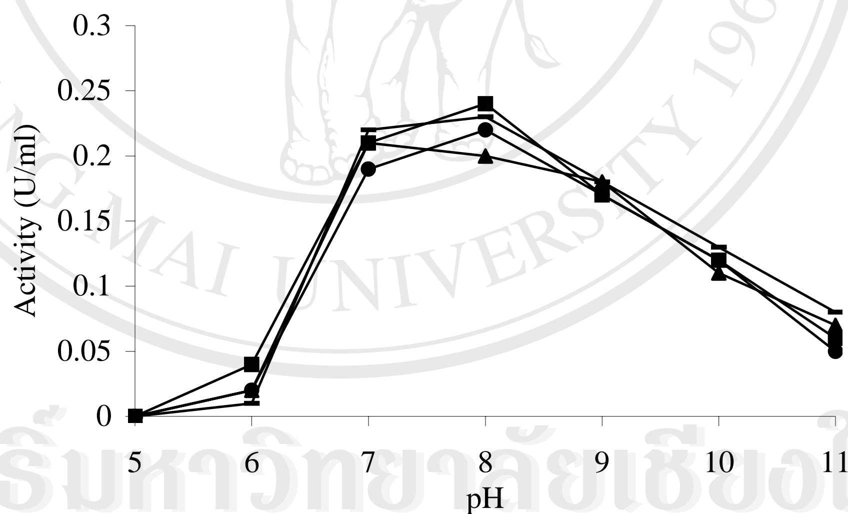

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Figure 3.5 Effect of pH on proteases activities from *Bacillus* sp., K52 (■), K53 (▲), K61 (-) and KK128 (●). The buffers used are citrate buffer (pH 5.0), phosphate buffer (pH 6.0, 7.0, 8.0), glycine/NaOH buffer (pH 9.0, 10.0), Na_2HPO_4/NaOH buffer (pH 11.0).

The effect of temperature on enzyme activity

The optimum temperatures of crude proteases activities from strains K52, K53, K61 and KK128 were at 55, 50, 55 and 55 ^{o}C, respectively (Figure 3.6).

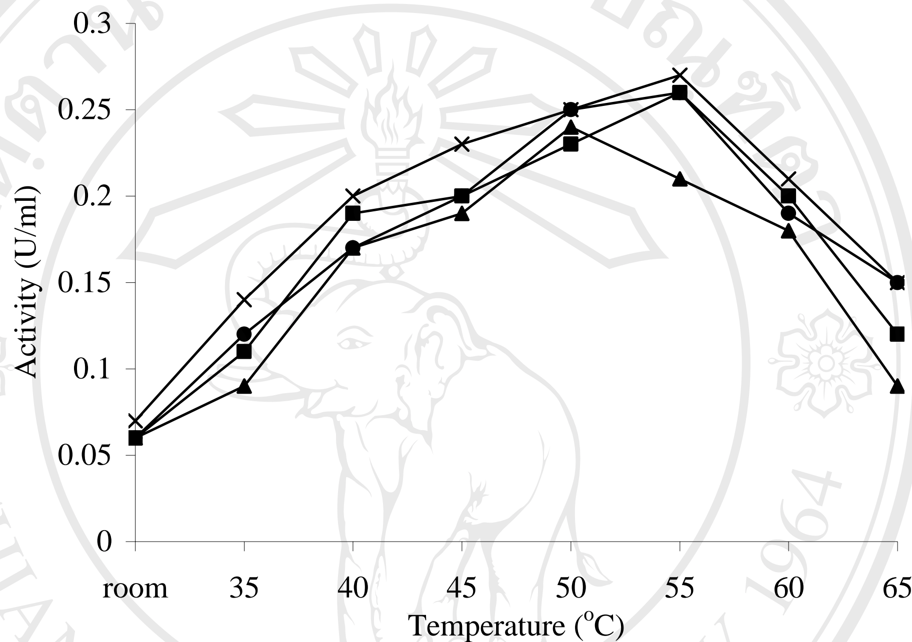

Figure 3.6 Effect of temperature on proteases activities from *Bacillus* sp., K52 (■), K53 (▲), K61 (✕) and KK128 (●). The enzyme activity was assayed at various temperatures (35-65 ^{o}C)

Thermal stability

The thermostabilities of crude proteases from *Bacillus* sp., K52, K53, K61 and K128 were investigated. The crude proteases was incubated at 40, 45, 50, 55 for 24 h and 60 °C for 1 h (Figure 3.7 a and b). It was found that the four crude proteasess were stable at 40 °C and 45 °C after incubated for 24 h, respectively, and at 50 °C and 55 °C retained the activities about 50 % after incubate for 12 and 2 h, respectively (Figure 3.7c and d). The resulted showed that this enzyme was stable at 55 °C for 1 h. These

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results indicated that the crude proteases from four bacteria were moderate thermotolerant. However, the crude proteases activities of *Bacillus* sp. strains K52, K53, K61 and KK128 decreased sharply after 30 min at 60 °C. Proteases from these bacteria metal ions (Mn^{2+}, Fe^{2+}, Zn^{2+}, Mg^{2+}, Ca^{2+}, Hg^{2+} on protease similar to the proteases of *Bacillus subtilis* strain 38 which isolated from traditionally fermented soybean showed unstable at 60 °C (Chantawannukul et al., 2002).

The effect of various reagents on enzyme activity

Table 3.13 showed the effect of chemical reagents and metal ion on the crude proteases activity. All of proteases were strongly inhibited by EDTA, SDS, Fe^{2+} and Hg^{2+} and moderately inhibited by DTT, PMSF and Cu^{2+}. With mercaptoethanol, Ba^{2+}, K^{+}, Zn^{2+}, a had slightly effect on proteases activity, whereas Co^{2+}, Glycerol, Mn^{2+}, K^{+}, and Ca^{2+} showed a small enhancement of proteases activity. These enzymes like most proteasess were stable and active in $CaCl_2$. Yang *et al*., 2000 studied the effects of metal ions (Mn^{2+}, Fe^{2+}, Zn^{2+}, Mg^{2+}, Ca^{2+}, Hg^{2+} on proteases activity by *Bacillus subtilis*. The proteases activity was activated by Mn^{2+}, Fe^{2+}, Zn^{2+}, Mg^{2+}, Co^{2+}, but inhibited completely by Hg^{2+}. The proteases was also inhibited by the metal-chelating agent such as EDTA, sulhydryl reagents such as, mercaptoethanol, and by cysteine hydrochloride, histidine, and glycerol. EDTA was the most effective inhibitor. This enzyme is a metal-chelator sensitive neutral proteases. The enzyme from *Bacillus pumilus* isolated from soybean food was activated by the addition of 1 mM Mn^{2+} and Ca^{2+} ion in the reaction mixture (Yasuda and Aoyama, 2000). The alkaline serine proteases from *Bacillus licheniformis* was activated by sodium chloride and stable in the presence of 0.7 % (w/v) $NaBO_3$, 0.5 %(w/v) NaCl and 3 % (w/v) H_2O_2 (Manachini and Fortina, 1998). Ba^{2+}, Ca^{2+}, Fe^{2+}, Mg^{2+}, Mn^{2+}, Zn^{2+} had the effect on the activities of proteases from *Bacillus latersporus* and *Flavobactereium* sp. Proteases from *B. laterosporus* was inhibited and by Fe^{2+}, Mg^{2+}, Mn^{2+} and Zn^{2+} ions but enhanced by Ba^{2+}, Ca^{2+} and Fe^{2+} (Sharma *et al*., 1996).

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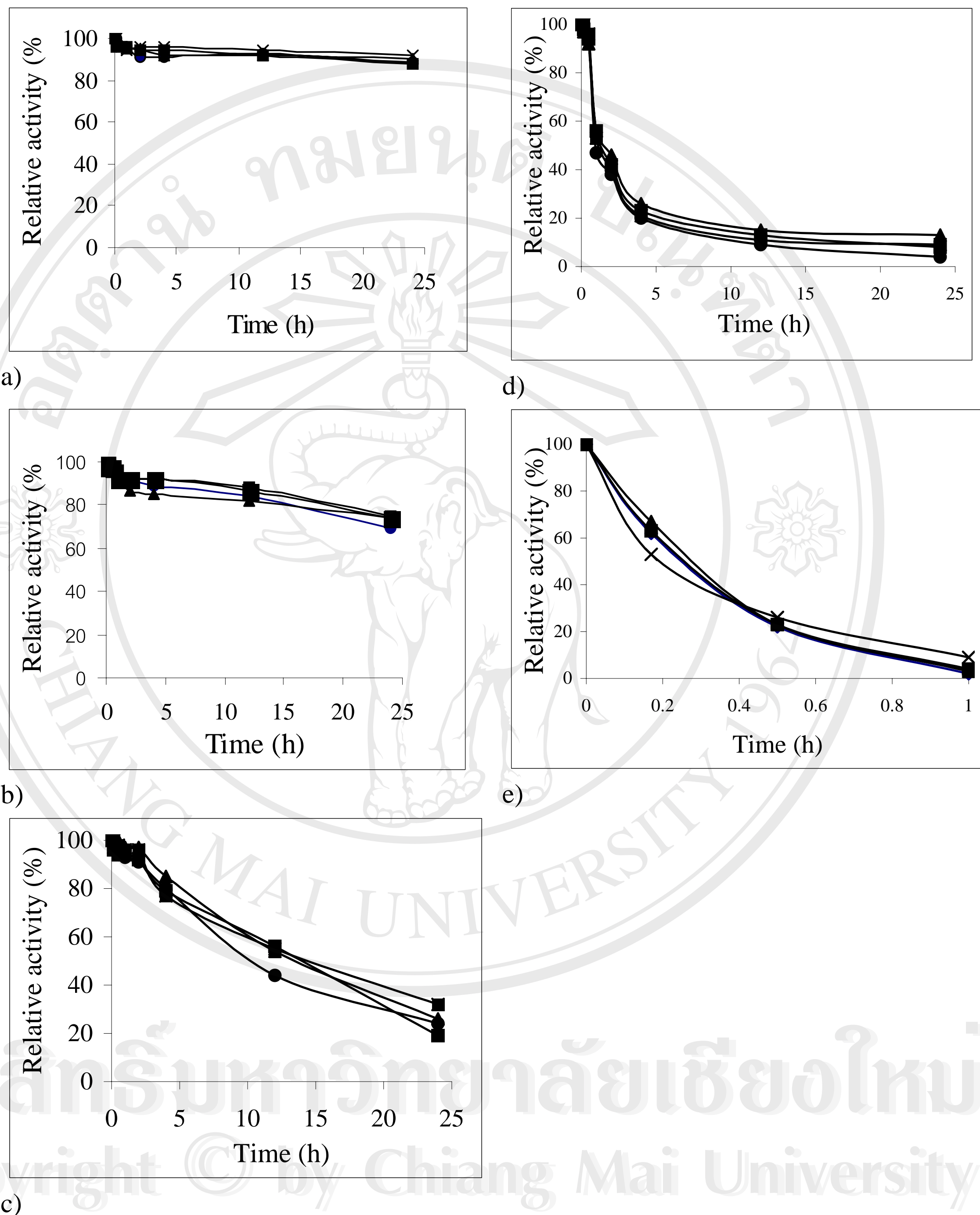

Figure 3.7 Effect of temperature on the stability of crude proteases from strains K52 (■), K53 (▲), K61 (✕) and KK128 (●). After the enzyme was treated at a) 40°C b) 45°C c) 50°C d) 55°C e) 60°C for 0.25-24 h before addition of substrate, the residual enzyme activity was assayed.

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Table 3.13 Effect of various reagents on crude proteases activities from *Bacillus* sp., K52, K53, K61and KK128

Reagents (5 mM)	**Relative activity (%)**			
	***Bacillus* sp.**			
	K52	**K53**	**K61**	**KK128**
None	100	100	100	100
EDTA	23	21	21	11
Mercaptoethanol	96	100	100	97
SDS	20	17	17	14
DTT	43	63	17	32
PMSF	50	13	59	43
$BaCl_2$	83	88	81	80
$CoSO_4$	101	78	111	111
$CuSO_4$	29	54	62	40
$(Fe)_2(SO_4)$	8	10	15	11
Glycerol	114	106	111	111
$MnSO_4$	110	103	107	109
$MgSO_4$	99	98	101	100
$HgCl_2$	27	26	24	11
KCl	112	96	113	91
$CaCl_2$	103	106	116	106
$ZnSO_4$	97	93	94	86
$K_3Fe(CN)_6$	97	80	92	93

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The proteases from *Pseudomonas* sp. was activated by Mg^{2+}, Co^{2+} and Ca^{2+}, and heavy metal ion such as Mg^{2+}, Cu^{2+} and Hg^{2+} inactivated the enzyme. Thiol reagents and diisopropyl fluorophosphate did not affect the enzymatic activity of the proteases. Metal reagent, ethylenediaminetetraacetic acid endo-phenanthroline, inhibited enzymatic activity, although citrate and oxalate did not affect it (Qua *et al*., 1981). Ferrous sulfate, copper sulfate, magnesium sulfate and sodium chloride had effect on proteases activity of *Pseudomonas aeroginosa* (Oh *et al*., 2000). The proteases activity of *Pseudoalteromonas* sp. strain CP76 was showed metalloproteases, strongly inhibited by EDTA and PMSF. No significant inhibition was detected with E-64, bestatin, chymostatin, or leupeptin (Porro *et al*., 2003). The proteases activity of *Flavobacterium* sp. was inhibited by Mg^{2+}, and Mn^{2+} ions but enhanced by Ba^{2+}, Ca^{2+} and $Fe^{2+.}$ The enzyme activity of the former was strongly inhibited by KCN, whereas that of the latter was only slightly inhibited by 8-hydroxyquinoline (Sharma *et al*., 1996).

The effect of concentration of NaCl on enzyme activity

The activity of the crude proteases from *Bacillus* sp., K52, K53, K61 and KK128 was decreased with increasing concentration of NaCl in all of strains (Figure 3.8). The results showed that the highest activity was obtained in the absence of sodium chloride, and that the activities were lost with increasing the concentration of NaCl. The results indicated that these enzymes were inhibited by the presence of high concentration of NaCl. The proteases activity from *Pseudoalteromonas* sp. strain CP76 showed high tolerant to a wide range of NaCl concentrations (0-4 M NaCl) and optimal activity at 7.5 % total salts (Porro *et al*., 2003). Thongthai and Siriwongpairat (1978) found that sodium chloride generally to suppress proteases activities, the higher the concentration the greater the degree of suppression. The fish proteases activity was suppressed by high salt concentration. The visceral enzymes of fresh water and marine fish were sensitive to NaCl suppression to a similar extent. One exception was the

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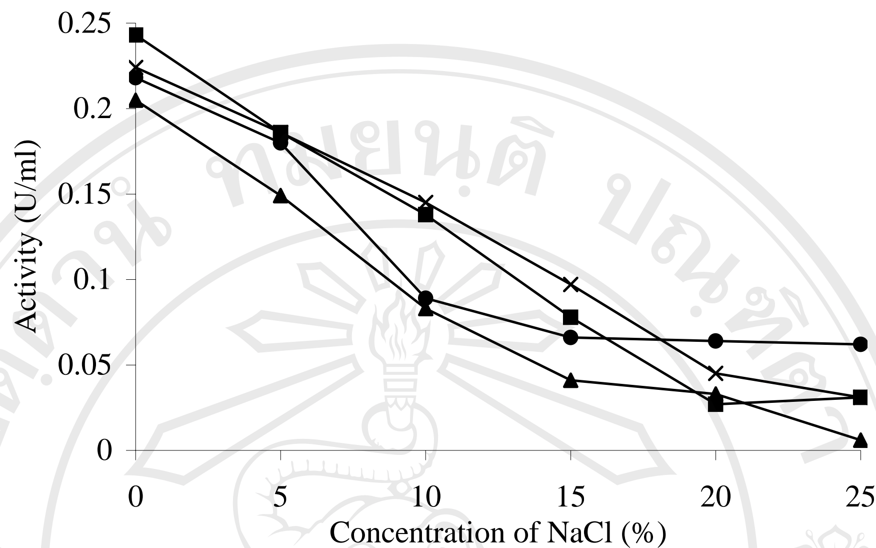

Figure 3.8 Effect of concentration of sodium chloride on crude proteases activities of strains K52 (■), K53 (▲), K61 (✕) and KK128 (●).

enzyme preparation from whole organs of marine fishes which seemed more resistant to NaCl suppression than its fresh water counterpart. The factor contributing to such resistance or protection of proteases activity is presently unclear.

Substrate specificity

Substrate specificity of the proteases from *Bacillus licheniformis* K61 towards diffirent substrates was shown in Table 3.14. The crude proteases from *Bacillus licheniformis* K61 was the most active on egg albumin with reducing effect on gelatin, Hoe, gluten, casein, whole egg and BSA. This proteases showed broad spectrum substrate utilization.

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Table 3.14 Substrate specificity of proteases of *Bacillus licheniformis* K61

Substrates	Relative activity (%)
Casein	100
BSA	43
Egg albumin	167
Hoe	133
Gluten	117
Whole egg	67
Gelatin	155

Proteases are difficult to characterize because of their diversity of action and structure. However, the enzyme in this study was not be purified. There are many factors in crude protease had effect on the characteristic of the enzyme. Therefore these proteases could not be clearly for classified.

Aminopeptidase are enzymes that catalyse the cleavage of amino acid residues at the N-terminal of peptides and proteins. The enzyme treatment of protein foods always found the formation of bitter peptides and it is the most serious problem in the practical use in food protein hydrolyzates. The use of peptidase enzyme to reduce or eliminate bitterness has increase significantly in recent years. Including, the thermostable enzyme are considerable interest to food industry.

Because of proteases of *Bacillus licheniformis* K61 in this study was not salt tolerant proteases, not thermotolerant enzyme. And *Bacillus* sp., K52, K53, K61 and KK128 were not showed the aminopeptidase activity. To develop a thermotolerant

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debittering enzyme, we decided to study the thermophilic bacteria *Geobacillus thermoleovorans* 47b that isolated from hot spring in Japan which supported by Department of Applied chemistry, Faculty of engineering, Oita University, Japan. This bacteria was screened by using bitter peptides as sole carbon and nitrogen source from hot springs in Oita, Japan. It was thermophilic bacteria that excellent grew at 60 ^{o}C and aminpeptidases activity were detected. So this strain was very interesting strain for application in food processing. The purification and characterization of aminopeptidase from *G. thermoleovorans* 47b and evaluated its usefulness in food processing were studied.

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