การผลิตและคุณสมบัติของเอนไซม์โปรติเอสที่ผลิตโดยแบคทีเรียทนร้อนที่คัดแยกได้จากกระบวนการหมักน้ำปลาไทย

Production and Properties of Protease Produced by Thermotolerant Bacteria Isolated from the Thai Fish Sauce Fermentation Process

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บทคัดย่อ

งานวิจัยนี้ได้คัดเลือกแบคทีเรียที่สามารถผลิตเอนไซม์โปรติเอสย่อยโปรตีนจากน้ำปลา คือ *Bacillus megaterium* ซึ่งสามารถผลิตเอนไซม์โปรติเอสได้สูงสุดในอาหารเหลวสูตร mM73 ที่อุณหภูมิ 45 องศาเซลเซียส ความเป็นกรด-ด่าง 9 ที่เวลา 24 ชั่วโมง ผลจากการศึกษาคุณลักษณะของเอนไซม์โปรติเอส แสดงว่าสภาวะเหมาะสมที่ *B. megaterium* ผลิตอัลคาไลน์โปรติเอส คือที่ความเป็นกรด-ด่าง 9 และอุณหภูมิ 55 องศาเซลเซียส เอนไซม์ มีความเสถียรที่ความเป็นกรด-ด่างและอุณหภูมิในช่วง 7-10 และ 30-50 องศาเซลเซียส ตามลำดับ โดยมีค่า relative activity มากกว่า 80% และ diisopropyl fluorophosphates เป็นตัวยับยั้งการทำงานของ crude enzyme ซึ่ง ลดกิจกรรมโปรติเอสได้ถึง 95% (relative activity คงเหลือ 5%) โดยเกลือโลหะที่สามารถยับยั้ง การทำงานของเอนไซม์โปรติเอส ได้แก่ Cd^{2+}, Cu^{2+}, Cu^{3+}, Hg^{2+} และ Zn^{2+} ทั้งนี้ Mn^{2+} และ Ca^{2+} มีผลทำให้กิจกรรมโปรติเอสเพิ่มสูงขึ้น

Abstract

Proteolytic bacteria were isolated from a fermented broth of Thai fish sauce. The highest protease production was found in mM73 liquid medium cultured with the isolate S2-1 (identified as *Bacillus megaterium*) at 45 ^{o}C, pH 9 for 24 h. The study of protease characterization and activity indicated that *B. megaterium* produced alkaline protease under optimum pH and temperature of pH 9 and 55 ^{o}C, respectively. This protease was stable in the pH and temperature ranges of 7 to 10 and 30 to 50 ^{o}C, respectively, resulting in relative activity of higher than 80%. A strong inhibitor was diisopropyl fluorophosphate which protease activity was decreased 95% (relative activity of 5%). Metal ions inhibiting protease activity were Cd^{2+}, Cu^{2+}, Cu^{3+}, Hg^{2+} and Zn^{2+}, while $Mn^{2+}$and Ca^{2+} enhanced protease activity.

คำสำคัญ: อัลคาไลน์โปรติเอส แบคทีเรียทนความร้อน กระบวนการหมักน้ำปลาไทย
Keywords: alkaline protease, thermotolerant bacteria, Thai fish sauce fermentation process

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Introduction

Fish sauce is one of the fermented sauces commonly used in every Thai household and most parts of Southeast Asia. The fish sauce fermentation process starts by mixing the fish and salts and then transferring to an underground fermentation tank. Normally the fish sauce fermentation process last about 8-12 months (Chaiyanan, 1992). The liquid obtained contains fish protein and is considered a good source of protein (Fukami et al., 2002).

The most important enzyme associated with fish sauce fermentation are amino acid degradation catalyzing enzymes called protease (Poralla, 1971). Protease is an enzyme which hydrolyzes protein into smaller peptide units or free amino acids and catalyzes peptide synthesis in organic solvents or in solvents with low water content. Proteases constitute a large and industrially important group of enzymes. They comprise about 60% of the total worldwide sales of enzymes (Rao et al, 1998).

In general, there are some micro-flora in the gut, gills and slime on the body of fish. These microorganisms possess the capability to produce protease for degrading protein in the fish body during the fish sauce fermentation process (Chaiyanan, 1992). During the process, the temperature may be continuously raised to 70 ^{o}C (Chaiyanan, 1992), and some normal flora will be killed at this high temperature. Thermo-tolerant microorganisms are the only group of normal flora which can survive and retain the capability to produce protease at high temperature. Therefore, there is an interest towards the isolation of a microorganism capable of producing a protease enzyme which can tolerate a high temperature during the fermentation process with the hope that this enzyme can shorten the time to ferment fish sauce.

Thus, this research was an attempt to isolate the thermo-tolerant microorganisms capable of producing protease in the fish sauce fermentation process. Additionally, the protease activity and characterization of this enzyme were investigated.

Material and Methods

Chemicals

Skim milk, diisopropyl fluorophosphate (DFP), $CaCl_2$, $HgCl_2$ were purchased from Wako Pure Chemical Industries, Ltd., Osaka, Japan. Yeast extract was purchased from Oxiod, Unipath Ltd., Basingstoke, Hampshire, England. $MnCl_2$, $CdCl_2$, $CuCl_3$, $CuSO_4$, $ZnCl_2$, NaCl were purchased from Kanto Chemicals Co. Ltd., Tokyo, Japan. All chemicals were of analytical grade.

Fish sauce fermented samples

The fish sauce fermented samples were collected from the Thai fish sauce fermentation process. The slurry parts of the fermented samples in the fermentation tank were collected at a depth of 30 cm from the surface. Three samples were obtained from the fish sauce factory in Samutsongkram province after fermentation for 2, 6 and 10 months. The fish used as raw materials in this fermentation process were anchovy (*Stolephorus indicus* Van Hasselt) and mackerel (*Scomberomorous guttatus* Bloch and Schneider). Four samples were obtained from the fish sauce factory in Keunubonrat District, Khon Kaen Province after being fermented for 2, 6, 36 and 48 months. Fresh-water fish i.e. Pla-soy (*Cirrhina jullieni* Sauvage) and Pla-nuanjun (*Cirrhina microlepis* Sauvage) were used as raw materials in this factory. These samples were placed in plastic bags and then kept at 4 ^{o}C prior to being used in the experiment.

Culture medium

Modified M73 (mM73) (Aroonpiroj, 1997) consists of 0.8% skim milk, 0.1% yeast extract, 1% $MgSO_4.7H_2O$, 0.5% KCl, 0.02% $CaCl.2H_2O$, 0.5% NaCl and 2.0% agar. All compounds except skim milk were mixed together in distilled water. The pH was adjusted to 7 before sterilization at 110 °C for 20 min. Skim milk solution was separately sterilized at 110 °C for 20 min. These two solutions were mixed aseptically before use.

Enzyme assay and protein determination

Alkaline protease activity was determined by measuring the release of trichloroacetic-acid soluble peptides from 1% casein in 10 mM borate buffer (pH 9) at 50 °C for 30 min (Aoyama et al., 2000). One unit of enzyme activity was defined as the amount of enzyme that releases 1 μg of tyrosine per ml per min under the above assay conditions. Specific enzyme activity was expressed as units/mg protein.

Protein was measured by the method of Lowry assay using the DC protein assay kit (Bio-Rad, CA, USA) with bovine serum albumin (BSA) as the standard protein.

Screening of protease producing micro-organism

The fish sauce samples were diluted tenfold in sterile saline solution. The diluted samples were placed on mM73 agar and then incubated at 37 °C for 24 h. A clear zone of skim milk hydrolysis gave an indication of protease producer microorganism. The single colonies presenting a clear zone of skim milk hydrolysis on mM73 agar were considered as the protease producing microorganisms. These microorganisms were spotted on mM73 agar containing 0.5, 5, 10, 15 and 20% NaCl and incubated at 45°C for 48 h to test for the halotolerant and thermotolerant abilities of each microorganism. The colonies which possessed a ratio of clear zone diameter (cm) to colony diameter (cm) of more than two were selected to be used in enzyme production and enzyme characterization.

Effect of pH on protease culture medium

The colonies with a ratio of clear zone diameter (cm) to colony diameter (cm) of more than two were cultured in mM73 broth at pH 7 and 9. The flasks were incubated at 45 °C, 200 rpm for 24 h. The cells were harvested by centrifugation at 10,000 g, 4 °C for 10 min. The supernatant was considered as crude enzyme and was dialyzed using a dialysis membrane with a 10,000 kDa molecular weight cutoff (Viskase Companies, Inc.) against 10 mM phosphate (pH 7) and borate buffer (pH 9) and then checked for protease activity and protein content. The isolate with the highest protease activity was considered as "the best" protease producer. This isolate was then identified and used in the next experiments.

Effect of carbon source on production of the enzyme

The "best" isolate identified as *Bacillus megaterium*, by the Thailand Institute of Scientific and Technology Research (TISTR), Bangkok, Thailand was cultured in a mM73 medium containing 0.5 and 1% of fructose, glucose, sucrose, maltose or soluble starch as a sole carbon source. Control was the mM73 medium without the addition of carbon source but skim milk was added at 0.8%. The culture condition was at 45 °C with shaking at 200 rpm for 24 h. After 24 h, the culture medium was centrifuged at 10,000 g for 10 min. The supernatant was then dialyzed against 10 mM borate buffer

(pH 9) and checked for protease activity and protein content.

Effect of nitrogen source on production of the enzyme

The effect of nitrogen source on protease enzyme production by *B. megaterium* was investigated in mM73 medium without yeast extract or skim milk. One percent of yeast extract, peptone, $(NH_4)_2SO_4$, skim milk or urea was then added to the medium as sole nitrogen source. Control was the mM 73 medium contained both 0.1% yeast extract and 0.8% skim milk. The culture condition was at 45 °C with shaking at 200 rpm for 24 h. After 24 h, the culture medium was centrifuged at 10,000 g for 10 min. The supernatant was then dialyzed against 10 mM borate buffer (pH 9) and checked for protease activity and protein content.

Optimal time for producing the enzyme

Optimal incubation time for producing a protease enzyme was found by culturing *B. megaterium* in mM73 medium. The culture condition was at 45 °C with shaking at 200 rpm. The culture broth was sampled every 6 h until 60 h of incubation to determine the growth curve of *B. megaterium*. The culture medium samples were centrifuged at 10,000 g for 10 min. The supernatant was dialyzed against 10 mM borate buffer (pH 9) and checked for protease activity and protein content.

Properties of the crude enzyme

The crude enzyme was the culture medium of *B. megaterium* in mM73 medium (pH 9) after incubation at 45 °C, 200 rpm for 24 h. The cells were harvested by centrifugation at 10,000 g, 4 °C for 10 min. The supernatant was considered as a crude enzyme and further used in the study of crude enzyme properties.

Optimal pH for enzyme activity

The enzyme activity of crude enzyme solution was determined by using 1% casein as a substrate dissolved in different pH of the buffer as follows: 10 mM McIlvaine (pH 2.2-8.0), borate buffer (pH 8.0-10.0), sodium borate buffer (pH 9.5-11.5) and sodium phosphate buffer (pH 11.0-12.0).

pH stability of the enzyme

To determine pH stability of an enzyme, a crude enzyme solution was dialyzed using a dialysis membrane with a 10,000 kDa molecular weight cutoff (Viskase Companies, Inc.) against different pH of the buffer as follows: 10 mM McIlvaine buffer (pH 2.2-8.0), citrate buffer (pH 4.0-7.0), phosphate buffer (pH 6.0-8.0), Tris-HCl buffer (7.0-10.0), borate buffer (pH 8.0-10.0), sodium borate buffer (pH 9.5-11.5) and sodium phosphate buffer (pH 11.0-12.0). The enzyme activity was then determined by using 1% casein as a substrate dissolved in 10 mM borate buffer (pH 9.0).

Optimal temperature for enzyme activity

Crude enzyme solution was dialyzed using a dialysis membrane with a 10,000 kDa molecular weight cutoff (Viskase Companies, Inc.) against 10 mM Tris-HCl buffer (pH 8.5). The enzyme activity was then determined by using 1% casein as a substrate dissolved in 10 mM borate buffer (pH 9.0) and incubating at various temperatures of 30, 40, 45, 50, 55, 60, 70, 80 and 90 °C.

Thermal stability of the enzyme

Crude enzyme solution was dialyzed using a dialysis membrane with a 10,000 kDa molecular weight cutoff (Viskase Companies, Inc.) against 10 mM Tris-HCl buffer (pH 8.5) and then incubating at various temperatures of 30, 40, 45,

50, 55, 60, 70, 80 and 90 °C for 30 min. The enzyme activity was further determined using 1% casein as a substrate dissolved in 10 mM borate buffer (pH 9.0) at 55 °C.

Substrate inhibitor of the enzyme

Crude enzyme solution was dialyzed using a dialysis membrane with a 10,000 kDa molecular weight cutoff (Viskase Companies, Inc.) against 10 mM Tris-HCl buffer (pH 8.5). The substrate inhibitor was added to a dialyzed enzyme before analyzing enzyme activity at a final concentration of 1 mM. The substrate inhibitors tested were ethylenediaminetetraacetic acid (EDTA), phenylmethylsulfonyl fluoride (PMSF), pepstatin A, E-64, trypsin inhibitor, $AgNO_3$, 1, 10-phenanthroline, p-Chloromercuribenzoic acid (PCMB), sodium dodecyl sulfate (SDS), Nα-tosyl-L-lysyl chloromethyl ketone (TLCK), L-1-tosyl-phenyllalanyl-chloromethyl ketone (TPCK), N-ethylmaleimide (NEM), chymostatin and diisopropyl fluorophosphate (DFP). The enzyme activity was then determined by using 1% casein as a substrate dissolved in 10 mM borate buffer (pH 9.0) at 55°C.

Effect of metal salts on enzyme activity

Crude enzyme solution was dialyzed using a dialysis membrane with a 10,000 kDa molecular weight cutoff (Viskase Companies, Inc.) against 10 mM Tris-HCl buffer (pH 8.5). The metal salt was added to a dialyzed enzyme before analyzing enzyme activity at a final concentration of 1 mM. The metal salts tested were $AlCl_3$, $CaCl_2$, $CdCl_2$, $CoCl_2$, $CuCl_3$, $CuSO_4$, $FeCl_3$, $HgCl_2$, $MgCl_2$, $MgSO_4$, $MnCl_2$, $NiCl_2$, $PbCl_2$, $ZnCl_2$. The enzyme activity was then determined by using casein as a substrate dissolved in 10 mM borate buffer (pH 9.0) at 55 °C.

Results and Discussion

Screening of protease producing micro-organism

Twenty-one isolates produced protease enzyme at 37 °C on mM73 agar and were tested for halo-tolerant and thermo-tolerant conditions. Only 3 isolates, S2-1, S6-1 and K36-2, showed ratio of clear zone diameter (cm) to colony diameter (cm) of more than 2 on mM73 agar containing 0.5% NaCl at 45 °C condition (Table 1) indicating a thermo-tolerant character and being a protease producer. These 3 isolates were tested for protease production in the culture broth.

Effect of pH on protease activity in culture broth

Three isolates with ratio of clear zone diameter (cm) to colony diameter (cm) of more than 2 obtained from screening experiment were cultured in mM73 broth at pH 7 and 9 to study the effect of pH on protease activity. The protease activity of crude enzyme produced by isolates S2-1, S6-1 and K36-2 at pH 7 were 136.28 mU/ml, 67.11 mU/ml and 15.96 mU/ml, respectively (Fig. 1). At pH 9, the protease activity of crude enzyme produced by isolates S2-1, S6-1 and K36-2 were 213.04 mU/ml, 75.29 mU/ml and 10.27 mU/ml, respectively. These results indicate that the isolate S2-1, identified as *B. megaterium*, has the highest capability to produce protease enzyme among these 3 isolates, with a maximum activity obtained at pH 9. Therefore, the isolate S2-1 was selected to be used in further experiments.

Effect of carbon source on production of protease

The isolate *B. megaterium* produced protease enzyme in all tested carbon sources. The maximum protease activity was obtained in control i.e., mM73 (contains skim milk without additional carbon source) (Fig. 2), indicating that *B. megaterium* has the

capability to use skim milk contained in a cultured medium as carbon source. Skim milk contains disaccharide lactose which has been reported to have positive effects on protease production by microorganisms. Naidu and Devi (2005) reported that lactose could be effectively used as a substrate for protease production by *Bacillus* sp. Alkaline protease production yield was increased with the addition of lactose to the cultured medium of *Aspergillus flavus* (Malathi and Chakraborty, 1991). Four proteolytic bacterial strains, *B. subtilis* EMCC 1020, *B. megaterium* EMCC 1057, *Serratia marcescens* EMCC 1247, and *Pseudomonas fluorescens* EMCC were reported to be able to utilize lactose in milk permeate (Ali and Roushdy, 1998). Donaghy and McKay (1993) and McKay (1992) discovered that whey, a waste by-product of the dairy industry containing mainly lactose and salts, could be used as a substrate for alkaline protease production by *Aureobasidium pullulans*.

Other carbon sources in this study might inhibit protease production (Fig. 2) of our isolate S2-1. The addition of glucose to culture medium was reported to reduce protease production by *B. subtilis* and *B. licheniformis* due to catabolic repression (Kole et al., 1988; Frankena, et al., 1986; Hanlon et al., 1982; Frankena et al., 1985).

Effect of nitrogen source on production of the enzyme

The isolate *B. megaterium* could produce protease enzyme using 1% skim milk and 1% yeast extract as nitrogen sources, with protease activity of 85.51 mU/ml and 25.49 mU/ml, respectively. However, the addition of 1% peptone, 1% $(NH_4)_2SO_4$ or 1% urea as a nitrogen source did not promote protease production by *B. megaterium* (Fig. 3). The control medium, mM73, consisting of skim milk and yeast extract exhibited a maximal protease activity (Fig. 3). These results reveal that *B. megaterium* can effectively use skim milk and yeast extract as nitrogen sources for protease production. The findings of Aroonpiroj (1997) support our results. They reported that bacteria selected from Thai fish sauce fermentation could produce protease enzyme on the medium containing 0.8% skim milk and 0.1% yeast extract. In addition, the organic nitrogen i.e. 1.0% yeast extract and 1.0% beef extract contained in the medium cultured with Bacillus sp. improved the protease activity from 36 U/ml to 65 and 75 U/ml, respectively (Naidu and Devi, 2005).

Optimal time for protease production

The growth of *B. megaterium* and protease activity were determined in mM73 medium for 60 h. *B. megaterium* grew rapidly and produced protease (Fig. 4). The enzyme activity and specific activity increased gradually in the exponential phase from 0-24 h and decreased in the stationary phase (24-42 h) with a maximum protease production reached at 24 h (Fig. 4). The enzyme activity and specific activity were 205.32 mU/ml and 1672.00 mU/mg protein, respectively, at 24 h. Our results correlate with Ward (1985) who reported that *Bacillus* sp. could produce protease during the late exponential phase. However, the research by Nascimento and Martins (2004) found that protease was produced by *Bacillus* sp. since the beginning of the cultivation was from 5 h after cultivation and reached a maximum within 9 h. In contrast, maximum growth of *Bacillus* sp. was observed at 24 h and protease was detected in culture medium throughout the fermentation (Chomsri, 2001).

Properties of the crude enzyme

Optimal pH for enzyme activity

The optimum pH for protease activity was observed as pH of 8-10 of borate buffer. The optimum pH was found to be 9 (Fig. 5) indicating that this enzyme might be an alkaline protease (Kumar and Takagi, 1999; Alencar et al., 2003). The same results, an optimum pH of 9, were observed in the protease produced by *B. licheniformis* (Al-Shehri et al., 2004), and *B. pumilus* (Aoyama et al., 2000).

pH stability of protease

A crude enzyme was dialyzed against different pHs, 2.2-12, of buffer to examine pH stability. Protease activity with relative activity of more than 80% was found when crude enzyme was dialyzed in the Tris-HCl buffer, pH range 7 to 10 (Fig. 6). However, within this pH range, the relative activities were observed to be less than 60% in McIlvaine, citrate and borate buffer (Fig. 6). It is important to note that at the same pH ranges but in different types of buffer, the protease activity depended on the type of buffer. Chomsri (2001) reported similar findings in which the protease activity was stable over the pH range of 7 to 10 with relative protease activity of 100% in phosphate and Tris-HCl buffer. Nascimento and Martins (2004) stated that the alkaline protease was active over a broad range of pH of 6 to 10 with relative activity retained in pH above 9.

Optimal temperature for protease activity

The optimum temperature for protease activity was determined at different temperatures from 30 °C to 90 °C using 1% casein as a substrate dissolved in borate buffer of pH 9. The protease activity was active from 30 °C to 60 °C but inactive in the range of 70 °C to 90 °C. The optimum temperature for protease activity was 55 °C (Fig. 7). Kumar and Takagi (1999) reported that the optimum temperature for alkaline protease ranged from 50 °C to 70 °C. Optimum temperature to produce protease by *B. licheniformis* and *B. pumilus* was at 55 °C (Kumar, 2002; Huang et al., 2003; Al-Shehri et al., 2004). However, the optimum temperature of 60 °C was observed in *Bacillus* sp. SMIA-2 (Nascimento and Martins, 2004). This result was similar to the reports of Banerjee et al. (1999) and Horikoshi (1990) in which the optimum temperature for *Bacillus* protease was 60 °C.

Thermal stability of the enzyme

The crude enzyme solution was incubated at various temperatures, 30 °C to 90 °C, for 30 min to determine the thermal stability. The results indicate that within 30 min, the enzyme was stable at 30 °C to 50 °C with relative activity of more than 95% (Fig. 8). Other research has found the same trend. For example, relative activity of protease enzyme produced by *B. clausii* was reported to be 100% after incubation of the enzyme over a temperature range from 30 °C to 65 °C for 60 min (Kumar et al., 2004). Protease produced by *Bacillus* sp. F603.1 was stable at 60 °C for 90 min (Chomsri, 2001) with a relative activity of 75%. *Bacillus* sp. SMIA-2 produced protease which was stable at 30 °C for 2 h with 100% relative activity (Nascimento and Martins, 2004). The proteases obtained from cultivation of *B. pumilus* were stable at 45 °C and 50 °C for 30 min with relative activity of 100% and 47%, respectively (Feng et al., 2001; Huang et al., 2003).

Substrate inhibitor of the protease

The inhibitory effects of various inhibitors on protease activity are shown in Table 2. The enzyme was strongly inhibited by 1 mM DFP resulting in

very low relative activity of 5% (Table 2). The protease was partially inhibited by PMSF, pepstatin A and SDS with relative activity of 39, 54 and 51%, respectively. In contrast, the activity of this enzyme was stimulated by the presence of E-64 and TLCK as indicated by relative activity of more than 100% i.e. 129 and 123%, respectively. Gold and Fahrney (1964) reported that alkaline protease was completely inhibited by PMSF and DFP. In this regard, PMSF sulfonated the essential serine in the active site and resulted in a complete loss of activity. The effect of inhibitors on protease produced by *B. subtilis* PE-11 reveals that protease is completely inhibited by PMSF, while 94% of this enzyme activity is inhibited by DFP (Adinarayana et al., 2003).

Effect of metal salts on enzyme activity

The protease activity was enhanced with the addition of Mn^{2+} and Ca^{2+} resulting in relative activity of 139 and 113%, respectively (Table 3), suggesting that metal ions have the capability to protect enzymes against denaturation. Nascimento and Martins (2004) reported that protease produced by *Bacillus* sp. SMIA-2 was enhanced by Mn^{2+} and Ca^{2+}. They explained that these metal ions protected the enzyme from thermal denaturation and maintained its active conformation at high temperature. Alkaline protease requires a divalent cation like Ca^{2+}, Mg^{2+} and Mn^{2+} or a combination of these cations for maximum activity. These cations enhance the stability of a *Bacillus* alkaline protease (Paliwal et al., 1994). An inhibitory effect on protease activity of our *B. megaterium* was observed in the presence of Cd^{2+}, Cu^{2+}, Cu^{3+}, Hg^{2+} and Zn^{2+}. The relative activities were 58, 54, 55, 43 and 63%, respectively (Table 3). Zn^{2+}, $Cu^{2+}$and Hg^{2+} were found to inhibit the catalytic activity of alkaline protease secreted by *B. brevis* (Banerjee et al., 1999).

Conclusion

This research successfully isolated a thermotolerant protease producer, *Bacillus megaterium*, from a fermented broth of Thai fish sauce. Optimal conditions for production of this protease were mM73 liquid culture at pH 9 and incubation for 24 h at 45 ^{o}C. Replacement of yeast extract and skim milk in mM73 medium by various types of carbon and nitrogen sources did not improve the protease production yield, indicating that yeast extract and skim milk were good carbon and nitrogen sources for producing protease by *B. megaterium*. Study of protease characterization and activity indicates that *B. megaterium* produces alkaline protease at optimum pH and temperature of pH 9 and 55 ^{o}C, respectively. This protease was stable in the pH and temperature range of 7 to 10 and 30 ^{o}C to 50 ^{o}C, respectively, resulting in relative activity of higher than 80%. A strong inhibitor was DFP in which protease activity was decreased by 95% (relative activity of 5%). Metal ions inhibiting protease activity were Cd^{2+}, Cu^{2+}, Cu^{3+}, Hg^{2+} and Zn^{2+}, while $Mn^{2+}$and Ca^{2+} enhanced the protease activity.

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Table 1. Isolates with ratio of clear zone diameter (cm) to colony diameter (cm) of more than 2.

Isolate	Diameter (cm)		ratio
	colony	clear zone	
S2-1	0.5	2.1	4.2
S6-1	1.5	3.0	2.0
K36-2	0.5	1.0	2.0

Table 2. Effect of various inhibitors on protease activity.

Inhibitor	Relative activity (%)
none	100
EDTA	99
PMSF	39
1,10 Phenanthroline	93
PCMB	84
pepstatin A	54
SDS	51
Chymostatin	72
E-64	129
Trypsin inhibitor	88
TLCK	123
TPCK	88
$AgNO_3$	87
NEM	89
DFP	5

Table 3. Effect of various metal salts on protease activity.

Metal salt	Relative activity (%)
None	100
$AlCl_3$	109
$CaCl_2$	113
$CdCl_2$	58
$CoCl_2$	88
$CuCl_3$	54
$CuSO_4$	55
$FeCl_3$	106
$HgCl_2$	43
$MgCl_2$	109
$MgSO_4$	104
$MnCl_2$	139
$NiCl_2$	109
$PbCl_2$	87
$ZnCl_2$	63

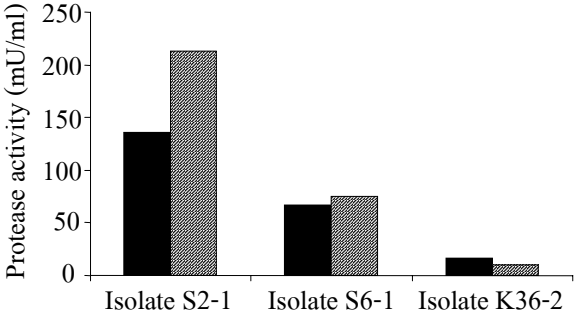

Fig. 1 Effect of pH 7 (■) and pH 9 (▨) on protease activity in culture broth.

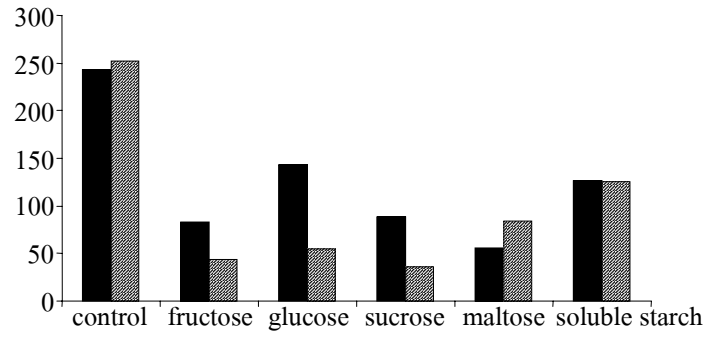

Fig. 2 Effect of carbon source on production of the enzyme (■) 0.5% carbon source, (▨) 1.0% carbon source.

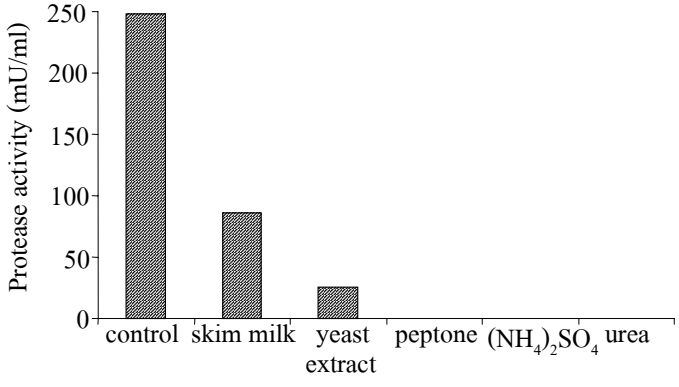

Fig. 3 Effect of nitrogen sources on the production of the enzyme.

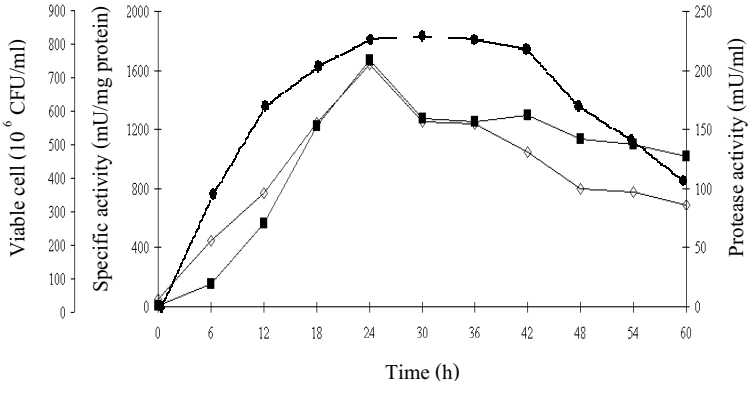

Fig. 4 Growth and protease production from *B. megaterium* (●) viable cell (10^6 CFU/ml), (□) protease activity (mU/ml), (■) specific activity (mU/mg protein).

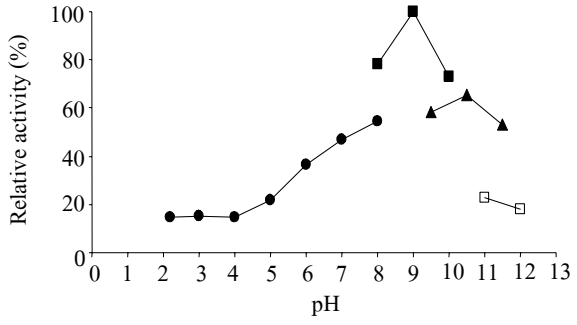

Fig. 5 Effect of pH on protease activity.

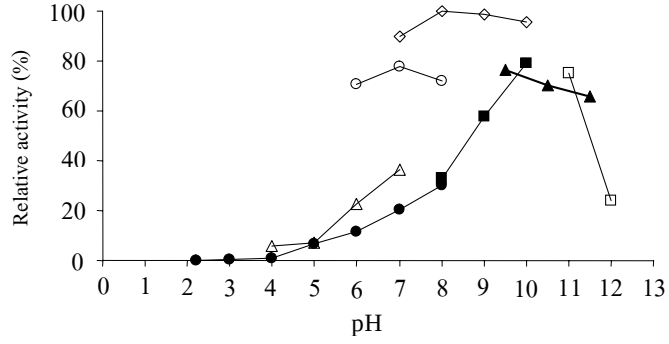

Fig. 6 Effect of pH stability on protease activity.

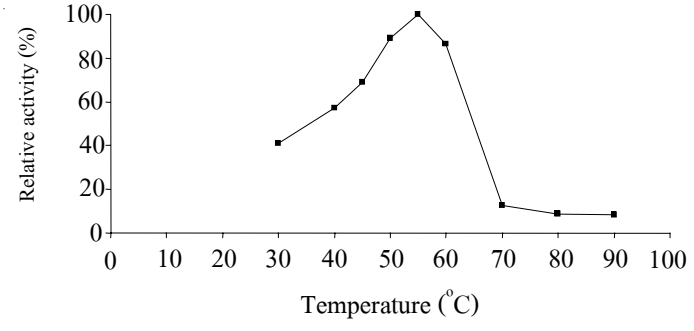

Fig. 7 Protease activity at various temperatures.

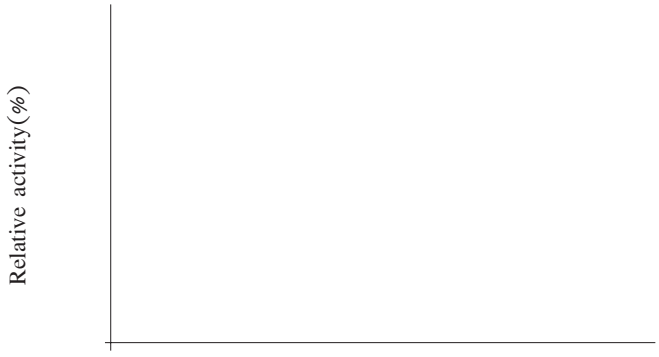

Fig. 8 Effect of thermal stability on protease activity.