รายงานวิจัยฉบับสมบูรณ์

โครงการ อัลคาไลด์โปรตีเนสของแบคทีเรียที่แยกได้จากมูลค้างคาว

(Alkaline proteinases of alkalophilic bacteria screened from bat faeces)

โดย ผศ. ดร. สมพร ตั๊นสกุล และคณะ

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สนับสนุนโดยสำนักงานคณะกรรมการการอุดมศึกษา และสำนักงานกองทุนสนับสนุนการวิจัย

บทคัดย่อ

จากการทดลองเก็บตัวอย่างมูลค้างคาวในวัดถ้ำสุวรรณคูหา อ. ตะกั่วทุ่ง จ. พังงา รวม 50 ตัวอย่าง สามารถแยกเชื้อรวม 75 สายพันธุ์ได้ภายหลังการทดสอบความสามารถในการสร้างและขับโปรตีเนสออกนอกเซลล์ด้วยวิธี agar plate assay โดยใช้ NA ที่มี pH 8.0 และมี skim milk 1% และภายหลังการทดสอบกิจกรรมโปรตีเนสโดยวิธี Folin-Ciocalteau assay พบว่าสายพันธุ์ PN51 (Phang Nga 51) มีกิจกรรมโปรตีเนสสูงสุด จากการวิเคราะห์ลำดับ 16S rDNA ของสายพันธุ์ดังกล่าว พบว่ามีความเหมือนถึง 100% กับ *Bacillus* sp. CNJ904 PLO4 สภาวะที่เหมาะสมในการผลิตโปรตีเนสคืออาหารที่ดัดแปลงสูตรมาจาก Lee ประกอบด้วยเปปโทน 1.0% สารสกัดยีสต์ 0.5% $CaCl_2$ 0.04% และ $MgCl_2$ 0.02% ซึ่งมีหัวเชื้อ 0.5% ที่ pH 8 โดยเขย่าที่ความเร็ว180 rpm ที่ $35^{o}C$ เป็นเวลา 22 ชั่วโมง พบว่าเอนไซม์จาก PN51 และถูกยับยั้งอย่างสมบูรณ์โดย PMSF และ chymostatin แต่ไม่ถูกยับยั้งโดย EDTA ดังนั้นเอนไซม์จาก PN51 เป็นเอนไซม์ชนิดเซรีนโปรตีเนส

Abstract

There were 75 bacterial strains producing proteinase outside the cells screened from 50 samples of bat faeces in Wat Suwankuha cave, Takua Thung District, Phang Nga, Thailand by agar plate assay, Nutrient agar plate, pH 8 containing 1% skim milk. The results showed that one strain named PN51 (Phang Nga 51) gave the highest proteinase activity after determined by Folin-Ciocalteau assay. The 16S rDNA sequence analysis of PN51 showed high homology of 100% correlation to that of *Bacillus* sp. CNJ904 PLO4. The optimum conditions for proteinase production of the strain were modified Lee's medium containing 1.0% peptone, 0.5% yeast extract, $CaCl_2$ 0.04% and $MgCl_2$ 0.02% with 0.5% starter at pH 8, 180 rpm, $35^{o}C$ for 22 hr. The enzyme activity was completely inhibited by phenylmethyl sulfonyl fluoride (PMSF), and chymostatin, but not by EDTA. Thus, the enzyme from PN51 was thought to be a serine-type proteinase.

Executive Summary

There were 75 bacterial strains producing proteinase outside the cells screened from 50 samples of bat faeces in Wat Suwankuha cave, Takua Thung District, Phang Nga, Thailand by agar plate assay, Nutrient agar plate, pH 8 containing 1% skim milk. The results showed that one strain named PN51 (Phang Nga 51) gave the highest proteinase activity after determined by Folin-Ciocalteau assay. The 16S rDNA sequence analysis of PN51 showed high homology of 100% correlation to that of *Bacillus* sp. CNJ904 PLO4. The optimum conditions for proteinase production of the strain were modified Lee's medium containing 1.0% peptone, 0.5% yeast extract, $CaCl_2$ 0.04% and $MgCl_2$ 0.02% with 0.5% starter at pH 8, 180 rpm, 35^{o}C for 22 hr.

The active fractions from Mono Q column had clearly separated into 4 peaks, indicating that the enzyme had at least four isoforms. Regarding the SDS-PAGE results, each isoforms of the enzyme was not pured or the autodegradation of the enzyme was occurred. These results need to be confirmed again. Some characteristics of the enzyme were studied. The enzyme activity was completely inhibited by PMSF and chymostatin. Therefore, the present study of the enzyme from PN51 was thought to be a serine-type proteinase. The amino-terminal amino acid sequence analysis of the enzyme is needed to be further investigated.

เนื้อหางานวิจัย

Introduction

Proteinases are applied in many fields, for example, wastewater treatment, food and medicinal industries. Although proteinases are produced by a wide range of microorganisms, plants and animals, most of proteinases are mainly from microorganisms, especially bacteria. Bacteria produce various kinds of extracellular proteinases to degrade proteins in their habitats (Branden, C. and Tooze, J., 1999). Bat faeces inside a limestone cave is one of unexpected habitats where can be screened bacteria producing alkaline proteinase. The bat faeces look like mouse dropping but are dry and powdery, containing insect exoskeletons, the wings of butterflies, moths or other food (http://www. rics-foundation.org/publish/document.aspx?did=3039&f=y) as protein sources to induce proteinase production of bacteria. There have been no report on bacteria producing alkaline proteinase screened from bat cave. The alkaline proteinases from the bacterial origin are the most important industrial enzymes, which contribute about 60% of the total world enzyme market (Ward, 1985; Kalisz, 1988; Outtrup, 1990). It may be applicable for detergent industries or waste treatment industries.

The aim of this first year study, was to screen, identify bacteria which could produce proteinase outside the cells and optimize the conditions for proteinase production of the bacteria.

Materials and methods

Materials

All purchased media and chemicals were commercial grades.

Screening

A total of 50 samples of bat faeces were collected from Wat Suwankuha cave, Takua Thung District, Phang Nga, Thailand. NA plate containing 1% of skim milk at pH 8.0 was used for screening the bacteria producting proteinase outside the cells.

Cultivation of bacteria

Tryptic Soy medium was used for cultivating the bacteria. The bacteria were inoculated 10% (v/v) in a tube containing 5 ml of Tryptic Soy Broth (TSB) and incubated on a reciprocal shaker at 35°C for 24 hr. The culture was then transferred to modified Lee's medium (Lee and Chang, 1990) containing glucose 2.0%, $CaCl_2$ 0.04% and $MgCl_2$ 0.02%. The modified Lee's medium was used for proteinase production of the bacteria on a reciprocal shaker at 35°C for 24 hr. The culture broth was centrifuged at 12,000 rpm for 5 min. The supernatant was used for determining extracellular proteinase activity.

Proteinase assay

Proteinase activity was determined by casein Folin-Ciocalteau assay. Casein was dissolved in 0.1 M Tris-HCl buffer at pH 8.0. The reaction mixture was incubated at 35°C for 30 min. One unit of enzyme activity was defined as the enzyme quantity that liberates 1 μg of tyrosine per ml of the reaction mixture per min.

Identification of the bacteria

Some taxonomic characteristics of PN51 were identified according to the Bergey's Manual of Systematic Bacteriology (Sneath *et al.*, 1986).

16S rDNA sequence analysis

PN51 was cultivated in Nutrient broth under aerobic condition at 35°C for 12 hr. Genomic DNA was extracted by using the standard method (Sambrook *et al.,* 1989) and then amplified by GeneAmp PCR System 9600. Universal primers were used; position 27 for forward and 1389 for reverse. The PCR product was 1.3 kb. The partial DNA sequencing was performed by starting at the region of 520 bp. The amplified DNA was sequenced by using API 377 DNA Sequencer.

Culture condition for proteinase production

TSB medium was used for the growth and modified Lee's medium was used for proteinase production of PN51. The effect of peptone, yeast extract, pH, the speed of a reciprocal shaker, starter, temperature and time course on proteinase production were studied. They were as follows.

1. Effect of peptone on proteinase production

PN51 was inoculated in TSB for 24 hr at 35°C on a reciprocal shaker. It was then transferred to modified Lee's medium containing 2% yeast extract, 0.04% $CaCl_2$, 0.02% $MgCl_2$ and various amount of peptone, 0.6, 0.8, 1.0, 1.2 and 1.4%, respectively. The proteinase activity was measured after cultivation for 24 hr.

2. Effect of yeast extract on proteinase production

The preculture of PN51 was prepared as mentioned above. It was then transferred to modified Lee's medium containing 1.0% peptone, 0.04% $CaCl_2$, 0.02% $MgCl_2$ and various amount of yeast extract, 0.3, 0.4, 0.5, 0.6, and 0.7%, respectively. The proteinase activity was measured after cultivation for 24 hr.

3. Effect of pH on proteinase production

The preculture of PN51 was transferred to modified Lee's medium containing 1.0% peptone, 0.5% yeast extract, 0.04% $CaCl_2$ and 0.02% $MgCl_2$ with pH at 6, 7, 8 and 9. The proteinase activity was measured after cultivation for 24 hr.

4. Effect of speed of shaker on proteinase production

The preculture was transferred to modified Lee's medium containing 1.0% peptone, 0.5% yeast extract, 0.04% $CaCl_2$ and 0.02% $MgCl_2$ at pH 8 on a reciprocal shaker at 160, 170, 180, 190 and 200 rpm. The proteinase activity was measured after cultivation for 24 hr.

5. Effect of temperature on proteinase production

After preparing the preculture of PN51, it was then transferred to modified Lee's medium containing 1.0% peptone, 0.5% yeast extract, 0.04% $CaCl_2$ and 0.02% $MgCl_2$ at pH 8 on a reciprocal shaker at pH 8, at 180 rpm., with different temperature, 25, 30, 35, 40, 45 and 50°C. The proteinase activity was measured after cultivation for 24 hr.

6. Effect of starter on proteinase production

The strain was inoculated in TSB for 24 hr at 35°C on a reciprocal shaker. It was then transferred to modified Lee's medium containing 1.0% peptone, 0.5% yeast extract, 0.04% $CaCl_2$ and 0.02% $MgCl_2$ at pH 8 on a reciprocal shaker at 180 rpm with various amount of starter, 0.3, 0.4, 0.5, 0.6 and 0.7%. The proteinase activity was measured after cultivation for 24 hr.

7. Time course of growth and proteinase production

PN51 was inoculated in TSB for 24 hr at 35°C on a reciprocal shaker. It was then transferred to modified Lee's medium containing 0.7% glucose, $CaCl_2$ and $MgCl_2$ at pH 8 on a reciprocal shaker at 180 rpm, 35° C. The growth and proteinase activity was measured after cultivation for 15, 18, 21, 22, 23, 24, 25, 26, 27, 45 and 48 hr.

Purification steps

All purification steps were carried out at 4°C except for Mono Q FPLC, which was performed at 25°C. Purification steps are $(NH_4)_2SO_4$ precipitation, DEAE-cellulose DE52 and Mono Q FPLC.

1. $(NH_4)_2SO_4$ precipitation

The culture supernatant was saturated overnight by 80% $(NH_4)_2SO_4$ in a cold room. The resultant precipitation was separated by centrifugation and then dissoled in 1/10-1/20 volume of 10 mM Tris-HCl at pH 8.0. The solution was then saturated by 20% $(NH_4)_2SO_4$ in the cold room. The precipitate was separated by centrifugation again and dissolved in a small volume of 10mM Tris-HCl at pH 7.5, followed by dialysis against the same buffer. The dialysate was centrifuged in order to get the supernatant. The volume, protein content, and proteinase activity of the supernatant were measured.

2. Anion exchange chromatography

2.1 DEAE-cellulose DE52 column chromatography

DEAE-cellulose DE52 column was first equilibrated with 10 mM Tris-HCl buffer at pH 8.0. The dialysate was loaded onto a DEAE-cellulose DE52 column and washed with 5 volume of the buffer at a flow rate of 1.0 ml/min. Proteinase was eluted with a linear gradient of NaCl from 0 to 1.0 M NaCl. Active fractions (3-ml each) were pooled and then dialyzed against the same buffer. The dialysate was concentrated by Centricon YM-30 (Amicon) and centrifuged to remove precipitate before loading onto a Mono Q column chromatography.

2.2 Mono Q column chromatography

Mono Q column was connected to a Fast Protein Liquid Chromatography (FPLC) system and equilibrated with 10 mM of Tris-HCl buffer at pH 8.6 containing 10 mM of $CaCl_2$. Active fractions from DEAE-cellulose DE52 column chromatography were loaded onto a Mono Q column and then the column was washed with the same buffer at a flow rate of 0.2 ml/min. A linear gradient of 1 M NaCl was used for elution of the proteinase. Active fractions (0.5-ml each) were pooled and concentrated by Centricon YM-30. The purity of the enzyme was checked by SDS-PAGE.

Protein determination

Protein concentration in the chromatography system was measured by a UV detector at an absorbance of 280 nm and whereas protein concentration of the purified enzyme was determined using bicinchoninic acid (BCA) with bovine serum albumin as a protein standard.

Effect of inhibitors

In order to identify the type of proteinase, the following combination of proteinase inhibitors were used: 1) serine proteinase inhibitor, PMSF, TLCK, TPCK, chymostatin, leupeptin and elastatinal. 2) metalloproteinase inhibitor, EDTA-2Na.

The enzyme and inhibitor was mixed and incubated at 30°C for 1 hr. The proteinse activity was then determined after incubation.

Results and conclusions

Screening

The agar plate assay was carried out for screening of bacteria producing proteinase. The results showed that 75 strains each gave a halo on NA-casein plate (data not shown).

Identification of the bacteria

Taxonomic characteristics of PN51 were identified and showed that there were some characteristics similar to *Bacillus* such as Gram staining, shape of cells (Fig. 1) and catalase test, but could not identified that it belongs to which species of *Bacillus* (Table1). The 16S rDNA fragment of PN51 was amplified by PCR and its sequence was determined by an automatic DNA sequencer (Fig. 2). As shown in Fig. 3, the partial conserved region in 16S rDNA sequence of PN51 gave a significant similarity to 5 bacterial strains. PN51 showed a high homology of 100% correlation to that of *Bacillus* sp. CNJ904 PLO4 (Gontang *et al*., 2006) The evolutionary tree between PN51 and other bacteria showed that PN51 has close evolution to *Bacterium* str. 47083 (96.5%), *Bacillus* sp. CNJ815 PLO4 (90.2%), *Bacterium* JL-74 (90.2%), and *Bacillus* sp. P01 (90.2%).

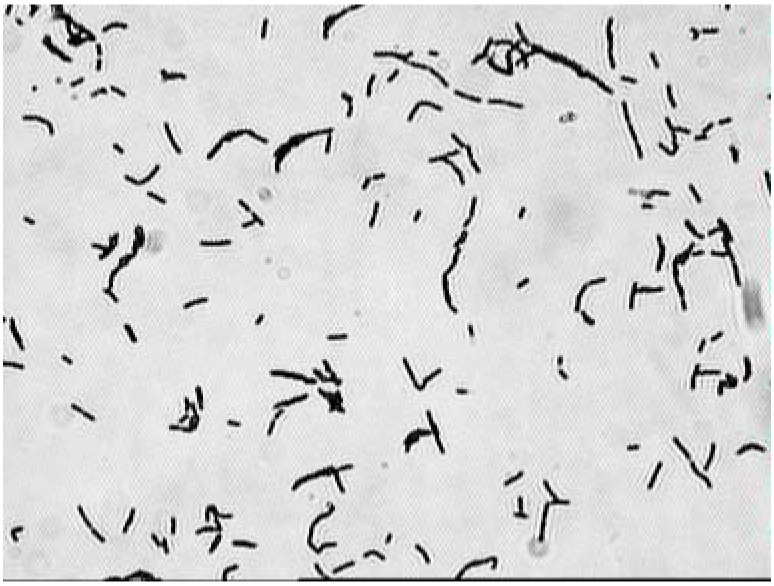

Fig. 1. Cell shape and Gram staining of PN51

Table 1. Characteristics of PN51

Characteristics	PN51
Gram staining	positive
Cell shape	rod
Endospores produced	-
Rods or filaments curved	+[a]
Motile	-
Catalase	-
NaCl and KCl required	-
Growth at pH	
6.8, NB	+
5.7, NB	-
Growth in NaCl	
2%	+
5%	+
7%	-
10%	-
Growth at	
8°C	-
10°C	-
30°C	+
35°C	+
40°C	-
50°C	-
55°C	-
65°C	-
Isolation source	bat cave

[a] Chains common

There was no published data of characteristics of *Bacillus* sp. CNJ904 PLO4 to compare with the strain PN51.

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1   ATTGGAAACT  GGGAGACTTG  AGTGCAGGAG  AGAAAAGTGG  AATTCCACGT
51  GTAGCGGTGA  AATGCGTAGA  GATGTGGAGG  AACACCAGTG  GCGAAGGCGG
101 CTTTTTGGCC  TGTAACTGAC  GCTGAGGCGC  GAAAGCGTGG  GGAGCAAACA
151 GGATTAGATA  CCCTGGTAGT  CCACGCCGTA  AACGATGAGT  GCTAGGTGTT
201 GGGGGGTTCC  ACCCTCAGTG  CTGAAGTTAA  CACATTAAGC  ACTCCGCCTG
251 GGGAGTACGA  CCGCAAGGTT  GAAACTCAAA  GGAATTGACG  GGGGCCCGCA
301 CAAGCAGTGG  AGCATGTGGT  TTAATTCGAA  GCAACGCGAA  GAACCTTACC
351 AGGTCTTGAC  ATCCTCTGAC  CACTCTAGAG  ATAGAGCTTT  CCCCTTCGGG
401 GGACAGAGTG  ACAGGTGGTG  CATGGTTGTC  GTCAGCTCGT  GTCGTGAGAT
451 GTTGGGTTAA  GTCCCGCAAC  GAGCGCAACC
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Fig. 2. 16S rDNA sequence of PN51

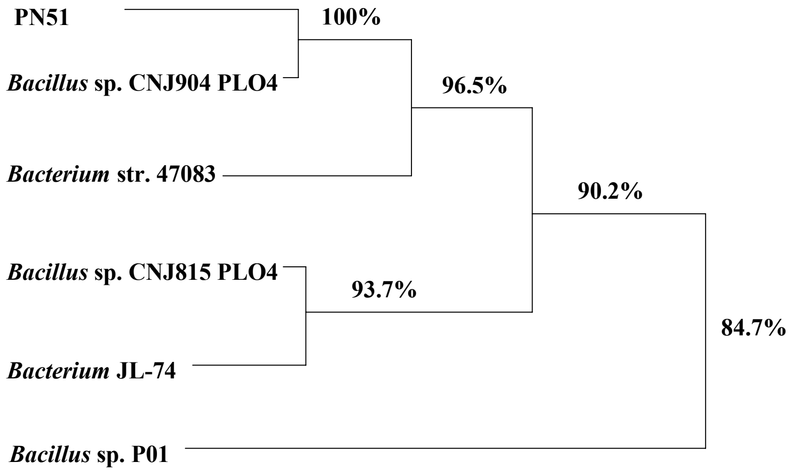

Fig. 3. The evolutionary tree between PN51 and other bacteria
The percentage indicated the similarity of 16S rDNA sequence of PN51 to those of others.

Culture condition for proteinase production

1. Effect of peptone on proteinase production

PN51 showed higher enzyme production in modified Lee's medium containing 1.0% peptone (Fig. 4).

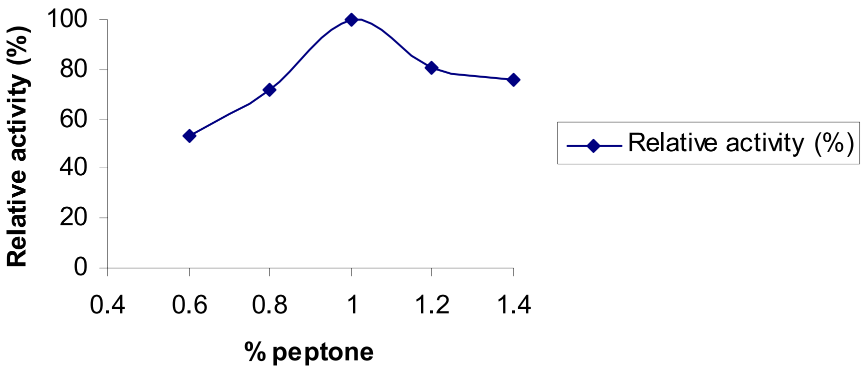

Fig. 4. The effect of peptone on proteinase production of PN51

2. Effect of yeast extract on proteinase production

The strain PN51 gave the highest proteinase production in modified Lee's medium containing 0.5% yeast extract (Fig. 5).

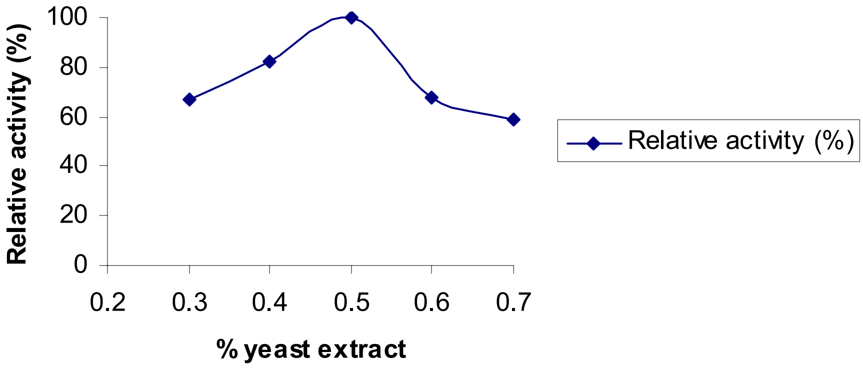

Fig. 5. The effect of yeast extract on proteinase production of PN51

3. Effect of pH on proteinase production

The pH in the medium which was suitable for the proteinase production of PN51 was 8.0 (Fig. 6).

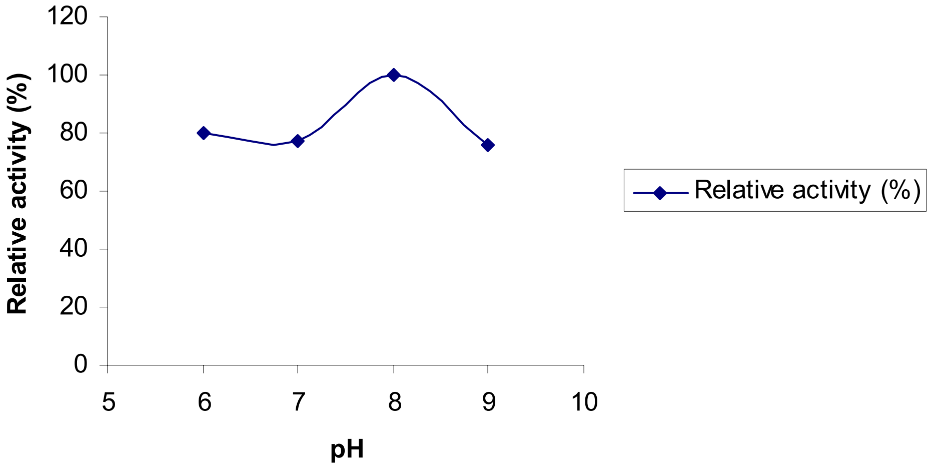

Fig. 6. The effect of pH on proteinase production of PN51

4. Effect of speed of shaker on proteinase production

The results showed that PN51 gave the highest proteinase production on a reciprocal shaker at 180 rpm (Fig. 7).

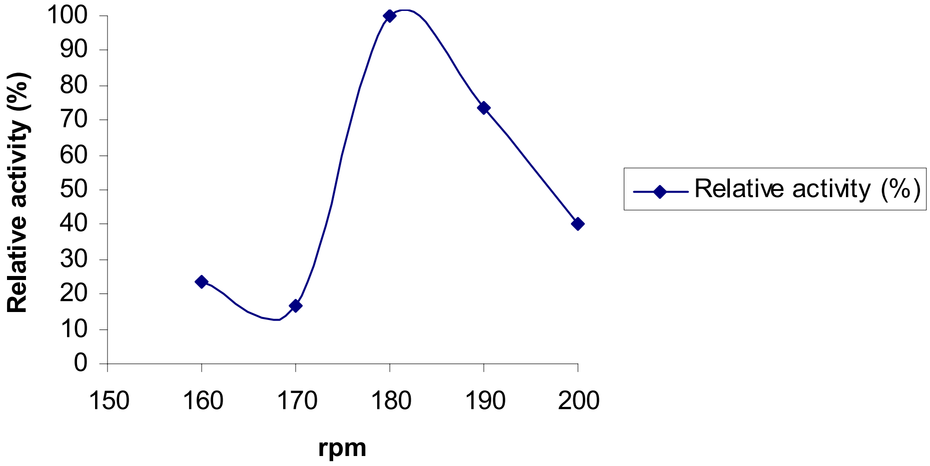

Fig. 7. The effect of the speed of a reciprocal shaker on proteinase production of PN51

5. Effect of temperature on proteinase production

The strain gave highest enzyme production at 35°C (Fig. 8).

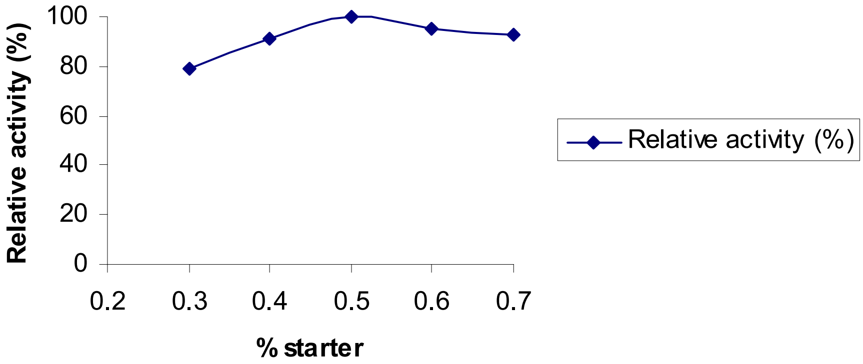

Fig. 8. The effect of starter on proteinase production of PN51

6. Effect of starter on proteinase production

The results showed that 0.5-0.7% of starter gave the maximum of proteinase production (Fig. 9).

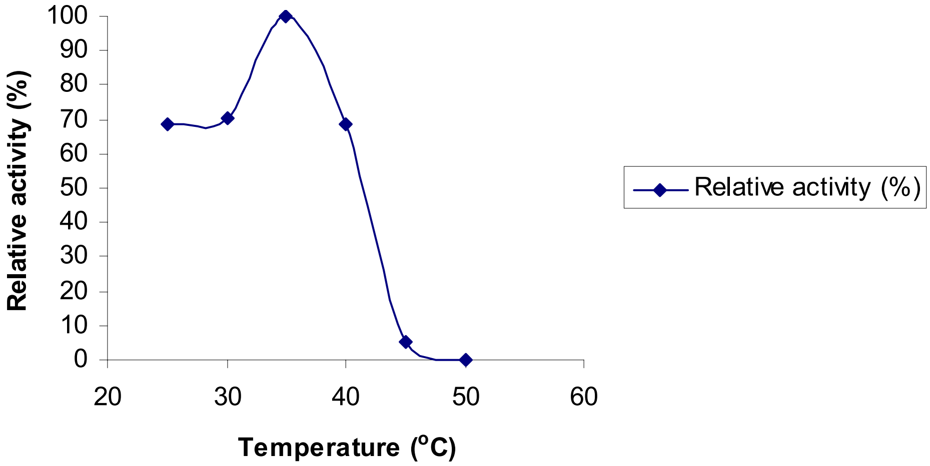

Fig. 9. The effect of temperature on proteinase production of PN51

7. Time course of growth and proteinase production

The 10% of PN51 was inoculated in modified Lee's medium containing 1.0% peptone, 0.5% yeast extract, 0.04% $CaCl_2$ and 0.02% $MgCl_2$ at 35°C, 180 rpm. The strain gave the highest proteinase production after cultivating for 22 hr (Fig. 10).

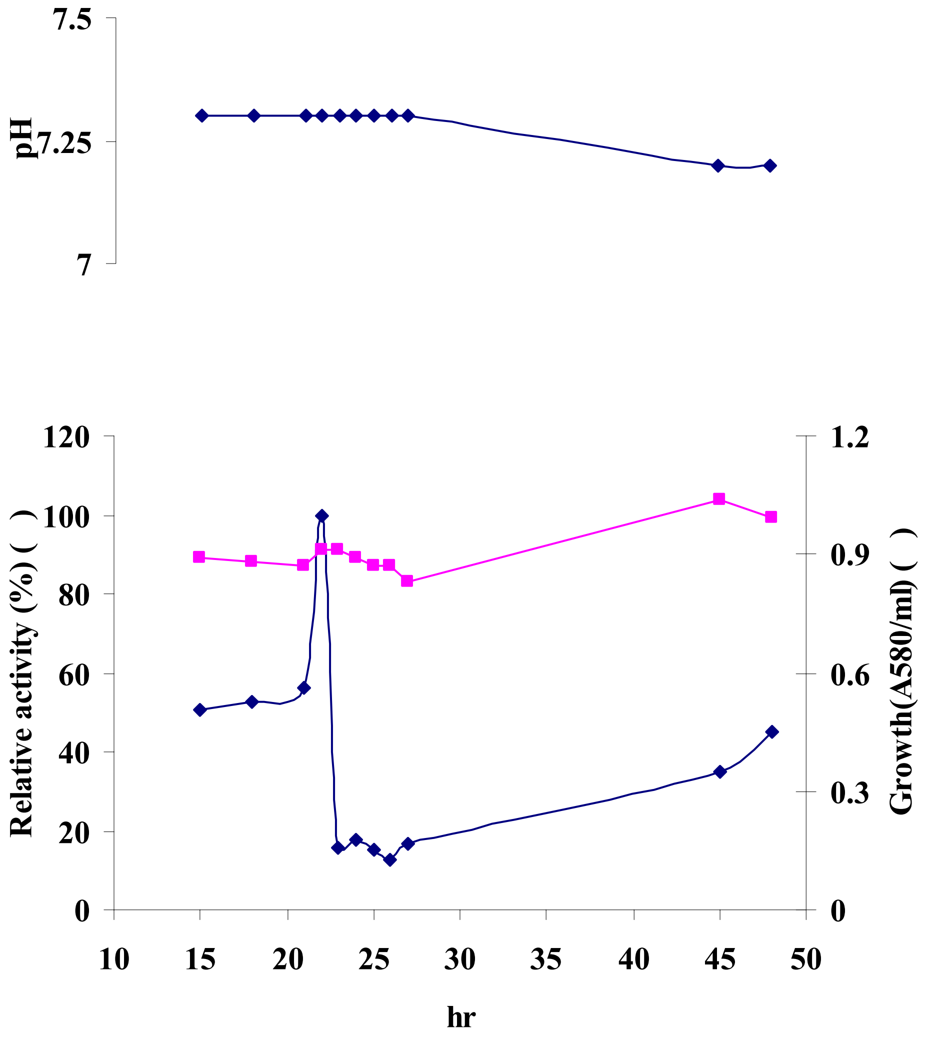

Fig. 10. Time course of proteinase production from PN51 in large scale culture (500 ml)

Enzyme purification

Culture filtrate from 1.9 litre-medium was precipitated with $(NH_4)_2SO_4$ at 20-80% saturation. The dialyzed crude enzyme was loaded onto a DEAE-cellulose DE52 column. Proteinase was eluted at the first peak of elution profile (Fig. 11). The active fractions were pooled and then loaded onto a Mono Q anion-exchange column. The active fractions were then eluted at 0.37-0.48 M NaCl (Fig. 12). The active fractions were fraction number 15, 16, 17 and 18 which came out at the resolution time, 41.2, 48.05, 56.72, 65.2 min, respectively. The purified enzyme did not show a single band on SDS-PAGE (Fig. 13). As shown in Table 3, 12 mg of the purified enzyme from 1.9 liter of culture supernatant was obtained, with 2.5% proteinase activity yield, and 28.4-fold purification (Table 3).

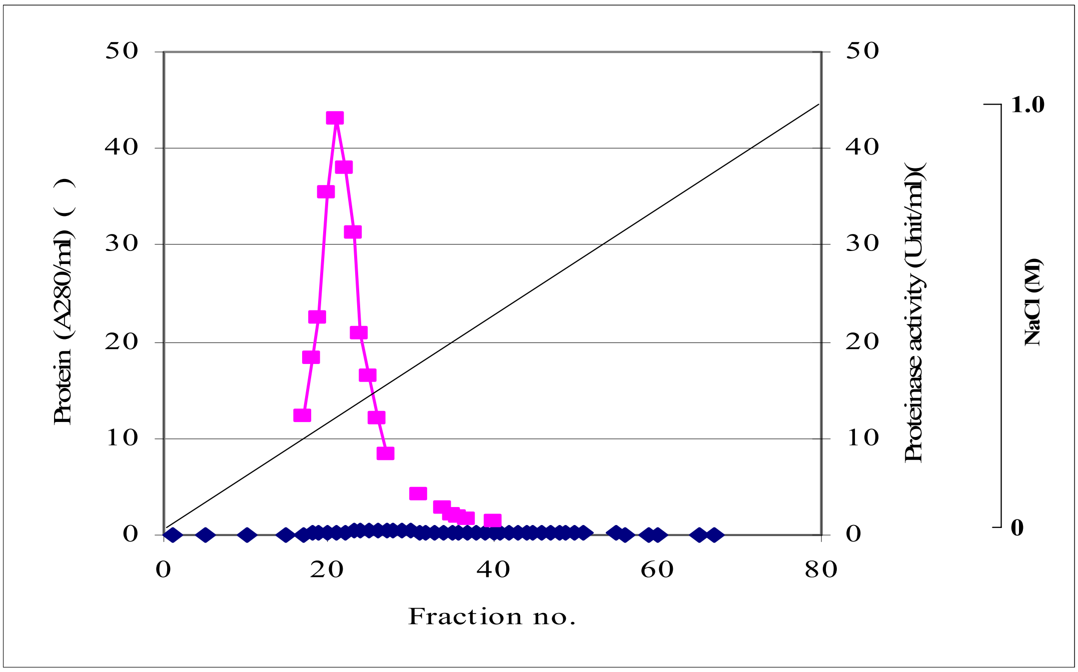

Fig. 11. Elution profile of proteinase from PN51 by DEAE-cellulose DE52

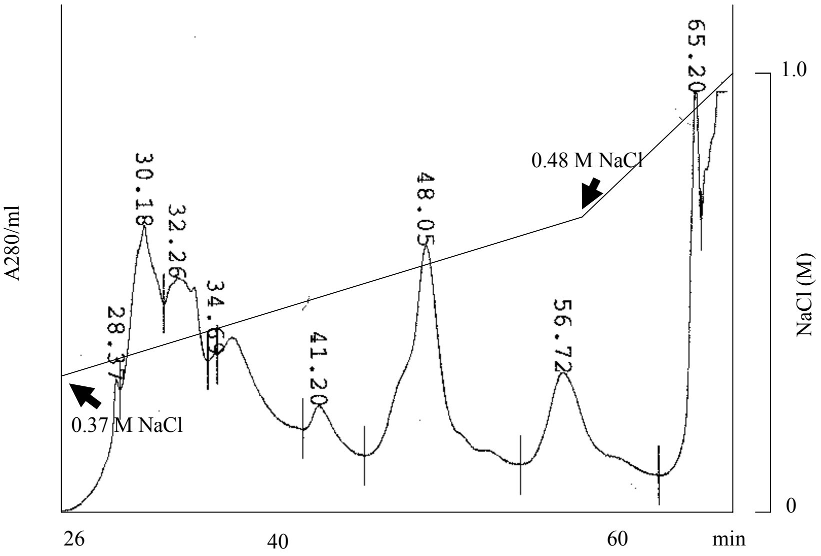

Fig. 12. Elution profile of proteinase from PN51 by Mono Q FPLC

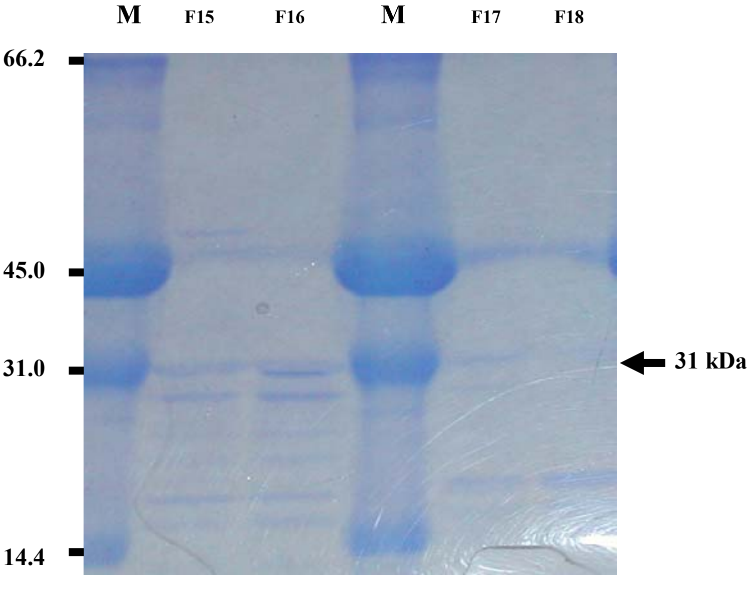

Fig. 13. SDS-PAGE analysis of the purified enzyme

Table 3. Purification of the extracellular proteinase from PN51

Step	Total proteinase act. (Unit)	Total Protein (mg)	Specific activity (Unit mg^{-1})	Yield (%)	Purification factor (fold)
1. Culture supernatant	24,356	13,870	1.76	100	1
2. 20-80% $(NH_4)_2SO_4$ ppt.	6,054	2,672	2.3	25	1.3
3. DEAE-cellulose DE52	3,040	120	25	12.5	14.4
4. Mono Q FPLC	600	12	50	2.5	28.4

Effect of inhibitors

The proteinase from PN51 could be inhibited by PMSF and chymostatin which had relative activity 0 and 7%, respectively (Table 4). It was inhibited a little by elastatinal and leupeptin which had relative activity decreased to 71 and 85%, respectively.

Table 4. Effect of inhibitors on the proteinase activity from PN51

Inhibitor	Concentration (mM)	Relative activity (%)
None	-	100
PMSF	1	0
TLCK	1	100
TPCK	1	101
Chymostatin	0.1	7
Elastatinal	0.1	71
Leupeptin	0.1	85

Discussion

The strain PN51 which gave the highest activity of proteinase was identified by taxonomic characteristics and 16S rDNA sequence analysis. The 16S rDNA sequence analysis of PN51 showed high homology of 100% correlation to that of *Bacillus* sp. CNJ904PLO4 isolated from tropical marine sediments (Gontang *et al.*, 2006 (unpublished data)). Caves are opening in bedrock caused by solution or erosion by percolating water, flowing water or wave action (Ulukanli and Digrak, 2002). Thus, some microorganisms from the marine could enter the cave and establish populations similar to those that exist in marine sediments. The enzyme was purified by ammonium sulphate precipitation, DEAE cellulose DE 52 and Mono Q column chromatography. The active fractions from Mono Q column had clearly separated into 4 peaks, indicating that the enzyme had at least four isoforms. Regarding the SDS-PAGE results, each isoforms of the enzyme was not pured or the autodegradation of the enzyme was occurred. These results need to be confirmed again. Some characteristics of the enzyme were studied. The enzyme activity was completely inhibited by PMSF and chymostatin. Therefore, the present study of the enzyme from PN51 was thought to be a serine-type proteinase. The amino-terminal amino acid sequence analysis of the enzyme is needed to be further investigated.

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Output ที่ได้จากโครงการ

เนื่องจากยังประสบปัญหาในขั้นตอนของ purification ดังนั้นยังไม่มี output ซึ่งต้องอาศัยเวลาและงบประมาณที่สำรองไว้ในการทำงานวิจัยนี้ต่อไป

ภาคผนวก

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รายงานการเงิน

ตามเอกสารที่แนบมา