PURIFICATION AND CHARACTERIZATION OF PROTEASE PRODUCED BY THE THERMOPHILIC FUNGUS, *ASPERGILLUS FUMIGATUS* SS0509

มหาวิทยาลัยศิลปากร สงวนลิขสิทธิ์

By
Sanirat Sangmuang

A Thesis Submitted in Partial Fulfillment of the Requirements for the Degree
MASTER OF SCIENCE
Department of Microbiology
Graduate School
SILPAKORN UNIVERSITY
2010

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การแยกบริสุทธิ์และศึกษาคุณสมบัติของเอนไซม์โปรติเอสจากราทนร้อน *Aspergillus fumigatus* SS0509

โดย

นางสาวสนิรัสมิ์ แสงเมือง

มหาวิทยาลัยศิลปากร สงวนลิขสิทธิ์

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรมหาบัณฑิต

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The Graduate School, Silpakorn University has approved and accredited the Thesis title of "Purification and characterization of protease produced by the thermophlic fungus, *Aspergillus fumigatus* SS0509" submitted by MISS Sanirat Sangmuang as a partial fulfillment of the requirements for the degree of Master of Science in MICROBIOLOGY

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SANIRAT SANGMUANG: PURIFICATION AND CHARACTERIZATION OF PROTEASE PRODUCED BY THE THERMOPHILIC FUNGUS, *ASPERGILLUS FUMIGATUS* SS0509. THESIS ADVISOR: ASST. PROF. EAKAPHUN BANGYEEKHUN, Ph.D. 106pp.

Eleven strains of thermophilic fungi were isolated from fertilized hay at Silpakorn University, Sanamchandra palace, Nakhorn Pathom. Of these, the fungus strain SS0509 showed maximum protease production on CHM media and was selected for further studied. This fungus was identified to be *Aspergillus fumigatus* based on morphology, growth characteristics in different culture media, ITS sequence and specific primers. The culture condition for protease production was studied in basal media, CHM, YHM and PHM at pH range from 5-10 and temperature range from 35-50°C for 3 days and the results indicated that the optimum culture condition was in YHM, pH 8 at 40°C. The enzyme activity assay was investigated at pH range from 5-10 and temperature range from 35-70°C. The result revealed that the optimum condition for protease activity assay was at pH 8 and 55°C. The protease was purified in a two-step procedure, (i) dialization and precipitation by polyethylene glycol 6000, and (ii) gel filtration, with a 4.2-fold increase in specific activity and the specific activity was 17.35 unit/mg proteins with 18.98% yield. The molecular weight of purified protease was estimated to be 42.4 kDa by SDS-PAGE and 83.6 kDa by Native-PAGE. These suggested that the purified protease might be a homodimeric protein. The effects of protease inhibitors showed that the enzyme appeared to be a serine protease, but neither trypsin nor chrymotrysin. Then, the purified protease was characterized. The optimum pH and temperature of the proteolytic activity was pH 8 and 55°C, respectively. The enzyme activity was stable at 55°C for 60 minutes and decrease afterward. The enzyme can be storage at -20°C and 4°C for at least 15 days.

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คำสำคัญ : *Aspergillus fumigatus* เอนไซม์ทนร้อน โปรติเอส การแยกบริสุทธิ์

สนิรัสมิ์ แสงเมือง : การแยกบริสุทธิ์และศึกษาคุณสมบัติของเอนไซม์โปรติเอสจากราทนร้อน *Aspergillus fumigatus* SS0509. อาจารย์ที่ปรึกษาวิทยานิพนธ์ : ผศ.ดร.เอกพันธ์ บางยี่ขัน. 106 หน้า.

แยกราทนร้อน 11 สายพันธุ์จากกองปุ๋ยหมัก มหาวิทยาลัยศิลปากร พระราชวังสนามจันทร์ จังหวัดนครปฐม ราสายพันธุ์ SS0509 สามารถสร้างเอนไซม์โปรติเอสในอาหาร CHM ได้มากที่สุดและนำไปใช้ในการศึกษา การศึกษาด้วยลักษณะทางสัณฐานวิทยา ลักษณะการเจริญในอาหารต่างๆ ลำดับกรดนิวคลีอิกของ ITS และการใช้ไพรเมอร์ที่จำเพาะเจาะจง ระบุได้ว่าราสายพันธุ์ SS0509 คือ *Aspergillus fumigatus* การศึกษาสภาวะสำหรับการเลี้ยงเชื้อที่เหมาะสมสำหรับการสร้างเอนไซม์ในอาหาร basal medium CHM YHM และ PHM ความเป็นกรดด่างที่ 5-10 และที่อุณหภูมิ 35-60 องศาเซลเซียส พบว่าสภาวะที่เหมาะสมคือในอาหาร YHM ความเป็นกรดด่างที่ 8 ที่อุณหภูมิ 40 องศาเซลเซียส ศึกษาสภาวะสำหรับการวัดกิจกรรมของเอนไซม์ที่ความเป็นกรดด่างที่ 5-10 และที่อุณหภูมิ 35-70 องศาเซลเซียส พบว่าสภาวะที่เหมาะสมคือที่ความเป็นกรดด่างที่ 8 และที่อุณหภูมิ 55 องศาเซลเซียส การแยกบริสุทธิ์เอนไซม์ผ่านสองกระบวนการคือ (1) ทำให้เข้มข้นขึ้นด้วยการแยกโดยผ่านเยื่อเลือกผ่านและโพลีเอทิลีนไกลคอล 6000 และ (2) เจลฟิลเตรชั่น พบว่าให้ค่ากิจกรรมคิดเป็น 4.2 เท่า โดยมีค่ากิจกรรมพิเศษเท่ากับ 17.35 หน่วยต่อมิลลิกรัมและได้ผลผลิตคิดเป็นร้อยละ 18.98 มวลโมเลกุลของโปรตีน จาก SDS-PAGE พบว่ามีขนาดประมาณ 42.4 กิโลดาลตัน และจาก Native-PAGE พบว่ามีขนาดประมาณ 83.6 กิโลดาลตัน แสดงว่าเอนไซม์นี้เป็นโปรตีนที่มีสายโพลีเปปไทด์ 2 หน่วยเหมือนกัน การศึกษาผลของสารยับยั้งโปรติเอสสามารถระบุได้ว่าเอนไซม์ที่แยกบริสุทธ์ได้นี้ จัดอยู่ในกลุ่มเซอรีนโปรติเอส แต่ไม่ใช่ ทริปซินหรือไคโมทริปซิน เมื่อศึกษาคุณสมบัติของเอนไซม์โปรติเอสบริสุทธิ์พบว่ากิจกรรมของเอนไซม์ทำงานเหมาะสมที่ความเป็นกรดด่างที่ 8 และที่อุณหภูมิ 55 องศาเซลเซียส เอนไซม์มีความเสถียรที่อุณหภูมิ 55 องศาเซลเซียสเป็นเวลา 15 ถึง 60 นาที และสามารถเก็บรักษาเอนไซม์ที่อุณหภูมิ -20 และ 4 องศาเซลเซียส เป็นเวลาอย่างน้อย 15 วัน

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ภาควิชาจุลชีววิทยา บัณฑิตวิทยาลัยมหาวิทยาลัยศิลปากร ปีการศึกษา 2553

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Sanirat Sangmuang

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CHAPTER I
INTRODUCTION

Proteases are enzymes that hydrolyze peptide bonds of protein into peptides and amino acids. That can be classified into four major groups according to the character of their catalytic active sites and action: serine protease, cysteine (thiol) protease, aspartic protease and metalloprotease. Proteases constitute one of the most important groups of industrial enzymes accounting for about 60% of the total worldwide enzyme market because of their various applications in many industries such as the detergent, food, leather, silk, dairy and pharmaceutical industry (Tunga et al. 2003: 1553-1558; Merheb et al. 2007: 127-131). The value of worldwide sales of industrial enzymes was estimated to be US$ 1.7-2.0 billion (Shankar et al. 2011: 579-585)

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Many industrial processes take place at high temperature and therefore, the thermostable proteases are suitable for these industries. For example, the detergent industry is performed at 20-60°C at a pH ranging of 7.5 to 10.5. The alkaline proteases are particularly important for this application because they are both stable and active under high temperature, alkaline condition and in the presence of surfactants (Tunga et al. 2003: 1553-1558). The proteases in detergent industries account for 30% of the total worldwide enzyme production and represent one of the largest and most successful applications of modern industrial biotechnology. An alkaline protease with elastolytic and keratinolytic activities was in leather processing, this enzyme plays an important role as catalysts in technical process at 50-70°C (Sexena et al. 2005: 289-290).

Microorganisms including bacteria, fungi and yeasts are the important sources for thermostable proteases. Many thermophilic fungi produced thermostable proteases, such as in genus *Aspergillus* (Shumi et al. 2004: 312-317; Tunga et al. 2003: 1553-1558), *Mucor* (Alves et al. 2005: 114-117), *Penicillum* (Djemal et al. 2009: 469-477; Hashimoto et al. 1972: 986-992), *Thermomyces* (Li

et al. 1997: 18-22) and *Rhizopus* (Ikasari and Mitchell 1996: 171-175; Kumar et al. 2005: 1701-1705). A large proportion of commercially available thermostable proteases were derived from *Bacillus* (Tunga et al. 2003: 1553-1558), an alkaline protease from *Bacillus licheniformis* AP1 was suitable for tannery industry (Tang et al. 2004: 1421-1424). Now, the thermostable protease from fungi having their major applications enzyme because fungal proteases offer a distinct advantage over the bacterial protease in terms of (1) easing the downstream processing, (2) the fungal cells can be easily removed from the final product by simple filtration, (3) ability of fungus to grow on cheap substrate, (4) easy immobilization of mycelium for repeated use, and (5) they can grow in a broad range of pH, 4-11 (Gupta et al. 2002: 15-32; Sharma et al. 2006: 1-14; Hussain et al. 2010: 986-992). In dairy industry, the rennin proteases derived from *Rhizomucor pusillus* and *R. miehei* are used in cheese making (Aehle 2004: 1-489). In pharmaceutical industry, protease from *A. fumigatus* use to improve diagnosis and monitoring of invasive aspergillosis (Schaal et al. 2007: 93-100). In commercial detergents, the alkaline protease from *A. niger* it suitability for application in detergent industry (Devi et al. 2008: 1-6). In food industry, the acid protease from *A. oryzae* is used for modification of food proteins and as digestive aids (Vishwanatha et al. 2009: 402-407).

Nowadays, the thermostable proteases are imported for industrial uses in Thailand. Since, Thailand located on highly biodiversity area, it is possible to search for new thermostable protease from thermophilic fungus and applied for industries in our country. The aims of this study are isolated, purify and characterize the protease from a thermoplilic fungus isolated from fertilized hay.

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CHAPTER II
REVIEW LITERATURE

1. *Aspergillus fumigatus*

Aspergillus fumigatus is a saprophytic fungus that can be found worldwide. Hence, it naturally inhabits in soil and decaying organic matter, such as fertilizer. *A. fumigatus* is a facultative thermophilic species. This fungus can grow from 12-55°C, spanning most of the mesophile-thermophile ranges. Conidia can survive at 70°C. This condition regularly encounters in self-heating compost heaps (Edwards 1990: 1-33; Deacon 1980: 121-135). *A. fumigatu*s is distinguished by rapid growing in turquoise to dark green shades, phialides curving to be roughly parallel to each other, the axis of the stipe, and small conidia borne in columns. A few isolates of *A. fumigatus* are pigmentless and produce white conidia. The chains of conidia are borne directly on broadly vesicles in the absence of metulae. Cleistothecium is absent. An identification of *Aspergillus* species using growth characteristic on different media is described according to Klich (2002). The growth characteristic of *A. fumigatus* is described here (Table 1).

Table 1 Growth characteristic of *A. fumigatus* in different media (Klich 2002:1-50)

	Media				
	CYA25	MEA	CYA37	CY20S	CZ
Diameter (mm)	(35)[*]40-70	(30)[*]45-70	(57)[*]60-70	(30)[*]40-70	45-60
Colors Conidia	Greyish turquoise Dark turquoise Dark green Dull green	Greyish turquoise Dark turquoise Dark green Dull green	Greyish turquoise Dark turquoise Dark green Dull green or Grayish brown	Greyish turquoise Dark turquoise Dark green Dull green	Greyish turquoise Dark turquoise Dark green Dull green
Mycelium	White	White	White	White	White
Exudate	present uncolored	present uncolored	present uncolored	absent	present uncolored
Reverse	Uncolored Yellowish Red brown green floccose, plane, radially furrowed	Uncolored Dull yellow grey	Uncolored Yellowish Red brown green floccose, plane, radially furrowed	Uncolored Yellowish Red brown green floccose, plane, radially furrowed	Uncolored Yellowish Red brown green floccose, plane, radially furrowed
Soluble pigment	absent	absent	absent	absent	absent
Cleistotheia	absent	absent	absent	absent	absent
Sclerotia	absent	absent	absent	absent	absent

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[*] Rare case

1.1 Identification of fungi

The identification of fungi based on morphology has historically been a very difficult task and required amount of experience to identify to the species level. In addition to morphological studies, the growth characteristic in different media can be used to identify the species *Penecillium* and *Aspergillus* (Samson et al. 1995: 1-248; Klich 2002: 1-115), the present of Q-10 ubiquinone system can be used for identify *A. fumigatus*, and patterns of glucose 6-phosphate dehydrogenase and glutamate dehydrogenase can be used for identification of clinical and nonclinical isolates of *Aspergillus* spp. (Matsuda et al. 1992: 1999-2005).

Nowadays, the identification of fungi base on conserved nucleotide sequence of the genome has been developed. Identification based on PCR amplification of conserved regions of the ribosomal DNA (rDNA) have been used in many fungi. rDNA consists of external transcribed spacer (ETS), 18S, internal transcribed spacers 1 (ITS1), 5.8S, internal transcribed spacers 2 (ITS2), and 28S tracts as a tandem repeated unit (Fig. 1). 18S and 28S ribosomal genes are the most conserved regions in eukaryotic cells and useful for identify fungal species with in genus (Korabecna 2007: 783-787). ITS regions are the non-functional sequence between 18S, 5.8S and 28S rDNA. There is a high degree of variation in these parts due to the accumulation of mutation and useful for identify fungal strain with in species. Many fungi were identified by using these parts. For example, Wu et al (2003) developed the nucleotide probes for the detection of common airborne fungi from 18S rDNA. The18S rDNA sequence represented species specific. The DNA probe based on18S rDNA sequences were also verified as genus or species specific. Seyfarth et al (2008) diagnosed an infection with *Fusarium proliferatum* based on ITS sequence.

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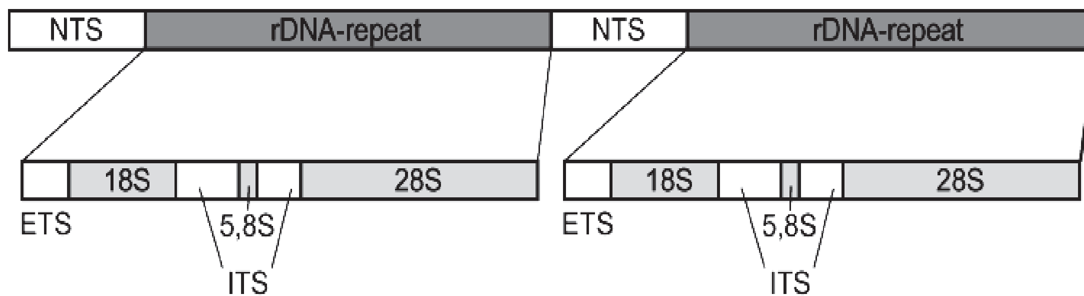

Figure 1 The gene segment of rDNA contains 18S, 5.8S, and 28S tracts and forms a tandem repetitive cluster. Non transcribed spacer (NTS), external transcribed spacer (ETS), internal transcribed spacers 1 and 2 numbered (ITS). (http://en.wikipedia.org/wiki/File:Eucaryot_rdna.png)

2. Protease

Proteases are enzyme that hydrolyzes peptide bonds. Their molecules are relatively small and compact. Proteases are a large group of enzyme, ubiquitous in nature and found in a wide variety of microorganisms. These enzymes play important roles in physiological processes; for example, they are involved in the regulation of metabolism and gene expression, enzyme modification, pathogencity and the hydrolysis of large proteins to smaller molecules for transport and metabolism (Rao et al. 1998: 597-635).

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Proteases are the important group of enzymes and represent nearly 60% of total enzymes saled since they are used in the detergent, food, beer, meat, leather, dairy, silk and pharmaceutical industries (Tunga et al. 2003: 1553-1558; Merheb et al. 2007: 127-131). The value of worldwide sales of industrial enzymes was estimated to be US$ 1.7-2.0 billion (Shankar et al. 2011: 579-585).

2.1 Classification of protease

Protease can be classified by various criteria. (I) Based on their site of action, enzyme that cleave the peptide bonds at terminal amino acid of a substrate chain are named exopeptidases, and enzyme that cleave peptide bonds distant from the terminal of a substrate chain are named endopeptidases. (II) Based on characterized and optimal pH ranges, proteases are classified as acid protease,

neutral protease or alkaline protease. (III) Based on the functional groups presented of the active site and catalytic mechanisms, the proteases are categorized into four groups; serine proteases, aspartic acid proteases, cysteine/thiol proteases and metalloproteases (Table2).

Table 2 The major classes of protease and their active sites (Neurath 1989: 1-12).

Protease class	Active sites
Serine protease I	His^{57}, Asp^{102}, Ser^{195} (pancreas bovine)
Serine protease II	Asp^{32}, His^{64}, Ser^{221}(fibrinogen bovine)
Cysteine protease	Cys^{25}, Asp^{158}, His^{159}(spleen bovine)
Aspartic acid protease	Asp^{32}, Asp^{213}(liver bovine)
Metalloproteases I	Zn, Try^{248}, Glu^{270}(pancreas bovine)
Metalloproteases II	Zn, Glu^{143,} His^{231}(insulin bovine)

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Exopeptidase. The exopeptidases act only near the ends of polypeptide chains. Based on their sites of action at the N and C terminus, can be classifies as aminopeptidase and carboxypeptidase, respectively. Aminopeptidases act at a free N terminus of a polypeptide chain to liberate single amino acid residual, dipeptide or tripeptide (Rao et al. 1998: 597-635).

Endopeptidase. Endopeptidases were classified by their action at the peptide bond in the inner regions of the polypeptide chain away from the N or C terminus. The endopeptidases were divided into four sub groups based on their catalytic mechanism, (I) serine protease, (II) aspartic protease, (III) cysteine protease and (IV) metalloprotease (Neurath 1989: 1-12)

(I) Serine protease. Serine proteases are a class of proteolytic enzymes characterized using specific active site, a serine residual (Ser), a histidine residual (His) and an aspartic residual (Asp). The specific inhibitors of serine protease are phenylmethanesulfonylfluoride (PMSF), Diisopropylfluorophosphate (DPF) or 3, 4-Dichloroisocoumarin (3, 4-DCI) (Salvesen and Nagase 1989: 83-102). Serine proteases were categorized into two families, chymotrypsin and

subtilisin. Of the chymotrypsin family are chymotrypsin, trypsin, and elastase were characterized (Neurath 1989: 1-12). Chymotrypsin cleaves peptide bonds after specific hydrophobic amino acid residues such as phenylalanine, tryptophan and tyrosine, and its activity is specifically inhibited by Chloro-3-tosylamido-4-phenyl-2-butanone (TPCK). Trypsin cleaves peptide bonds following a positive-charged amino acid residue such as arginine and lysine, and its activity is specifically inhibited by 1-Chloro-3-tosylamido-7-amino-2-heptanone HCl (TLCK). Elastase cleaves peptide bonds next to a small amino acid residue like alanine, glycine, and valine and its activity is specifically inhibited by elastinal. Unlike chymotrypsin family, the arrangement of catalytic triad on active site of subtilisin family is Asp-32, His-64, and Ser-221. The subtilisin cleaves peptide bonds of any amino acid but, aromatic or hydrophobic amino acid (Bond, 1989: 145-161). Subtilisin is divided into classes I and II, based on amino acid sequence pattern. Class I subtilisins are extracellular enzymes found in bacteria, and class II subtilisins are extracellular enzymes found in fungi. Both play a role in nutritional uptake (Monod et al. 1991: 23-28). All fungal subtilisins characterized to date are homologous with proteinase K from *Tritirachium album*. Various physiological roles have been suggested, or demonstrated, for subtilisin-like proteases (Seger et al. 1999: 395-402).

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(II) Aspartic protease. Aspartic protease, commonly known as acidic proteases, is a kind of endopeptidase is depended on aspartic acid residues for its catalytic activity. Acidic protease has been grouped into three families, pepsin (A1), retropepsin (A2) and enzyme from pararetroviruses (A3). The members of families A1 and A2 were known to be related to each other, while those of family A3 showed less relatedness to A1 and A2. The aspartic protease was inhibited by pepstain (Dunn 1989: 57-79). Most aspartic proteases showed maximal activity at low pH (pH 3-4), isoelectric points in the range of pH 3 to 4.5. And molecular masses of 30 to 45 kDa. Microbial aspartic proteases can be divided into two groups, (i) pepsin-like enzymes from *Aspergillus*, *Penicillium*, *Rhizopus*, and *Neurospora* and (ii) rennin-like enzymes from *Endothia* and *Mucor* (Rao et al. 1998: 597-635).

(III) Cysteine protease. Cysteine protease can be found in both prokaryotes and eukaryotes. Approximately 20 families of cysteine protease have been recognized. Typically activity cysteine proteases depends on cysteine and histidine residual. The order of Cys and His (Cys-His or His –Cys) residues differed among the family. Generally, cysteine protease is actived only in the presence of reducing agents such as cysteine. The cysteine protease is inhibited specifically by Iodoacetic acid. Papain was the best-known cysteine protease (Maheshwari et al. 2000: 461-488). Some cysteine proteases have neutral pH optimum (Rao et al. 1998: 597-635). The protease from *Humicola lanuginosa* preferentially cleaves at the C-terminal end of hydrophobic amino acid residues. This protease differed from the papain group, the plant enzyme, in its interaction with three affinity matrices and its substrate specificity towards synthetic substrates. This enzyme represents a unique example of a cysteine protease obtained from fungi (Shenolikar et al. 1982:147-152).

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(IV) Metalloprotease. Metalloprotease is the most diverse of the catalytic types of protease. They are characterized by the requirement for a divalent metal ion for their activity. The metal ion is coordinated to the protein via three ligands. The ligands co-ordinated the metal ion can vary with histidine, glutamate, aspartate, lysine, and arginine. About 30 families of metalloprotease have been recognized. Seventeen families are endopeptidase, 12 families are exopeptidase and only 1 family is endopeptidase and exopeptidase. All of them are inhibited by chelating agents such as EDTA but not by sulfhydryl agents or of DFP. The examples of metalloprotease were described. *Thermoascus aurantiacus* metalloprotease (Dini et al. 2009: 9210-9217), *Aspergillus oryzae* metalloprotease (Sumantha et al. 2005: 313-319).

2.2 Protease inhibitor

An enzyme inhibitor is a molecule that binds to enzymes and decreases their activities. The binding of the inhibitor can competitive a substrate to enter to the enzyme's active site or hinder the enzyme from catalysis. Binding of inhibitor is either irreversible or reversible.

Irreversible inhibitors often contain reactive functional groups such as nitrogen mustards, aldehydes, haloalkanes, alkenes, michael acceptors, phenyl sulfonates, or fluorophosphonates. These electrophilic groups react with amino acid side chain to form covalent adducts. The residues modified are those with side chains containing nucleophiles such as hydroxyl or sulfhydryl groups; these include the amino acids serine, cysteine, threonine or tyrosine. Irreversible inhibitors are generally specific for one class of enzyme. They do not destroy the protein structure but specifically alter the active site of their target. Irreversible inhibitors display time-dependent inhibition because the amount of active enzyme at a given concentration of irreversible inhibitor will be different depending on how long the inhibitor is pre-incubated with the enzyme (Salto et al. 1994: 10691-10698). Diisopropylfluorophosphate (DFP), phenylmethanesulfonylfluoride (PMSF), Chloro-3-tosylamido-4-phenyl-2-butanone (TPCK), 1-Chloro-3-tosylamido-7-amino-2-heptanone HCl (TLCK) and Iodoacetic acid are shown as an example of irreversible protease inhibitors (Neurath 1989: 1-12).

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Reversible inhibitors bind to enzymes with non-covalent interactions such as hydrogen bonds, hydrophobic interactions and ionic bonds. Multiple weak bonds between the inhibitor and the active site combine to produce strong and specific binding. Unlike irreversible inhibitors, reversible inhibitors generally do not undergo chemical reactions when bound to the enzyme and can be easily removed. There are four kinds of reversible enzyme inhibitors. They are classified according to the effect of varying the concentration of the enzyme's substrate on the inhibitor. Ethylenediaminetetraacetic acid (EDTA), Elastinal and Pepstatin are shown as an example of reversible protease inhibitors (Samuels and Paterson 1995: 661-669).

2.3 Sources of protease

Proteases can be isolated from a variety of sources such as plants animals and microorganisms. Microbial proteases have been used in biological application since they possess almost the characteristic desire. Microbes serve as

the preferred sources of protease because of their rapid growth and the limited space required for their cultivation (Sharma et al. 2006: 1-14).

Microbial proteases account for approximately 40% of the total worldwide enzyme sales. A large proportion of commercially available alkaline protease is derived from *Bacillus* strains, such as the thermostable serine alkaline protease from *Bacillus subtilis* PE11. This protease is stable at alkaline pH, at high temperature, is compatible with commercial and local detergents (Adinarayana et al. 2003: 1-9). Serine protease produced from *Bacillus* strain HS08 is a thermostable enzyme and used in the detergent industry (Guangrong et al. 2006: 2433-2438). Subtilisin JB1 produced by *B. subtilis* JB is used for fishery waste degradation (Sung et al. 2010: 900-911).

Although the potential use of several fugal sources is increasing, proteases from fungi having their major applications. Fungal proteases offer distinct advantages over the bacterial proteases in terms of (1) easing the downstream processing, (2) the fungal cells can be easily removed from the final product by simple filtration, (3) the ability of fungi to grow on cheap substrates, (4) easy immobilization of mycelium for repeating use, and (5) they can grow in a broad range of pH, 4-11 (Gupta et al. 2002: 15-32; Sharma et al. 2006: 1-14; Hussain et al. 2010: 497-504).

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In dairy industry, the rennin proteases derived from *Rhizomucor pusillus* and *R. miehei* are used in cheese making (Aehle 2004: 1-489). The acid protease from *A. oryzae* is used for modification of food proteins and as digestive aids (Vishwanatha et al. 2009: 402-407). In pharmaceutical industry, protease from *A. fumigatus* is used for diagnosis and monitoring of invasive aspergillosis (Schaal et al. 2007: 93-100). The alkaline protease from *A. niger* is applied for detergent industry (Devi et al. 2008: 1-6).

2.4 Protease from thermophilic fungi

Thermophilic fungi are a small assemblage in Eukaryota that normally have a unique mechanism of growing at temperature range from 20-50°C or above (Maheshwari et al. 2000: 461- 488). It is well known that the thermophilic fungi are found from soils, composts, piles of hays, stored grains, wood chip piles,

nesting material of birds, snuff, municipal refuse, and other accumulations of organic matter (Salar and Aneja 2007: 77-107).

Thermophilic fungi are known to produce thermostable enzymes. The use of these enzymes may present many advantages, for example the food industry, due to the high processing temperatures that could be applied. They can increase the reaction rates, improve solubility of reagents, and decrease of contamination mesophilic microorganisms. Besides thermal stability, these enzymes also exhibit higher stability towards other protein denaturating conditions such as ionic detergents and organic solvents, when compared to mesophilic enzymes (Macchione et al. 2008: 223-230). Several thermophilic fungi have been isolated and studied for produce protease production (Table 3).

Table 3 Proteases produced from thermopilic fungi

Thermophilic fungi	Protease	MW(kDa)	Characteristics
Thermomyces lanuginosus[1]	serine proteases	-	$70^{o}C$, pH 5, 9
Thermoascus aurantiacus[2]	proteases	-	$60^{o}C$, pH 5.5
Thermoascus aurantiacus[3]	metalloprotease	24.5	$55^{o}C$, pH 7.5
Penicillium duponti[4]	acid protease	-	45-$50^{o}C$, pH 4.7
Penicillium expansum[5]	alkaline protease	20.5	$35^{o}C$, pH 10.5
Penicillium sp.6	acid protease	-	32oC, pH 3.5
Aspergillus fumigatus7	subtilisin	33	30oC, pH 9.0
Aspergillus fumigatus CBS113.268	serine proteases	33	37-42oC, pH 9
Aspergillus fumigatus TKU0039	serine proteases	124	40oC, pH 8.0

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Table 3 Proteases produced from thermopilic fungi (continuous)

Thermophilic fungi	Protease	MW(kDa)	Characteristics
Aspergillus fumigatus[10]	protease	-	$30^{o}C$, pH 5
Aspergillus fumigatus[11]	elastase	32	$45^{o}C$ pH7.4
Aspergillus niger[12]	alkaline protease		$40^{o}C$, pH 9.0
Aspergillus niger l1[13]	aspatic protease	50	$60^{o}C$, pH 3
Aspergillus niger[14]	alkaline protease	34	$60^{o}C$ pH8
Aspergillus niger[15]	alkaline protease	38	$45^{o}C$ pH8.5
Aspergillus Oryzae AWT 20[16]	alkaline protease	33	$55^{o}C$ pH9
Aspergillus oryzae[17]	alkaline protease		-
Aspergillus oryzae MTCC5341[18]	aspatate protease	47	$55^{o}C$, pH 3-4
Aspergillus parasiticus[20]	alkaline protease	23	$40^{o}C$, pH 8.0
Aspergillus clavatus ES1[21]	serine protease	32	$50^{o}C$, pH 8.5
Aspergillus nidulan HA-10[22]	serine protease	42	$35^{o}C$, pH 8.0
Aspergillus flavus[23]	protease	-	$30^{o}C$, pH 8
Aspergillus terreus[24]	serine protease	35	$60^{o}C$, pH 7-11
Mucor pusillus25	acid proteases	-	35-45oC
Malbranchae pulchella var. sulphurea26	alkaline protease		45oC
Humicola lanuginosa27	thiol proteinase	23	45oC, pH 7.0

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Table 3 Proteases produced from thermopilic fungi (continuous)

Thermophilic fungi	Protease	MW (kDa)	Characteristics
Piromyces sp.[28]	serine proteases		50°C, pH7.9
Scytalidium thermophilum[29]	alkaline protease	-	37-45°C, pH 6.5-8
Rhizopus oryzae[30]	aspartic proteases	34	60°C, pH 5.5

[1]Li et al. 1997; [2]Merheb et al. 2007; [3]Dini et al. 2009; [4]Hashimoto et al. 1972; [5]Dahot 1994; [6]Dajmel et al. 2009; [7]Monod et al. 1991; [8]Larcher et al. 1992; [9]Wang et al. 2005; [10]Oyeleke et al. 2010; [11]Frosco et al.1992 ; [12]Coral et al. 2003; [13]Siala et al. 2009 ; [14]Kim 2004; [15]Devi et al 2008; [16]Sharma et al. 2006; [17]Gua and Ma 2008; [18]Vishwanatha et al. 2009; [19]Shumi et al. 2004; [20]Tunga et al. 2003; [21]Hajji et al. 2007; [22]Charles et al. 2008; [23]Oyeleke et al. 2010, [24]Hussain et al. 2010; [25]Arima et al. 1968; [26]Ong and Gaucher 1973 ;[27]Shenolikar 1982; [28]Asao et al. 1993; [29]ÏFrij and Ögel 2002, [30]Kumar et al. 2005.

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CHAPTER III
MATERIALS AND METHODS

1. Isolation and screening of the thermophilic fungi

1.1 Fertilized hay was collected from Silpakorn University, Sanamchandra palace, Nakhorn Pathom. The fungi were isolated by dilution method and spread on potato dextrose agar plate (PDA). Then, the cultures were incubated at 40°C for 3 days.

1.2 Pure cultures were obtained by subculturing the isolated colonies to new PDA plate and incubated at 40°C for 3 days.

1.3 Pure isolated thermophilic fungi were transferred to casein hydrolysis agar medium and incubated at 40°C for 3 days. Protease production was determined by measuring the clear zone.

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1.4 The thermophilic fungi were maintained on casein hydrolysis agar medium at 4°C as the stock cultures.

2. Morphology and physiology

2.1 The fungal mycelium disc was prepared by culturing on PDA at 40°C for 3 days.

2.2 The followed morphology of fungi was observed.

1. Length, width and surface texture of stipe
2. Dimeter and shape of vesicle
3. Uniseriate and biseriate of seriation
4. Length, shape and surface texture of conidia
5. Dimeter, shape, color and surface cell- hyphae/ parcenchyma of cleistothecia/Sclerotia (if present)
6. Day to Maturation, length, width, surface texture and farrows/flanges of ascospore

2.3 Fungal growths were examined on different culture media and temperatures as followed. One loop of conidia from agar slant was mixed 0.2%

agar and 0.05% tween 80. Then, 0.2 µl aliquots were placed on each of three equidistant points on the agar plate.

1. Czapek agar (CZ25) at 25°C
2. Czapek Yeast agar (CYA25) at 25°C
3. Czapek Yeast agar (CYA37) at 37°C
4. Czapek Yeast Sucrose agar (CYAS25) at 25°C
5. Malt extract agar (MEA25) at 25°C

2.4 Identification of fungi based on morphological and growth characteristics on different culture media were performed according to Klich (2002).

3. DNA extraction

3.1 Mycelium was collected from liquid culture by filtration and rinsed three times with sterile water.

3.2 The mycelium was ground in liquid nitrogen using ceramic mortar and pestle.

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3.3 300 µl of cell lysis solution and 1.5 µl of Protenase K solution (20 mg/ml) were added to 1.5 ml tube. The 10-20 mg ground mycelium was added to the cell lysis/ Protenase K solution and vortexed.

3.4 Cell lysate was incubated at 55ºC for 1 hour.

3.5 1.5 µl of RNase A solution was added to the cell lysate. The sample was mixed by inverting the tube 25 times and incubated at 37ºC for 15 minutes.

3.6 The sample was cooled down to room temperature. 100 µl of protein precipitation solution was added uniformly with the cell lysate by vortexing each tube at high speed for 20 second.

3.7 The tube was centrifuged at 13000-16000 rpm for 3 minutes.

3.8 The supernatant containing the DNA was poured into a new 1.5 ml centrifuge tube containing 300 µl of isopropanal and mix the sample by inverting gently 50 times.

3.9 The tube was centrifuged at 13000-16000 rpm for 1 minute. Then, the supernatant was discarded.

3.10 300 µl of 70% ethanol was added and then, the tube was mixed by inverting several times.

3.11 The tube was centrifuged at 13000-16000 rpm for 1 minute.

3.12 The tube was inverted, drained and air dried for 15 minutes.

3.13 50 µl of DNA hydration solution was added and incubated for 1hour at 55ºC.

3.14 The tube was centrifuged at 13000-16000 for 5-10 minutes and then the supernatant containing the DNA was transferred to a clean tube.

3.15 The DNA concentration and purity was determined by spectrophotometer at 260 and 280 nm.

3.16 DNA was stored at 2-8ºC.

4. PCR amplification

4.1 PCR amplification of the ITS region

4.1.1 The PCR amplification of ITS region was performed in 50 µl aliquot containing 10 mM Tris-HCl (pH8.8), 50 mM KCl, 2 mM $MgCl_2$, 0.2 mM of each dNTP, 0.2 µM of forward primer (ITS1; TCC GTA GGT GAA CCT GCG G), 0.2 µM of reward primer (ITS4; TCC TCC GCT TAT TGA TAT GC), 100ng/µl of fungal genomic DNA, 36.5 µl of water and 2.5 unit of Taq polymerase.

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4.1.2 PCR condition was 1 cycle of predenaturation at 95ºC for 2 minute followed by 40 cycle of denaturation at 94ºC for 30 second, DNA annealing at 55ºC for 30 second, extension at 72ºC for 30 second and 1 cycle of final extension at 72ºC for 10 minute.

4.1.3 The PCR reaction was analyzed in 1% agarose gel with 100 bp molecular weight marker.

4.1.4 The PCR products of ITS region were sent to First Base Labaratory, Malasia for DNA sequencing. The DNA sequence was analyzed using Chromaslite 201, and compared with database from National Center for Biotechnology Information (Alschul et al. 1997: 3389-3402).

4.2 PCR amplification of specific rDNA-ITS regions for *Aspergillus fumigatus* (Zhao 2001).

4.2.1 The PCR amplification of specific DNA for *A. fumigatus* was performed in 50 µl aliquot containing with 10 mM Tris-HCl (pH 8.8), 50 mM KCl, 2 mM $MgCl_2$, 0.2 mM of each dNTP, 0.2 µM of forward primer (Asp5; GAT AAC GAA CGA GAC CTC GG, or Asp1; CGG CCC TTA ATT AGC CCG GTC), 0.2 µM of reward primer (AFUM2; ACC TTA GAA AAA TAA AGT TGG GTG, AFUM1;TTA CGA TAA TCA ACT CAG ACT GCA TA), 100 ng/µl of fungal genomic DNA, 36.5 µl of water and 2.5 unit of Taq polymerase.

4.3.2 The first PCR was done by using primer Asp5 and AFUM2. The second PCR was perform by using primer Asp1 and AFUM1.

4.2.3 PCR condition was 1 cycle of predenaturation at 95ºC for 5 minute followed by 40 cycle of denaturation at 95ºC for 30 second, DNA annealing at 58ºC for 30 second, extension at 72ºC for 5 minute and 1 cycle of final extension at $72^{o}C$ for 5 minute.

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4.2.4 The PCR reaction was analyzed in 1% agarose gel using 100 bp molecular weight marker.

5. Purification of PCR products using gel extraction kit (Fermentas, EU)

5.1 The expected PCR product from ITS region in agarose gel was excised using a clean scalpel and weighed in a microcentrifuge tube.

5.2 Three volumes of binding solution and 10 µl of silica powders was added to the tube and incubated at $55^{o}C$ for 5 minutes. Mix by vortex every 2 minutes.

5.3 The tube was centrifuged at 13,000 rpm at room temperature for 5 seconds. The supernatant was removed.

5.4 The DNA sample was washed with wash buffer 3 times.

5.5 The tube was incubated at room temperature for 15 minutes.

5.6 The DNA sample was resuspended in TE buffer, incubated at $55^{o}C$ for 5 minutes and eluted by centrifugation at 13,000 rpm for 1 minute, respectively.

5.7 The DNA solution was transferred to a new microcentrifuge tube.

6. Fungal growth measurement

6.1 Fungal colonial diamater measurement

6.1.1 The fungal mycelium discs were prepared by culturing on casein hydrolysis agar medium (CHM) at 40°C for 3 days.

6.1.2 The 0.5 mm mycelium disc was transferred to the center of basal medium (0.1% KH_2PO_4, 0.05% KCl, 0.02% $MgSO_4.7H_2O$, 0.01% $CaCl_2.2H_2O$, 1% Glucose, 1.2%Agar), CHM (basal medium containing 15% (v/v) skim milk), YHM (basal media containing 15% (v/v) yeast extract) and PHM (basal medium containing 15% (v/v) peptone) and incubated at 35, 40, 45, and 50°C for 5 days.

6.1.3 Quantitative measurement of the hyphal growth was performed by measurement of colony diameter in triplicate.

6.2 Fungal dry weigh measurement

6.2.1 The fungal mycelium discs were prepared by culturing on CHM at 40°C for 3 days.

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6.2.2 The 0.5 mm mycelium disc was transferred in basal medium, CHM, YHM, and PHM at pH 5, 6, 7, 8, 9 or 10 with shaking at 120 rpm at 35, 40, 45 or 50°C for 5 days.

6.2.3 Quantitative measurement of the hyphal growth was performed by dry weight in triplicate.

7. Effect of culture medium on protease production

7.1 The fungal mycelium discs were prepared by culturing on CHM at 40°C for 3 days.

7.2 The fungus was grown in basal medium, CHM, YHM, and PHM at 40°C for 3 days.

7.3 The fungus growth was determined as described in 6.2 and protease activity was measured as described in 10.

8. Effect of temperature on protease production

8.1 The fungal mycelium disc were prepared by culturing on CHM at 40°C for 3 days.

8.2 The fungus was grown in YHM broth at 35, 40, 45, 50, 55 and 60°C for 3days.

8.3 The fungus growth was determined as described in 6.2 and protease activity was measured as described in 10.

9. Effect of pH on protease production

9.1 The fungal mycelium discs were prepared by culturing on CHM at 40°C for 3 days.

9.2 The fungus was grown in YHM broth, pH 5, 6, 7, 8, 9 or 10 at 40°C for 3 days.

9.3 The fungus growth was determined as described in 6.2 and protease activity was measured as described in 10.

10. Protease activity measurement

10.1 The fungus was cultured in liquid medium.

10.2 Supernatant was filtrated and stored on ice.

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10.3 0.5 ml of culture filtrate and 0.5 ml of Tris-HCl (pH 8) were incubated with 0.5 ml of 1% casein (pH 7) at 55°C for 30 minutes. (In blank, 2 ml of 5%trichloroacetic acid was added before incubation)

10.4 The reactions were precipitated with 2 ml of 5% trichloroacetic acid.

10.5 The reaction tubes was incubated at 4°C for 10 minutes and centrifuged at 3000 rpm for 10 minutes.

10.6 The supernatant was collected and protein concentration determined as described in 12.

10.7 The activity of protease was calculated according to the following formula: One unit (U) of enzyme activity is defined as the amount of enzyme that liberates peptide fragment equivalent to 1 mg of BSA under the assay conditions.

11. Protein concentration measurement

11.1 Standard curve

11.1.1 The concentration of bovine serum albumin at 0, 10, 20, 30, 40, and 50 μg/ml of were prepared (Bradford 1976: 248-254).

11.1.2 800 µl of BSA solution of each concentration was mixed with 200 µl of coomassie brilliant blue.

11.1.3 The reaction tube was incubated for 2 minutes.

11.1.4 The supernatant was collected and determined by spectrophotometer at 595 nm.

11.2 Protein concentration measurement

11.2.1 800 µl of sample was mixed with 200 µl of coomassie brilliant blue.

11.2.2 Reaction tube was incubated for 2 minutes.

11.2.3 The supernatant was collected and determined by spectrophotometer at 595 nm.

11.2.4 Protein concentration was calculated according to the standard curve.

12. Effect of temperature on protease activity

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12.1 The fungus was grown in YHM broth pH 8 at 40°C for 3 days and the culture filtrate was taken.

12.2 The protease activity measurement was performed as described in 10 at different temperatures (35, 40, 45, 50, 55, 60, 65 and 70°C).

13. Effect of pH on protease activity

13.1 The fungus was grown in YHM broth pH 8 at 40°C for 3 days and the culture filtrate was taken.

13.2 The protease activity measurement was performed as described in 11 at 55°C with the pH of the reaction mixture adjusted using one of the following buffer; 0.1 M sodium acetate for pH 5, 0.1 M sodium phosphate for pH 6-7, 0.1 Tris-HCl for pH 8-9 or 0.1 M glycine- NaOH for pH 10.

14. Purification of the thermostable protease.

14.1 Acetone Precipitation

14.1.1 One volume of culture filtrate was added to two volumes of cold (-20°C) acetone in test tube.

14.1.2 The tube was mixed by vortex and incubated at -20°C for 60 minutes.

14.1.3 The tube was centrifuged at 13,000-15,000 × *g* for 10 minutes.

14.1.4 The supernatant was removed properly and allowed the acetone to evaporate from the uncapped tube at room temperature for 30 minutes.

14.1.5 The buffer was added and mixed by vortex thoroughly to dissolve protein pellet.

14.1.6 The protease activity and protein concentration of supernatant was measured.

14.2 Ammonium sulfate Precipitation

14.2.1 Ammonium sulfate was added to the culture filtrate to the final 80% saturation at 4°C with continuous stirring for overnight.

14.2.2 The resulting precipitate was centrifuged at 10,000 × *g* for 30 minutes at 4°C.

14.2.3 The protein pellet was then dissolved in 10 ml of 100 mM Tris-HCl buffer.

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14.2.4 The concentrate solution was dialyzed against the same buffer for overnight at 4°C (dialysis membrane with a molecular weight cut off of 6-8kDa)

14.2.5 The protease activity and protein concentration of supernatant was measured.

14.3 Polyethylene glycol 6000 Precipitation

14.3.1 The culture filtrate was dialyzed against 100 mM Tris-HCl buffer overnight at 4°C with continuous stirring (dialysis membrane with a molecular weight cut off of 6-8 kDa).

14.3.2 The dialysis bag was placed on crystalline Polyethylene glycol 6000 for 90 minutes, or until the volume was one-tenth of original volume. Then, the Polyethylene glycol was removed.

14.3.3 The protease activity and protein concentration of supernatant was measured.

14.4 Filtration

14.4.1 The culture filtrate is added into the concentrator. (membrane with a molecular weight cut off of 10 kDa).

14.4.2 The column was centrifuged at 10,000 × *g* for 10 minutes.

14.4.3 The protease activity and protein concentration of filtrated was measured as described in 10.

14.5 Size exclusion chromatography

14.5.1 One ml of the concentrated sample (0.2 mg/ml) was applied to a column of Superdex 200 HR 10/30 column (Amersham Bioscience).

14.5.2 The column was equilibrated with 0.2 column volumes 100 mM Tris-HCl buffer (pH 8.0) and washed 2 column volumes with same buffer.

14.5.3 Proteins were eluted with 100 mM Tris-HCl buffer (pH 8.0). One ml eluted fractions was collected.

14.5.4 The protein concentration was determined by spectrophotometer at 280 nm.

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14.5.5 The protease activity and protein concentration of fractions was measured as described in 10.

14.6 Native-Polyacrylamide Electrophoresis

14.6.1 The protein samples were separated in 8.0 % polyacrylamide gels. Buffer system that stacks at a pH of 6.8 and resolves at a pH of 8.8 as standard protocol (Laemmli 1970: 680-685).

14.6.2 Electrophoresis was carried out at a constant current of 100 V/mA per gel until the tracking dye (bromophenol blue) reached the bottom of the gel.

14.6.3 Gel was stained in 0.25% Coomassie brilliant blue R reagent in 50% methanol and 10% acetic acid and destained in 15% methanol and 10% acetic acid.

14.7 SDS-Polyacrylamide Electrophoresis

14.7.1 The protein samples were separated in 12.0 % polyacrylamide gels. Buffer system that stacks at a pH of 6.8 and resolves at a pH of 8.8 as standard protocol (Laemmli 1970: 680-685).

14.7.2 Electrophoresis was carried out at a constant current of 100 V/mA per gel until the tracking dye (bromophenol blue) reached the bottom of the gel.

14.7.3 Gel was stained in 0.25% Coomassie brilliant blue R reagent in 50% methanol and 10% acetic acid and destained in 15% methanol and 10% acetic acid.

15. Effect of temperature, pH, stability and storage stability on the purified protease activity

15.1 Effect of the protease activity of purified enzyme was measured as described in 10 by incubating the reaction mixture at different temperatures ranging from 35 to 70°C and the pH of the reaction mixture range from pH 5-10.

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15.2 To study the time- dependent thermal stability of the purified enzyme was incubated at 55°C for 0, 15, 30, 45, 60 and 90 minutes. Then, the activity was measured as described in 10.

15.3 To study the storage stability, purified enzyme was store at different temperatures (-20, 4 and 25°C) for 0, 1, 7 and 15 days. Then, the activity was measured as described in 10.

16. Effect of protease inhibitors on the purified protease activity

16.1 To study the effect of protease inhibitors, 10mM EDTA, 100μM TLCK, 1mM PMSF, 100μM TPCK, 1μM Pepstatin A and 100μM Iodoacetic acid, was incubated with purified enzyme at 30°C for 15 minutes.

16.2 The protease activity was measured as described in 10.

17. Statistical Analysis

Data were analyzed by Analysis of Variance (ANOVA) and Turkey's HSD Test using statistical software SPSS 11.5.

CHAPTER IV
RESULTS

1. Isolation and screening of the thermophilic fungi

To isolate thermophilic fungi, the fertilized hay was suspended and serially diluted using sterile water. Then the solution was spreaded on potato dextrose agar medium and incubated at 40°C for 3 days. Eleven pure isolated fungi were obtained. The fungal colonies were transferred to casein hydrolysis medium to determined protease production by measure the clear zone after incubation for 3 days. The results showed that casein hydrolysis index of the 11 fungal strains were statistically different (Table 4). Of these, five strains, SS0109-SS0509, were selected for further assay the protease activities. The fungus strain SS0509 yielded the highest protease production (Fig. 2).

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Table 4 Casein hydrolysis index of thermophilic fungal strains producing protease

Strains	Colonial dimeter (cm)±SD	Clear zone (cm) ±SD	Casein hydrolysis index±SD
SS0109	5.98±0.08	5.98±0.08	0.00±0.00^{a}
SS0209	5.83±0.18	6.38±0.09	0.54±0.09^{bc}
SS0309	5.99±0.05	6.50±0.01	0.50±0.06^{bc}
SS0409	6.02±0.03	6.46±0.09	0.44±0.06^{bc}
SS0509	6.01±0.06	6.77±0.10	0.75±0.10^{c}
SS0609	5.76±0.33	6.24±0.05	0.48±0.30^{bc}
SS0709	5.17±0.53	5.64±0.51	0.47±0.02^{bc}
SS0809	5.97±0.14	6.33±0.09	0.35±0.05^{ab}
SS0909	5.88±0.27	6.19±0.19	0.30±0.09^{ab}
SS1009	5.94±0.11	6.28±0.05	0.34±0.16^{ab}
SS1109	5.91±0.18	6.32±0.09	0.40±0.16^{bc}

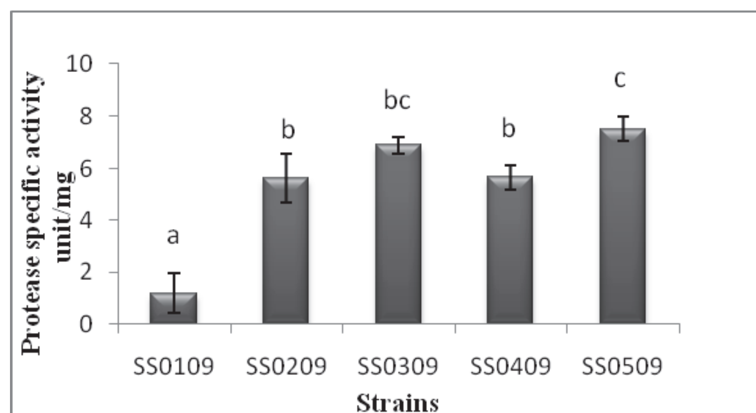

Figure 2 Protease activity of fungi isolated from fertilized hay. The values represented means of triplicates experiments, error bars indicated SD and the letters “a”, “b” and “c” revealed the statistic groups.

2. Identification of the thermophilic fungus

Identification of the thermophilic fungus strain SS0509 based on morphological and growth characteristics was performed according to Klich (2002). The results showed that this fungus is *Aspergillus fumigatus*. The description of *A. fumigatus* based on this living specimen is described below.

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Colony diameters at 7 days: CYA25 35-40 mm, MEA 43-45 mm, CYA37 49-50 mm, CY20S 30-40 mm, CZ 15-20 mm. Colony colors and textures show in (Fig 3 a-j and Table 5). On CYA25, conidia dark turquoise; mycelium white, exudates present and uncolored; reverse radials and yellowish, texture radials furrowed. On MEA, conidia colored as on CYA25; mycelium white; exudates present and uncolored; reverse radials and dull yellow, texture as on CYA25. On CYA37, conidia dark green; other characters as on CYA25. On CY20S, exudates absent, otherwise colors and texture as on CYA25. Colony characters on CZ similar to those on CY20S.

Microscopic characteristics: conidial heads predominantly columnar; stipes uncolored, smooth walled 300-350 µm x 5-7 µm (Fig. 4a) , expanding gradually in to pyriform vesicles 10-15 µm in diameter; uniseriate; phialides 5-8 µm x 2-3 µm, over upper half to two-third of the vesicle; conidia globose, rough, 2-3 µm in diameter (Fig. 4b-4c).

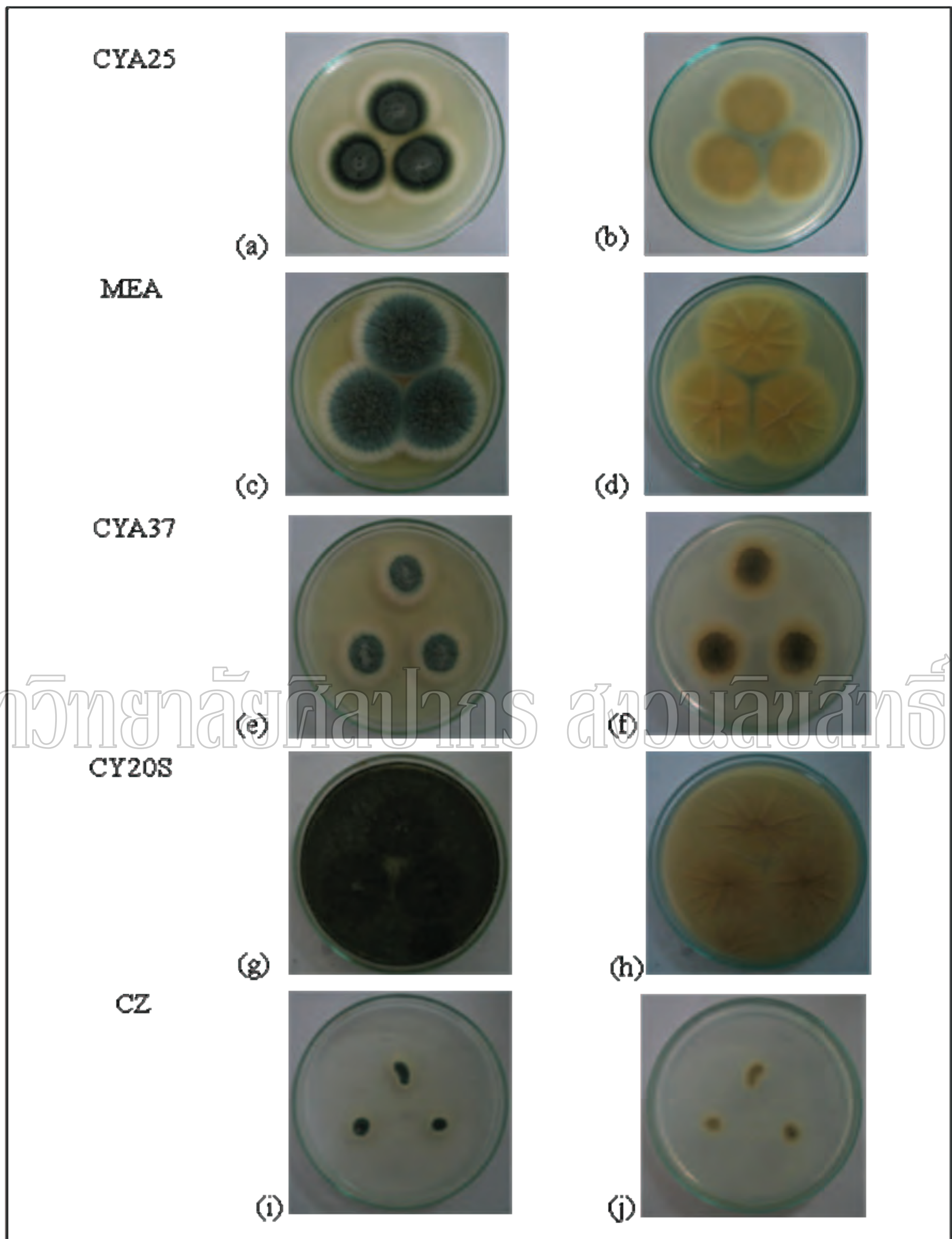

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Figure 3 Seven day old colonies and reverse on media indicated: (a) and (b) on CYA25; (c) and (d) on MEA; (e) and (f) on CYA37; (g) and (h) on CY20S; (i) and (j) on CZ.

Table 5 Growth of *Aspergillus fumigatus* in different media CYA25, MEA, CYA37, CYA20S and CZ

	CYA25	MEA	CYA37	CY20S	CZ
Diameter (mm)	35-40	43-45	49-50	30-40	15-20
Colors Conidia Mycelium	 Dark turquoise White	 Dark turquoise White	 Dark Green White	 Dark turquoise White	 Dark turquoise White
Exudate	Present uncolored	Present uncolored	Present uncolored	absent	absent
Reverse	radials yellowish	radials Dull yellow	radials	radials	radials
Soluble pigment	absent	absent	absent	absent	absent
Cleistotheia	absent	absent	absent	absent	absent
Sclerotia	absent	absent	absent	absent	absent

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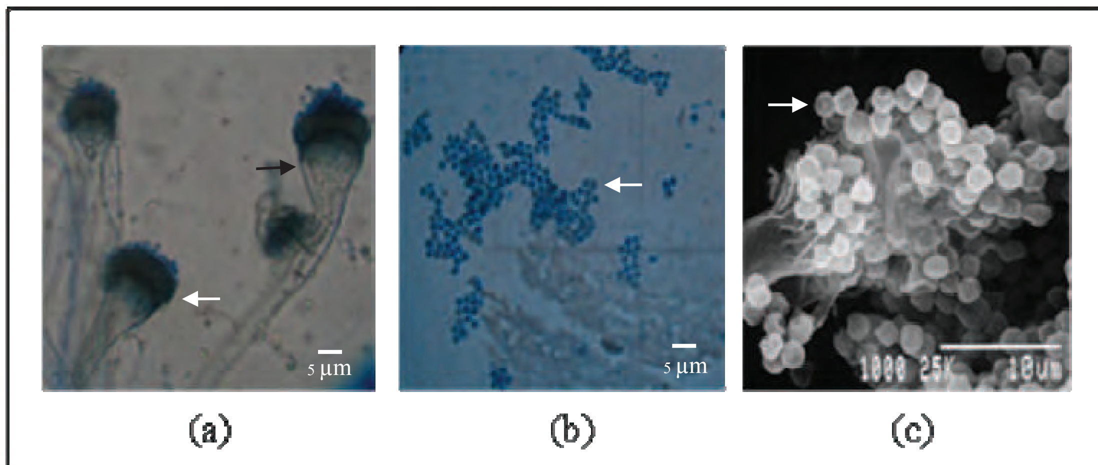

Figure 4 (a) Conidial heads predominate columnar as shown by white arrow and vesicle are shown by black arrow, (b) Conidia are shown by white arrow, (c) Scanning electron micrograph (SEM)* of conidia are shown by white arrow .

*SEM was kindly performed by Miss Maliwan Kankaew when she was a trainee at the Department of Medical Science, Ministry of Public Health of Thailand.

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To confirm the identification based on morphology and physiology, the internal transcribed spacer region was amplified. An approximate 600 bp DNA fragment was obtained from PCR (Fig. 5) and DNA sequencing revealed a size of 556 bp. By compared with GenBank published sequence (Altschul et al. 1997: 3389-3402), indicated that the sequence was ITS with 97% similarity to *Aspergillus fumigatus* isolate F8 (EF151433), *A. fumigatus* strain Z1 (HQ331439) and *A. fumigatus* strain CBMAI 1007 (FJ669802), 92% to *A. clavatus* strain UWFP 730 (AY214442) and 90% to *A. cervinus* strain SRRC 3719 (AY373845) and *A. pallidus* strain IMI 129967(AF400606). Multiple sequence alignment was performed with clustralW2 program (Fig. 6) and the phylogenetic tree was constructed by using UPGMA method (Fig. 7).

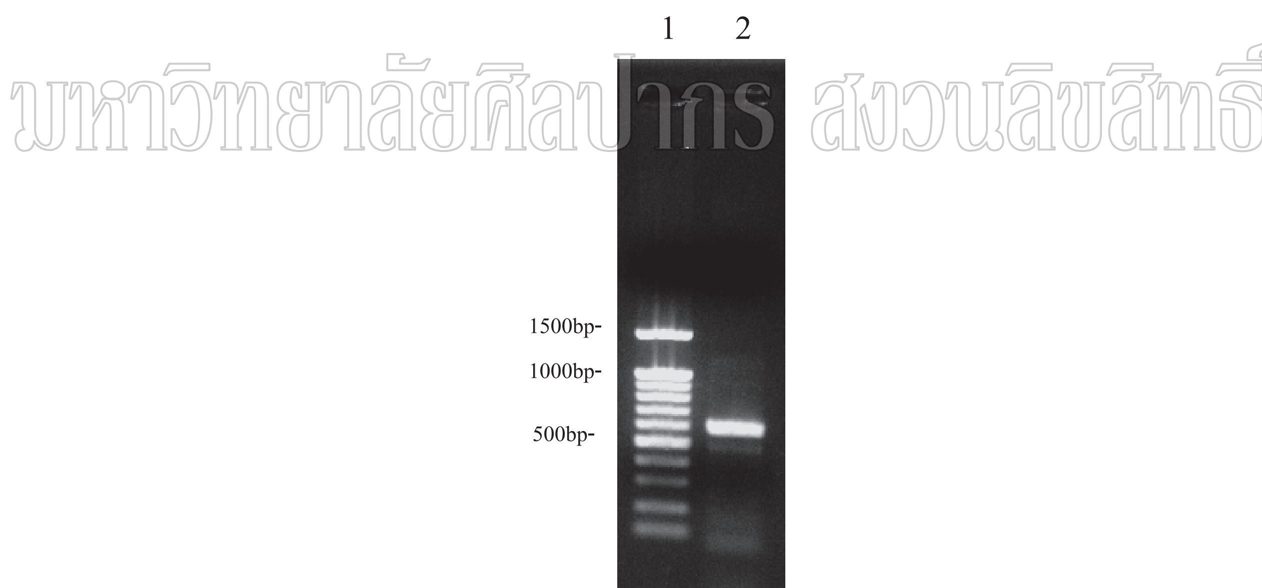

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Figure 5 PCR product of the internal transcribed spacer region from *A. fumigatus* SS0509. Lane 1, 100 bp+1500 bp molecular weight marker, lanes 2, PCR product

```
AFSS0509        CACCCGTGTCTATCGTACCTT-GGTGCTTCGGCGGGCCCACCGCTTTC-GACGGCCACCG 100
AF1             ----CGTGTCTATCGTACCTT-GTTGCTTCGGCGGGCCCGCCGCCTTC-GACGGCCGCCG 54
AF2             CACCCGTGTCTATCGTACCTT-GTTGCTTCGGCGGGCCCGCCGT-TTC-GACGGCCGCCG 87
AF3             CACCCGTGTCTATCGTACCTT-GTTGCTTCGGCGGGCCCGCCGTTTC--GACGGCCGCCG 104
ACl             CACCCGTGTTTATCGTACCTT-GTTGCTTCGGCGGGCCCGCCGTCTTCGGACGGCCGCCG 119
AP              ------------------------------GGCGGGCCCGCTGTCTTCGGACGGCCGCNG 30
ACe             CACCCGTGTCTATTGTACCTTTGTTGCTTCGGCGGGCCCGCCGTCA------GGCCGCCG 114
                                  *********  * *        ****  * *

AFSS0509        GGGAGGCCTG-GCGCAACCGGGCCCGCGCCCGCCGAAGACCCCAACATGAACGCTGTTCT 159
AF1             GGGAGGCCTT-GCGCCCCCGGGCCCGCGCCCGCCGAAGACCCCAACATGAACGCTGTTCT 113
AF2             GGGAGGCCTT-GCGCCCCCGGGCCCGCGCCCGCCGAAGACCCCAACATGAACGCTGTTCT 146
AF3             GGGAGGCCTT-GCGCCCCCGGGCCCGCGCCCGCCGAAGACCCCAACATGAACGCTGTTCT 163
ACl             GGGAGGCCTCCGCGCCCCCGGGCCCGCGCCCGCCGAAGACCACAACATGAACTCTGTTCT 179
AP              GGGAGGCTTCNGCGCCCCCGGGCCCGCGCCCGCNGAAGACCACAACATGAANTCTGTTNT 90
ACe             GGG-GGCATTCG--CCCCCGGGCCCGCGCCCGCCGAAGACCCCAACACGAACACTGTTCT 171
                *** *** *  *  *  **************** ******* ***** ***  ***** *

AFSS0509        GAAAGTATGCAGTCTGAGTTGATTATCGTAATCAGTTAAAACTTTCAACAACGGATCTCT 219
AF1             GAAAGTATGCAGTCTGAGTTGATTATCGTAATCAGTTAAAACTTTCAACAACGGATCTCT 173
AF2             GAAAGTATGCAGTCTGAGTTGATTATCGTAATCAGTTAAAACTTTCAACAACGGATCTCT 206
AF3             GAAAGTATGCAGTCTGAGTTGATTATCGTAATCAGTTAAAACTTTCAACAACGGATCTCT 223
ACl             GAAGTTTTGCAGTCTGAGTTGATTATCATAATCAGTTAAAACTTTCAACAACGGATCTCT 239
AP              GAAGTTTTGCAGTCTGAGTTGATTATCATAATCAGTTAAAACTTTCAACAACGGATCTCT 150
ACe             GAA---TTGCAGTCTGAGTTGATTATTA-AATCAATTAAAACTTTCAACAACGGATCTCT 227
                ***    *******************   ***** *************************

AFSS0509        TGGTTCCGGCATCGATGAAGAACGCAGCGAAATGCGATAAGTAATGTGAATTGCAGAATT 279
AF1             TGGTTCCGGCATCGATGAAGAACGCAGCGAAATGCGATAAGTAATGTGAATTGCAGAATT 233
AF2             TGGTTCCGGCATCGATGAAGAACGCAGCGAAATGCGATAAGTAATGTGAATTGCAGAATT 266
AF3             TGGTTCCGGCATCGATGAAGAACGCAGCGAAATGCGATAAGTAATGTGAATTGCAGAATT 283
ACl             TGGTTCCGGCATCGATGAAGAACGCAGCGAAATGCGATAACTAATGTGAATTGCAGAATT 299
AP              TGGTTCCGGCATCGATGAAGAACGCAGCGAAATGCGATAACTAATGTGAATTGCAGAATT 210
ACe             TGGTTCCGGCATCGATGAAGAACGCAGCGAAATGCGATAACTAATGTGAATTGCAGAATT 287
                **************************************** *******************

AFSS0509        CAGTGAATCATCGAGTCTTTGAACGCACATTGCGCCCCCTGGTATTCCGGGGGGCATGCC 339
AF1             CAGTGAATCATCGAGTCTTTGAACGCACATTGCGCCCCCTGGTATTCCGGGGGGCATGCC 293
AF2             CAGTGAATCATCGAGTCTTTGAACGCACATTGCGCCCCCTGGTATTCCGGGGGGCATGCC 326
AF3             CAGTGAATCATCGAGTCTTTGAACGCACATTGCGCCCCCTGGTATTCCGGGGGGCATGCC 343
ACl             CAGTGAATCATCGAGTCTTTGAACGCACATTGCGCCCCCTGGTATTCCGGGGGGCATGCC 359
AP              CAGTGAATCATCGAGTCTTTGAACGCACATTGCGCCCCCTGGTATTCCGGGGGGCATGCC 270
ACe             CAGTGAATCATCGAGTCTTTGAACGCACATTGCGCCCCCTGGTATTCCGGGGGGCATGCC 347
                ************************************************************

AFSS0509        TGTCCGAGCGTCATTGCTGCCCTCAAGCACGGCTTGTGAGTTGGACCCAAAACCCC-TC 398
AF1             TGTCCGAGCGTCATTGCTGCCCTCAAGCACGGCTTGTGTGTTGGGCCCCCGTCCCCC-TC 352
AF2             TGTCCGAGCGTCATTGCTGCCCTCAAGCACGGCTTGTGTGTTGGGCCCCCGTCCCCC-TC 385
AF3             TGTCCGAGCGTCATTGCTGCCCTCAAGCACGGCTTGTGTGTTGGGCCCCCGTCCCCC-TC 402
ACl             TGTCCGAGCGTCATTGCTGCCCTCAAGCACGGCTTGTGTGTTGGGCCCCCGTCCCCGGTT 419
AP              TGTCCGAGCGTCATTGCTGCCCTCAAGCACGGCTTGTGTGTTGGGCCCCCGTCCCCGGTT 330
ACe             TGTCCGAGCGTCATTGCTGCCCTCAAGCACGGCTTGTGTGTTGGGCCCCCGTCCCCGGTC 407
                *************************************  ****** ***    ***  *

AFSS0509        TCCCGGGGGACGGGCCCGAAAGGCAGCGGCGGCACCGCGTCCGGTCCTCGAGCGTATGGG 458
AF1             TCCCGGGGGACGGGCCCGAAAGGCAGCGGCGGCACCGCGTCCGGTCCTCGAGCGTATGGG 412
AF2             TCCCGGGGGACGGGCCCGAAAGGCAGCGGCGGCACCGCGTCCGGTCCTCGAGCGTATGGG 445
AF3             TCCCGGGGGACGGGCCCGAAAGGCAGCGGCGGCACCGCGTCCGGTCCTCGAGCGTATGGG 462
ACl             TCCC-GGGGACGGGCCCGAAAGGCAGCGGCGGCACCGCGTCCGGTCCTCGAGCGTATGGG 478
AP              TCCC-GGGGACGGGCCCGAAAGGCAGCGGCGGCACCGCGTCCGGTCCTCGAGCGTATGGG 389
ACe             TCCCCGGGGACGGGCCCGAAAGGCAGCGGCGGCACCGTGTCCGGTCCTCGAGCGTATGGG 467
                **** ******************************** **********************

AFSS0509        GCTTTGTCACCTGCTCT-GTAGGCCCGGCCGGCGCCAGCCGACACCCAACTTTA-TTTTT 516
AF1             GCTTTGTCACCTGCTCT-GTAGGCCCGGCCGGCGCCAGCCGACACCCAACTTTA-TTTTT 470
AF2             GCTTTGTCACCTGCTCT-GTAGGCCCGGCCGGCGCCAGCCGACACCCAACTTTA-TTTTT 503
AF3             GCTTTGTCACCTGCTCT-GTAGGCCCGGCCGGCGCCAGCCGACACCCAACTTTA-TTTTT 520
ACl             GCTTTGTCACCCGCTCTTGTAGGGCCGGCCGGCGCCTGTCGACACCAACCC--AATTTTT 536
AP              GCTTTGTCACCCGCTCTTGTAGGGCCGGCCGGCGCCTGTCGNCACCAACCC--A-TTTTT 446
ACe             GCTTTGTCACCCGCTCT-GTAGGTCTGGCCGGCGCCTGTCGACACCAACCCCTAATTTTT 526
                *********** ***** ***** * ********** * ** **** * *   * *****
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Figure 6 Comparison of nucleic acid sequence of ITS region of *A. fumigatus* strain SS0509 (AFSS0509) with *A. fumigatus* isolate F8 (AF1; EF151433), *A. fumigatus* strain Z1 (AF2; HQ331439), *A. fumigatus* strain CBMAI 1007 (AF3; FJ669802), *A. clavatus* strain UWFP 730 (ACl; AY214442), *A. pallidus* strain IMI 129967(AP; AF400606), *A. cervinus* strain SRRC 3719 (Ace; AY373845).

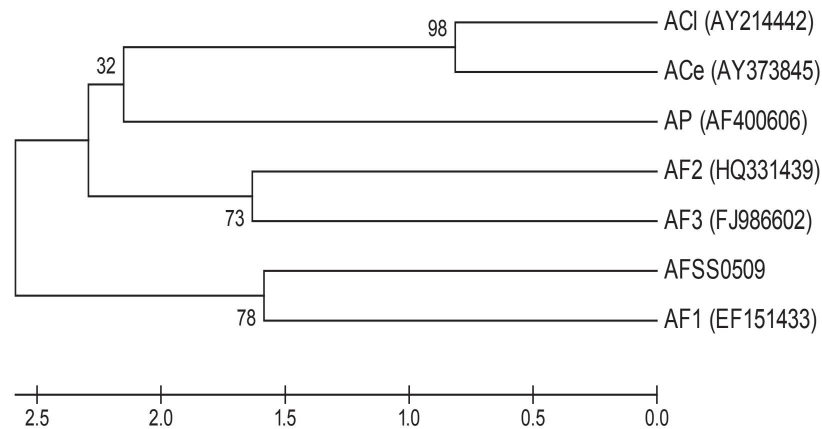

Figure 7 Phylogenetic tree based on ITS region from *Aspergillus fumigatus* SS0509 and *A. fumigatus* isolate F8 (AF1; EF151433), *A. fumigatus* strain Z1 (AF2; HQ331439), *A. fumigatus* strain CBMAI 1007 (AF3; FJ669802)*, A. clavatus* strain UWFP 730 (ACl; AY214442), *A. pallidus* strain IMI 129967 (AP; AF400606), *A. cervinus* strain SRRC 3719 (Ace; AY373845).

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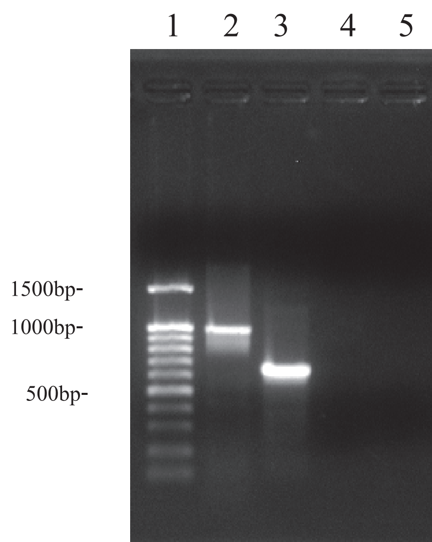
Zhao (2001) reported the used of nested PCR and specific primers to ribosomal DNA-ITS regions of *A. fumigatus*. The first PCR could amplified 996 bp products from *A. fumigatus*-relate species. While, the second PCR could generate 643 bp DNA fragment of *A. fumigatus*.

In this study, the genomic DNA of *Aspergillus fumigatus* SS0509*, A. flavus* TISTR 3366 and *A. niger* TISTR 3257 were amplified by using the specific primers reported by Zhao (2001). The PCR profile of SS0509 DNA showed the same results as observed in *Aspergillus fumigatus* (Fig. 8).



Figture 8 Nested PCR for identification *of Aspergillus fumigatus*. Lane 1, 100-bp+1500 bp molecular weight marker; lanes 2, the first PCR of *A. fumigatus* SS0509; lanes 3, the second PCR of *A. fumigatus* SS0509; lanes 4, the first PCR of *A. flavus* TISTR 3366; lanes 5, the first PCR of *A. niger* TISTR 3257.

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According to morphology, physiology, ITS sequence and specific primer for identification of *A. fumigatus*, the results revealed that the fungus strain SS0509 is *A. fumigatus.*

3. Effect of culture media on fungal growth and protease production

The effect of various media (basal media, CHM, YHM and PHM) on fungal growth and protease production were studied at pH 5.4 and 40°C for 3 days. The fungal growth rate was determined by dry weight and the results showed that the maximum growth rate was in YHM (128.92 mg/ml; Fig. 9). The protease productions in CHM, YHM or PHM were not statistically different but, the protease activity in basal medium was undetectable (Fig. 10).

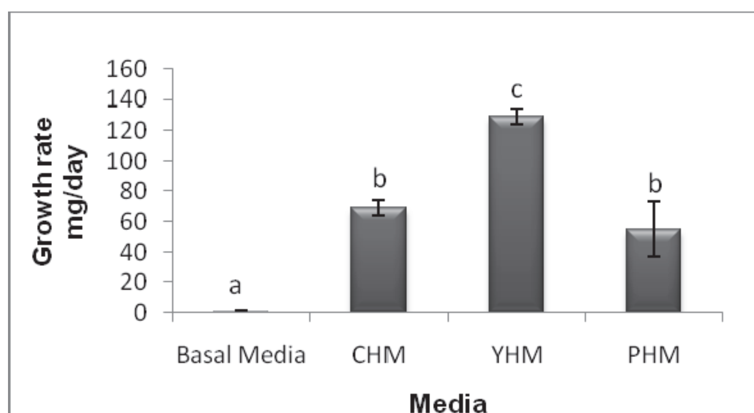

Figure 9 The effect of different culture media on growth of *Aspergillus fumigatus* SS0509. The values represented means of triplicates experiments, error bars indicated SD and the letters “a”, “b” and “c” revealed the statistic groups.

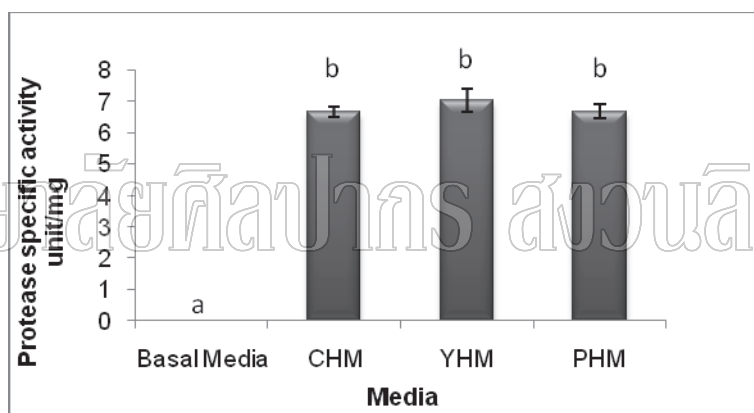

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Figure 10 The effect of different culture media on protease production of *Aspergillus fumigatus* SS0509. The values represented means of triplicates experiments, error bars indicated SD and the letters “a” and “b” revealed the statistic groups.

4. Effect of temperature on fungal growth and protease production

To study the effect of temperature on fungal growth and protease production, the fungus was cultured in YHM, pH 5.4 at temperature range from 35-50^{o}C for 3 days. The mycelium dry weight was not statistically different when culture at different temperatures (Fig. 11). The maximum production of protease (6.85 U/mg) was obtained at 40^{o}C and protease production statistically deceased when culture at temperature higher than 40^{o}C (Fig. 12).

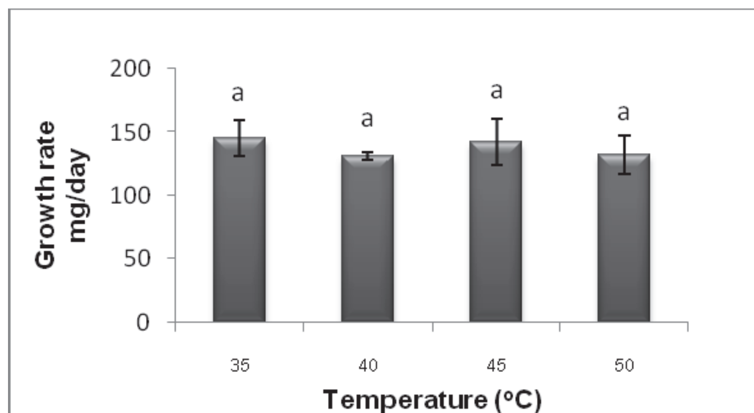

Figure 11 The effect of temperature on growth of *Aspergillus fumigatus* SS0509 in YHM pH 5.4 at 35-50°C. The values represented means of triplicates experiments, error bars indicated SD and the letter "a" indicates the statistic groups.

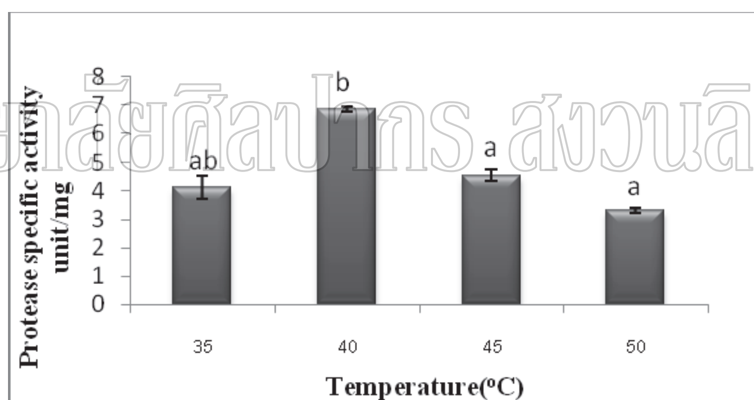

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Figure 12 The effect of temperature on protease production of *Aspergillus fumigatus* SS0509 at 35-50°C. The values represented means of triplicates experiments, error bars indicated SD and the letters "a" and "b" revealed the statistic groups.

5. Effect of pH on fungal growth and protease production

To study the effect of pH on fungal growth and protease production, the fungus was cultured in YHM, pH ranges from 5-10 at 40^{o}C for 3 days. The mycelium dry weight was not statistically different when culture at various pH (Fig. 13). Protease production of *A. fumigatus* SS0509 was undetectable when cultured at pH 5 while, was not statistically different at pH 6-10 (Fig. 14).

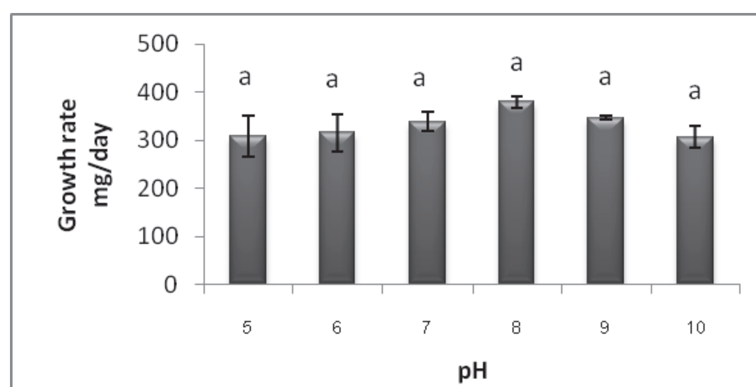

Figure 13 The effect of pH on growth of *Aspergillus fumigatus* SS0509 in YHM pH 5-10 at 40^{o}C. The values represented means of triplicates experiments, error bars indicated SD and the letter “a” revealed the statistic group.

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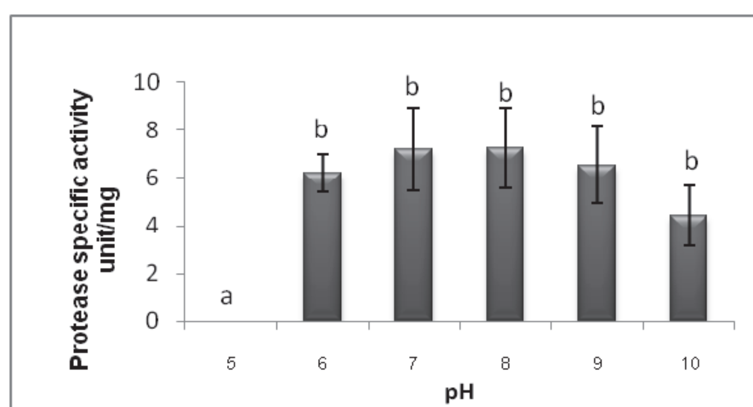

Figure 14 The effect of pH on protease production of *Aspergillus fumigatus* SS0509 at pH 5-10. The values represented means of triplicates experiments, error bars indicated SD and the letters “a” and “b” revealed the statistic groups.

6. Effect of temperature and pH on protease activity assay

The effect of temperature on the protease activity assay of the *A. fumigatus* SS0509 culture filtrate was examined when the fungus was culture in YHM pH 8 at 40^oC for 3 days. The enzyme activities were investigate at pH 7 and temperature range from 35-70^oC. The enzyme activity was high between 45-60^oC, but could not be detected at 70^oC (Fig. 15).

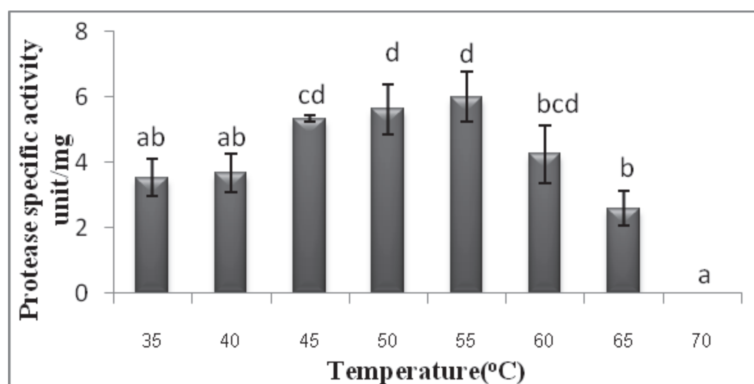

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Figure 15 The effect of temperature on the protease activity of the *Aspergillus fumigatus* SS0509 culture filtrate. The values represented means of triplicates experiments, error bars indicated SD and the letters "a", "b", "c" and "d" revealed the statistic groups.

To study the effect of pH on the protease activity assay, the enzyme activity was assayed at pH range from 5-10 at 55^oC. Protease activity of the *A. fumigatus* SS0509 culture filtrates was not statistically different at pH 6-10. No activity was detected at pH 5. (Fig. 16).

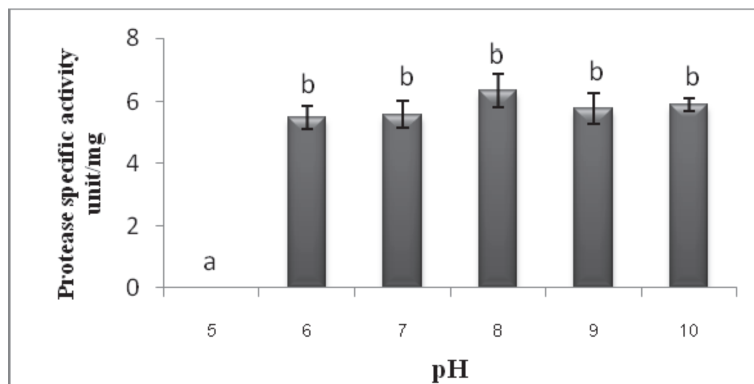

Figure 16 The effect of pH on the protease activity of the *Aspergillus fumigatus* SS0509 culture filtrate. The values represented means of triplicates experiments, error bars indicated SD and the letters “a” and “b” revealed the statistic groups.

7. Purification of protease

7.1 Separation by precipitation

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Four methods of protein precipitation were studied. Firstly, acetone precipitation showed specific activity of 8 unit/mg, 1.93-fold purification fold and 15.62% yield. Secondly, ammonium sulfate precipitation at 80% saturation yielded specific activity of 6.29 unit/mg, 1.52-fold purification fold and 13.28% yield. Thirdly, dialyzation and precipitation by polyethylene glycol (PEG) 6000 indicated the maximum specific activity by 16.33 unit/mg, purification fold - 3.96-fold and 19.14% yield. Fourthly, concentration of filtration using concentrator exhibited specific activity of 6.24 unit/mg, 1.51-fold purification fold and 16.09% yield.

Since the enzyme activity was dramatically decreased after precipitation, it is possible that some factors effecting to the enzyme activity might be lost during precipitation. To last this hypothesis, the concentrate part of filtration was mix with filtrate part, culture filtrate or YHM medium and assay the protease activity. Unfortunately, the specific activity could not be better (Table 6)

Table 6 Separation by precipitation step

Precipitation step	Total volume (ml)	Total protein (mg)	Total enzyme activity (unit)	Specific activity (unit/mg)	Purification fold	Yield (%)
Culture filtrate	3500	108.5	448	4.13	1	100
Acetone precipitation	350	8.75	70	8	1.93	15.62
Ammonium sulfate precipitation	350	9.45	59.5	6.29	1.52	13.28
Dialyzation and Precipitation by polyethylene glycol 6000	350	5.25	85.75	16.33	3.96	19.14
Filtration						
Concentrate	350	11.55	72.1	6.24	1.51	16.09
Filtrate	3150	126	252	2	0.48	56.25
Concentrate + Filtrate	350	9.1	42	4.61	1.12	9.38
Concentrate + culture filtrate	350	6.3	37.45	5.94	1.44	8.36
Concentrate + YHM medium	350	4.9	37.45	7.64	1.85	8.36

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7.2 Separation by gel filtration

0.2 mg of protein after dialyzation and precipitation by polyethylene glycol 6000 was applied to a Superdex 200 HR gel filtration (10/30) column. The enzyme was eluting by 100 mM Tris-HCl buffer (pH 8). There are two peaks found in fraction number 16-21 with a total protein of 0.15 mg. The first peak showed specific activity of 3.46 unit/mg while, the second peak revealed specific activity of 17.35 unit/mg (Fig. 17). The molecular weight of second peak of the purified protease was determined by SDS-PAGE and Native-PAGE and estimated to be 42.4 kDa and 83.6 kDa, respectively (Fig. 18). This suggested that the purified protease is homodimeric protein. In conclusion, the enzyme from *A. fumigatus* SS0509 was purified by two step procedures. By dialyzation and polyethylene glycol 6000 precipitation, the total enzyme was 85.75 units and the specific activity was 16.33 unit/mg. By Superdex 200 HR gel filtration, the total enzyme was 85.05 units and the specific activity was 17.35 unit/mg. The results of the purification in each step are summarized in Table 7.

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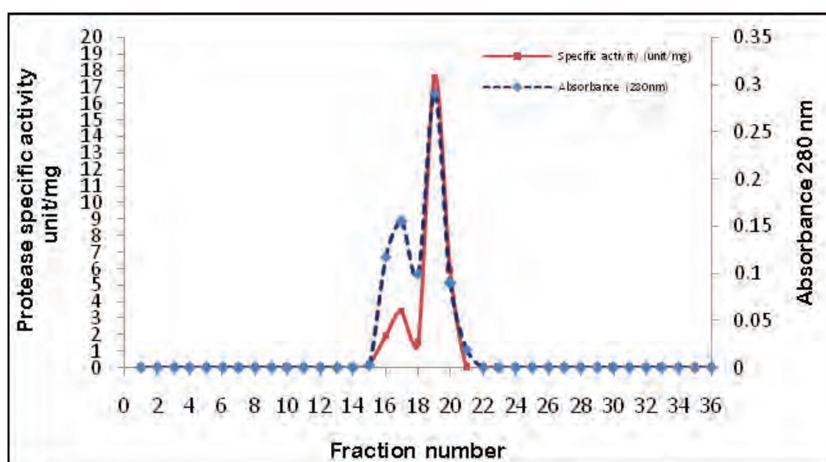

Figure 17 Elution profile of *Aspergillus fumigatus* SS0509 protein fractionated on Superdex 200 HR columns. (0.2 mg/ml, 1 ml) was applied to a column (10 x 300 mm) of Suphedex pre-equilibrated and eluted with 100 mM Tris-HCl, pH 8. One ml fractions were collected at a flow rate of 0.300 ml/minutes and assayed for absorbance at 280 nm (-----) and specific protease activity (——).

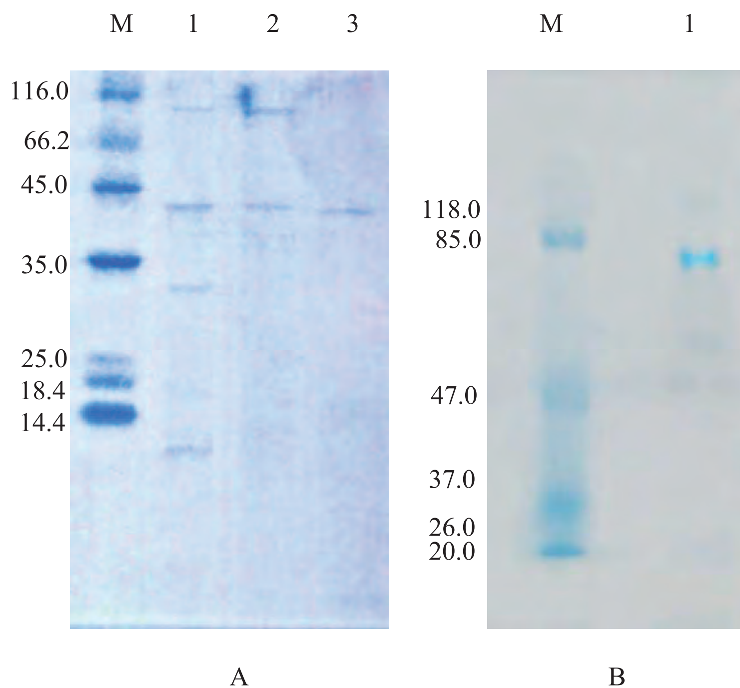

Figure 18 SDS-PAGE and Native PAGE of purified protease from *A. fumigatus* SS0509. (A) SDS-PAGE, lane M: Protein Molecular wt. Marker, lane 1: Culture filtrate, lane 2: Fraction after dialyzed and precipitation by polyethylene glycol 6000, lane 3: Fraction after Superdex 200 HR column. (B) Native-PAGE, lane M: Prestained Protein Molecular wt. Marker, lane 1: Fraction after Superdex 200 HR column.

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Table 7 The purification steps of the protease

Purification step	Total volumn (ml)	Total protein (mg)	Total enzyme activity (unit)	Specific activity (unit/mg)	Purification fold	Yield (%)
Culture filtrate	3500	108.5	448	4.13	1	100
Dialyzation and precipitation by polyethylene glycol 6000	350	5.25	85.75	16.33	3.96	19.14
Superdex 200 HR	350	4.9	85.05	17.35	4.2	18.98

8. Effect of protease inhibitors on the purified protease activity

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In order to identify the purified protease, the enzyme activity was measured in the presence of different specific inhibitors, i.e. EDTA, PMSF, TLCK, TPCK, Iodoacetic acid and Pepstatin A . The PMSF could completely inhibit the protease activity. The TPCK showed partial inhibition of approximately 4%, while, EDTA, TLCK, Iodoacetic acid and Pepstatin A had no inhibition effect on enzyme activity. This result indicated that the purified enzyme is a serine protease but not a member of neither trypsin nor chymotrypsin (Table 8).

Table 8 Effect of inhibitors on the purified protease activity

Inhibitors	Concentration	Specific activity (unit/mg)	Relative activity (%)
No inhibitor	-	14.8 ± 0.23	100
EDTA (metalloprotease inhibitor)	10 mM	14.8± 0.35	100
PMSF (serine protease inhibitor)	1 mM	0.00± 0.00	0
TLCK (trypsin-like serine protease inhibitor)	100 µM	14.8± 0.05	100
TPCK (chymotrypsin-like serine protease inhibitor)	100 µM	14.2± 0.23	95.94
Iodoacetic acid (cysteine protease inhibitor)	100 µM	14.8± 0.27	100
Pepstatin A (aspatic acid protease inhibitor)	1 µM	14.8± 0.32	100

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9. Effect of temperature, pH, storage stability, and time- dependent thermal stability on the purified protease activity from *A. fumigatus* SS0509

The effect of temperature on the purified protease activity was examined at temperature range from 35-70°C, pH 8. The enzyme activity could be detected from 35-60°C, but not higher than 60°C. The activity at 35-40°C was lower than 45-60°C. The maximum specific activity was at 55°C (8.11unit/mg; Fig. 19).

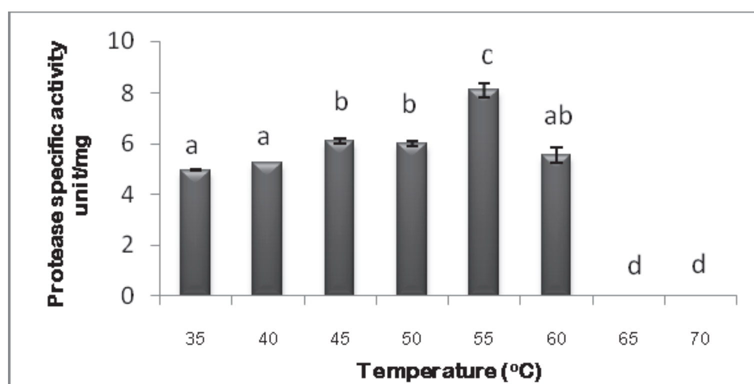

Figure 19 The effect of temperature on the purified protease activity of *Aspergillus fumigatus* SS0509. The values represented means of triplicates experiments, error bars indicated SD and the letters “a”, “b”, “c” and “d” revealed the statistic groups.

To study the effect of pH on protease activity of the purified protease, the enzyme assay was tested at 55°C pH range from 5-10. The maximum activity was found at pH 8 but, was undetectable at pH 5 (Fig. 20).

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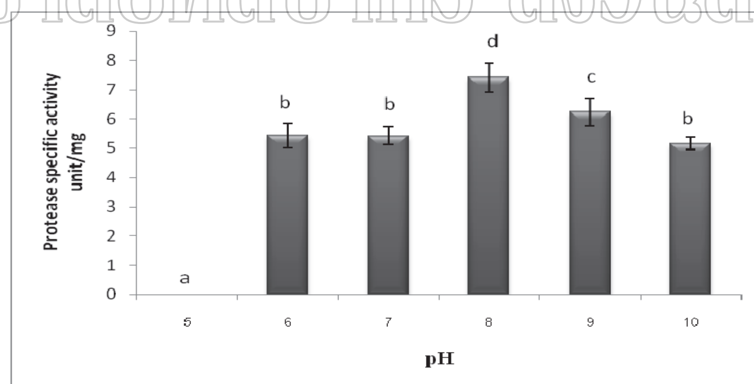
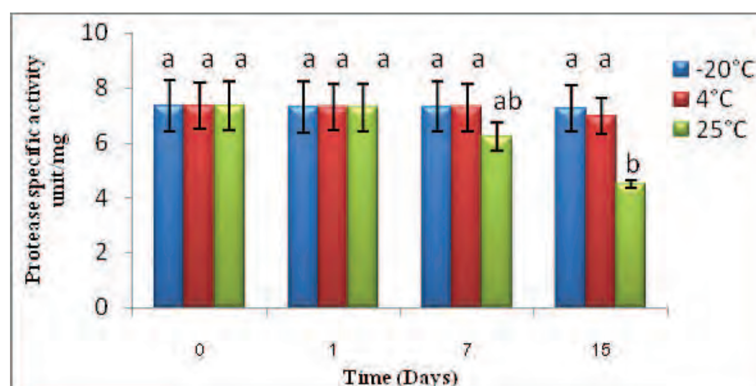


Figure 20 The effect of pH on the purified protease activity of *Aspergillus fumigatus* SS0509. The values represented means of triplicates experiments, error bars indicated SD and the letters “a”, “b”, “c” and “d” revealed the statistic groups.

The storage stability of purified protease was determined. The purified protease storage at -20, 4 and 25°C for 0, 1, 7 and 15 days. The enzyme activity was stable at -20°C and 4°C up to 15 days. However, at 25°C, the activity was decrease after 7 days (Fig. 21).



Figures 21 Storage stability of purify protease from *Aspergillus fumigatus* SS0509. The values represented means of triplicates experiments, error bars indicated SD and the letters "a" and "b" revealed the statistic groups.

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The time-dependent thermal stability of purified protease was determined. The purified protease was incubated at 55°C for 0 to 90 minutes. The results revealed that the enzyme was stable at 55°C 15 minutes up to 60 minutes but the stability decrease 17.3% after 90 minutes (Fig. 22).

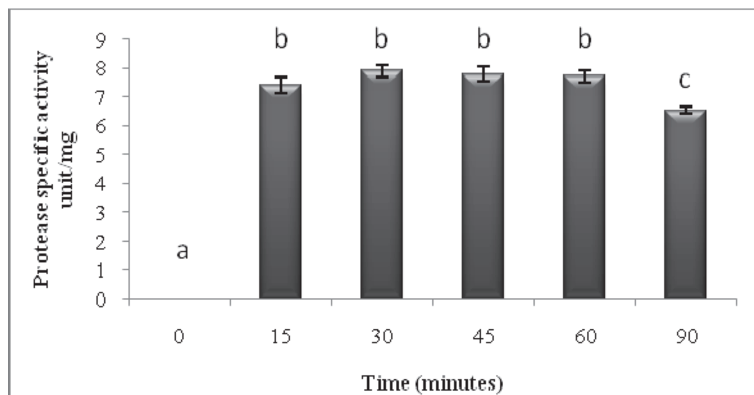

Figure 22 The time-dependent thermal stability of purified protease from *Aspergillus fumigatus* SS0509. The values represented means of triplicates experiments, error bars indicated SD and the letters “a”, “b” and “c” revealed the statistic group.

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CHAPTER V
DISCUSSION

Isolation and identification of the thermophilic fungus

The eleven strains of fungi were isolated from fertilized hay at Silpakorn University, Sanamchandra palace, Nakhorn Pathom. Of these, the fungal isolate SS0509 exhibited the largest zone of hydrolysis on CHM and the maximum protease production. Hence, this isolated was chose for further studies. To identify the fungus, the morphological observation and growth characteristic on different culture media was performed as described by Klich (2002). The fungus strain SS0509 was identified as *Aspergillus fumigatus* and this species is distinguished by rapid growing in turquoise to dark green shades, phialides curving to be roughly parallel to each other and the axis of the stipe, and small conidia borne in columns. However, colony diameter of *A. fumigatus* SS0509 on Dox Agar, Czapek Yeast Agar is smaller than the species reported by Klich (2002). The identification was confirmed by ITS region and DNA amplification by using specific primers for *A. fumigatus* (Zhao et al. 2001: 2261-2266) and strongly insisted that this fungus is *A. fumigatus*. *A. fumigatus* is a saprophytic fungus, which naturally inhabited in soil and decaying organic matter, such as fertilized hay, stored grains, wood chip piles and other accumulations of organic matter, wherein the warm, humid, and aerobic environment providing the basic physiological conditions for its development. *A. fumigatus* is a thermopile species, growth at temperature range from 20 to 50^{o}C or higher (Edwards. 1990: 1-33; Maheshwari et al. 2000: 461-488). Several thermophilic fungi were isolated and studied for the protease production, such as *Thermomyces lanuginosus* (Li et al. 1997: 18-22), *A. fumigatus*, *A. niger*, *A. clavatus*, *A. oryzae* (Monod et al. 1991: 23-28; Coral et al. 2003: 491-498; Hajji et al. 2008: 791-797; Guo et al. 2008: 301-308), *Penicillium duponti*, *P. expansum*, *P.* sp. (Hashimoto et al. 1972: 986-

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992; Dahot 1994: 100-105; Dajmel et al. 2009: 469-477), and *Rhizopus oryzae* (Kumar et al. 2005: 1701-1705).

Optimization of conditions for fungal growth and protease production

To optimize the conditions for fungal growth and protease production, the different culture media were examined. By comparative study among basal medium, CHM, YHM and PHM, the protease production in those media was not statistically different. While, the maximum growth rate of fungi was found in YHM media. The effect of yeast extract in culture medium on growth and protease production was also discovered in other fungi. Rosenberg reported that nearly half of the species of thermophilic and thermotolerant fungi tested required 0.01% of yeast extract for stimulated their growth (Maheshwari et al. 2000: 461-488). *Thermomyces langinosus* and *A. fumigatus* required 4% and 0.5% yeast extract for protease production, respectively (Li et al. 1997: 18-22; Shumi et al. 2004: 312-317).

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A. fumigatus is a facultative thermophile which can grow rang at temperature range of 20-50^{o}C (Maheshwari et al. 2000: 461-488). In this study, the temperature at 40^{o}C could maximize both growth rate and protease production. The result revealed that environmental temperatures did not affect on growth rate of organism but markedly exhibited influence on the levels of protease production. This result agrees with other fungi. The suitable temperature for growth and protease production of *A. niger* was between 35-45^{o}C (Devi et al. 2008: 1-6), and *T. langinosus* grew well at 40-55^{o}C (Li et al. 1997:18-22).

The optimum of pH on growth and protease production was examined in the pH range of 5.0-10.0. The fungal growth rate was not statistically different at all pH tested. The protease production was found at pH 6.0-10.0 but not at pH 5.0. In other *Aspergillus* species, the best protease production by *A. funiculosus, A. clavatus and A.niger* was at pH 7.0, 8.5 and 9.0, respectively (Hajji et al. 2007: 791-797; Devi et al. 2008: 1-6; Coral et al. 2003: 491-498). Interestingly, the extracellular protease productions are strictly pH regulated, for example alkaline proteases are only expressed at alkaline condition. Moreover, the pH of culture

pH also strongly affects to many enzymatic processes and transport of various components across the cell membrane (Coral et al. 2003: 491-498).

Optimization of condition for protease activity assay

The optimum temperature and pH on the protease activity assay of *A. fumigatus* SS0509 was studied using casein as a substrate. Protease activity was investigated at temperature range from 35-70°C. The enzyme activity of *A. fumigatus* SS0509 was high at a range of temperature from 45-60°C and completely inactived after 70°C. The maximum enzyme activity was found at 55°C. The optimum temperature for protease activity assay of *A. fumigatus* SS0509 was higher than other thermoplilic *Aspergillus* species. The protease activity assay from *A. niger* was 45°C, *A. clavatus* was 50°C, *A. fumigatus* CBS113.26 was 42°C, *A. parasiticus* was 40°C, *A. fumigatus* TKU003 was 40°C, and *A. nidulan* was 35°C (Devi et al. 2008: 1-6; Hajji et al. 2007: 791-797; Lercher et al. 1992: 65-69; Tunga et al. 2003: 1553-1558; Wang et al. 2005: 660-665; Charles et al. 2008: 347-352). However, the optimum temperature for protease activity assay of *Thermoascus auranticus* was 60°C (Merheb et al, 2007: 127-131).

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The optimum pH on the protease activity of *A. fumigatus* SS0509 was studied of various pH at 55°C. Protease activity is active in the pH rang 6.0-10.0, and the highest activity was fond at pH 8.0. This finding is in accordance with several reports. The pH for protease activity assay of several fungi, *A. fumigatus* (Monod et al. 1991: 23-28) *A. fumigatus* CBS113.26 (Lercher et a. 1992: 65-69) *A. parasiticus* (Tunga et al. 2003: 1553-1558) *A. fumigatus* TKU003 (Wang et a. 2005: 660-665) *A. nidulan* HA-10 (Charles et al. 2008: 347-352) *A. niger* (Devi et al. 2008: 1-6), were between pH 8.0-9.0.

Purification of protease

Four methods of protein precipitation were examined in this study. (I) Acetone precipitation was done by addition of cold acetone to culture filtrate. Acetone is widely used as a solvent. It can be completely water-miscible,

unreacting with protein, and easy to remove from a protein fraction. (II) Ammonium sulfate precipitation was performed by addition of 80% saturation ammonium sulfate to culture filtrate with constant stirred at 4^{o}C. The solubility of proteins varies according to the salt concentration of the solution. At low salt concentrations, the solubility of the protein increases and this is termed "salting in". At high salt concentration, the solubility of the protein decreases and this is called "salting out". At sufficiently high ionic strength, the proteins are almost completely precipitated from the solution. This technique is useful to quickly remove large amounts of contaminant proteins. (III) Filtration or ultrafiltration is a system that water is forced through the membrane and left the more concentrated protein solution behind. (IV) Precipitation with polymer is a method that the water is absorbed from protein solution by polymer such as Polyethylene glycol or carboxymethyl cellulose. Polyethylene glycol of molecular weight 4,000 or greater is the most effective. However, the Polyethylene glycol 6,000 or 20,000 is commonly used for protein precipitation (Scopes 1993: 1-307). In this study, the precipitation of all methods showed low specific activity and yield, suggesting that proteases are lost in precipitation processes.

Then the sample after protein precipitation was injected to Superdex 200 HR column chromatography. The principles of gel filtration are based on sized molecules. This technique use cross-linked dextrans and involved a partition of molecules between two liquid volumes, the volumes of mobile phase and the accessible volumes contained within the stationary porous bead. Molecules percolate through and around the porous beads in the column and are separated according to their mass and shape. After gel filtration the enzyme showed specific activity of 17.35 unit/mg and yield 18.98 %. The molecular weight of purified protease was determined by SDS-PAGE and Native-PAGE estimated to be 42.4 kDa and 83.6 kDa, respectively. This suggested that the purified protease is a homodimeric protein. According to my knowledge, the proteases purified from *A.fumigatus* were 32 kDa serine protease (Reichard et al. 1990: 61-74), 33 kDa alkaline protease (Monod et al. 1991: 23-28), 33 kDa serine protease (Lercher et al. 1992: 65-69), 34.4 kDa aspartic protease (Reichard et al. 1995: 61-74) and 124

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kDa serine protease (Wang et al. 2005: 660-665). This indicated that the protease purified from *A. fumigatus* in this study has never been reported.

Characterization of purified protease

In order to determine the class of protease, purified protease was measured in the present of different specific inhibitors. The PMSF could inhibit the protease activity completely. The TPCK could inhibit only about 4%, while, EDTA, TLCK, Iodoacetic acid and Pepstatin A had no inhibition effect on enzyme activity. This result indicated that the purified enzyme is a serine protease but not a member of trypsin and chymotrypsin. Probably, this protease is a subtilisin- like serine protease. To insist this hypothesis, the study of substrate specificity and amino acid sequence should be performed.

The effect of temperature on the purified protease activity was examined at temperature range from 35-70^{o}C. The enzyme activity could be detected from 35-60^{o}C, but not higher than 60^{o}C. The maximum proteolytic activity was fond at 55^{o}C and showed specific activity of 8.11unit/mg. The purified protease exhibited optimum pH at alkaline condition, pH 8. Several fungi have also been reported to produce extracellular alkaline proteases (Coral et al. 2003: 491-498).

The storage stability of purified protease was determined. The enzyme activity was stable at -20^{o}C and 4^{o}C up to 15 days. However, at 25^{o}C, the activity was decrease since 7 days. The time – dependent thermal stability of purified protease was studies and revealed that the enzyme was stable at 55^{o}C for 60 minutes but the activity decreased 17.3% after 90 minutes. The result indicated that this enzyme is an alkaline thermostable protease and stable at high temperature for such a long period. Therefore, this enzyme is probably applied in the industry.

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CHAPTER VI
CONCLUSION

1. The fungus strain SS0509 produce extracellular protease was isolated from fertilized hay from Silpakorn University, Sanamchandra palace, NakornPathom. Identification base on Morphology, physiology, ITS sequence and specific primers for identification of *A. fumigatus* indicated that this fungus is *A. fumigatus*.

2. The optimized conditions for fungal growth and protease production was on yeast hydrolysis medium (YHM), pH 8 at 40^{o}C. The optimized condition for protease production assay was pH 8 at 55^{o}C.

3. Protease was purified by dialyzation and precipitation by polyethylene glycol 6000 and Superdex 200 HR column chromatography. The enzyme was purified to 4.2 folds and the specific activity was 17.35 unit/mg proteins with 18.98% yield.

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4. The molecular weight of purified protease as determined by SDS-PAGE was estimated to be 42.4 kDa and by Native-PAGE was estimated to be 83.6 kDa. This suggested that the purified protease is a homodimeric protein.

5. The PMSF could inhibit the protease activity completely. The TPCK showed partial inhibit by 4%, while, EDTA, TLCK, Iodoacetic acid and Pepstatin A had no inhibition effect on enzyme activity. This result indicated that the purified enzyme was a serine protease but not a member of trypsin and chymotrypsin.

6. The purified enzyme showed maximum activity at 55^{o}C and pH 8. The enzyme activity was stable at -20^{o}C and 4^{o}C up to 15 days. However, at 25^{o}C, the activity was decrease since 7 days. The stability of enzyme at 55^{o}C for 60 minutes then, the activity decreased afterward.

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APPENDIXES

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Appendix A: Culture medium preparation

1. **Potato dextrose agar (PDA)**

Medium composition

Potato	200.0 g
Dextrose	20.0 g
Agar	13.0 g
Distilled water	to 1 litre

The final medium should be adjusted to pH 6.5 and sterilized by autoclaving at 121°C for 15 minute. The cooled medium should be poured into Petri dishes.

2. **Casein hydrolysis medium (CHM)**

Medium composition

KH_2PO_4	1.0 g
KCl	0.5 g
$MgSO_4.7H_2O$	0.2 g
$CaCl_2.2H_2O$	0.1 g
15% Skim milk	25.0 ml
Glucose	10.0 g
Agar	13.0 g
Distilled water	to 1 litre

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15% Skim milk is made by dissolving 3.75g Skim milk in 25 ml distilled water. This should be fully dissolved to a creamy texture prior to adding to the medium. The final medium should be adjusted to pH 5.4 and sterilized by autoclaving at 105°C for 30 minute. The cooled medium should be poured into Petri dishes.

3. **Czapek Concentrate**

Medium composition

$NaNO_3$	30.0 g
KCl	5.0 g
$MgSO_4.7H_2O$	5.0 g
$FeSO_4.7H_2O$	0.1 g
$ZnSO_4.7H_2O$	0.1 g
$CuSO_4.5H_2O$	0.05 g
Distilled water	100 ml

4. **Czapek Yeast Agar (CYA25, CYA37)**

Medium composition

K_2HPO_4	1.0 g
Czapek Concentrate	10.0 ml
Powdered Yeast Extract	5.0 g
Sucrose	30.0 g
Agar	15.0 g
Distilled water	to 1 litre

5. **Czapek Yeast Agar with 20% Sucrose (CY20S)**

Medium composition

K_2HPO_4	1.0 g
Czapek Concentrate	10.0 ml
Powdered Yeast Extract	5.0 g
Sucrose	200.0 g
Agar	15.0 g
Distilled water	to 1 litre

6. **Czapek Dox Agar (CZ)**

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Medium composition

Czapek Concentrate	10.0 ml
Sucrose	30.0 g
Agar	17.5 g
Distilled water	to 1 litre

7. **Malt Extract Agar (MEA)**

Medium composition

Powdered Malt Extract	20.0 g
Peptone	1.0 g
Glucose	20.0 g
Agar	17.5 g
Distilled water	to 1 litre

These media should be sterilized by autoclaving at 121^{o}C for 15 minute. The cooled twenty- five ml medium should be poured into Petri dishes. The volume is important since media depth or head space difference can lead to morphological changes.

Appendix B: Reagent

1. **Bradford Dye Stock**

100 mg Coomassie Blue G
50 ml Methanol
100 ml 85% Phospholic acid
H_2O 50 ml

The solution should be dark red and have a pH of -0.01 and stored at 4°C

2. **0.1M Tris –HCl, pH8**

12.11 g Tris (FW121.1)
Add 800 ml ddH_2O
Adjust to pH 8.0 with HCl
ddH_2O to 1000ml

The final solution should be adjusted to pH 8.0 and sterilized by autoclaving at 121°C for 15 minute. The cool solution should be filtrate and degas.

3. **5%Tricholo acetic acid (TCA)**

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5.0 g Tricholo acetic acid
ddH_2O to 100ml

4. **4X Running Gel Buffer (1.5M Tris-HCl, pH8.8)**

36.3 g Tris (FW121.1)
Add 150 ml ddH_2O
Adjust to pH8.8 with HCl
ddH_2O to 200ml
Store up to 3 months at 4°C in the dark.

5. **4X Stacking Gel Buffer (0.5M Tris-HCl, pH6.8)**

3.0 g Tris (FW121.1)
Add 40 ml ddH_2O
Adjust to pH6.8 with HCl
ddH_2O to 50ml
Store up to 3 months at 4°C in the dark.

6. **10% SDS**

10 g SDS
ddH_2O to 50ml
Store up to 6 months at room temperature.

7. **10% Ammonium Persulfate(Initiator)**
   0.1 g Ammonium perulfate
   0.2 ddH_2O to 1.0ml
   Use fresh; do not store.

8. **2X Treatment Buffer(0.125M Tris-HCl, 4% SDS, 20%v/v Glycerol, 0.2M DTT, 0.02% Bromophenol Blue, pH6.8) for SDS PAGE**
   2.5 ml 4X Stacking Gel Buffer
   4.0 ml 10% SDS
   2.0 ml glycerol
   2.0 ml bromophenol blue
   0.31 g dithiothretiol (DTT; FW 154.2)
   ddH_2O to 10.0 ml
   Store 0.5 ml aliquots at -20^{o}C for up to 6 months.

9. **Tank Buffer(0.025M Tris, 0.192M Glycine, 0.1%SDS, pH8.3) for SDS PAGE**
   30.28 g Tris (FW 121.1)
   144.13 g glycine
   10 g SDS
   dd$H_2$0 to 1L

   This solution can be made up directly in large reagent bottle because it is not necessary to check the pH. Store at room temperature for up to 1month.

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10. **2X Treatment Buffer(0.125M Tris-HCl, 20%v/v Glycerol, 0.02% Bromophenol Blue, pH6.8) for Native PAGE**
    2.5 ml 4X Stacking Gel Buffer
    2.0 ml glycerol
    2.0 ml bromophenol blue
    ddH_2O to 10.0 ml
    Store 0.5 ml aliquots at -20^{o}C for up to 6 months.

11. **11.Tank Buffer(0.025M Tris, 0.192M Glycine, pH8.3) for Native PAGE**
    30.28 g Tris (FW 121.1)
    144.13 g glycine
    dd$H_2$0 to 1L
    This solution can be made up directly in large reagent bottle because it is not necessary to check the pH. Store at room temperature for up to 1month.

12. **12.Staining Solution**

    Methanol CP 45 ml
    Acetic Acid CP10 ml
    H_20 45 ml
    Coomasie Brilliant Blue R 0.2 g
    Store up at room temperature in the dark.

13. **Destaining Solution**

    Methanol CP 25 ml
    Acetic Acid CP10 ml
    H_20 65 ml
    Store up at room temperature in the dark.

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Appendix C: Standard curve of Bradford Protease Assay

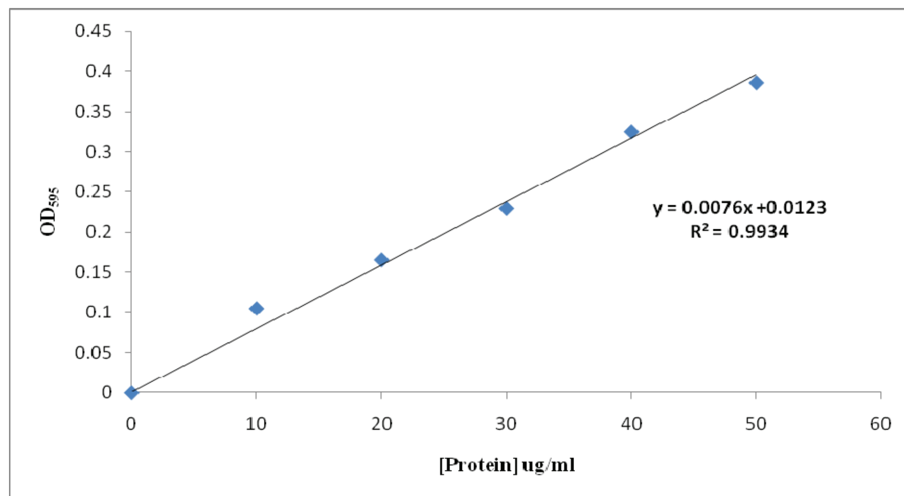

Figure 23 Bradford Protein Assay (Micro assay)

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Appendix D: Plot of relative mobility on Native and polyacrylamide sodium dodecyl sulfate gel electrophoresis

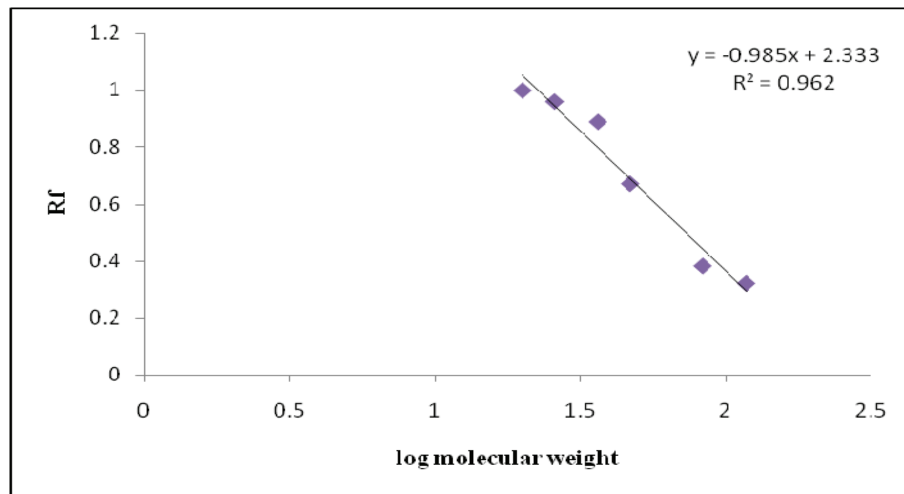

Figure 24 Relative mobility of protein marker and sample from Native polyacrylamide gel electrophoresis

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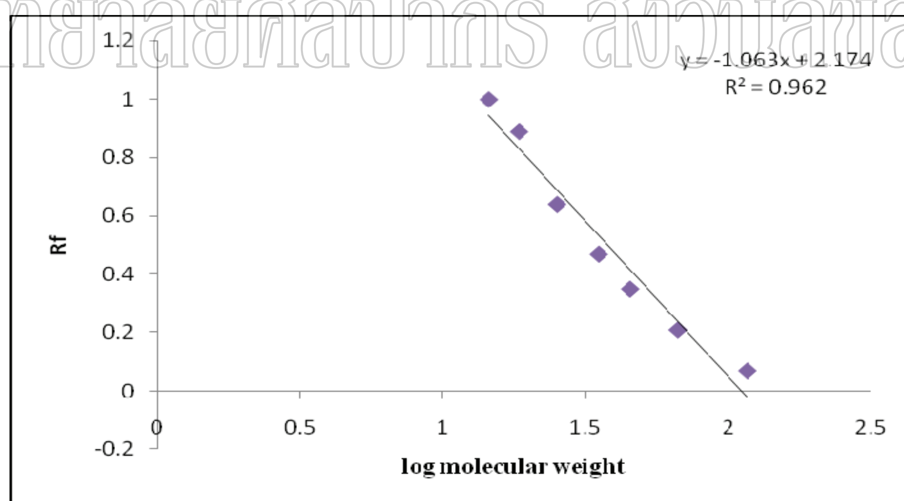

Figure 25 Relative mobility of protein marker and sample from polyacrylamide sodium dodecyl sulfate gel electrophoresis.

Appendix E: Protein concentration and protease activity

Table 9 Protease concentration of fungi isolated from fertilized hay

The fungi strains SS0109-SS0509 were cultured in 3-separeted flasks on CHM media, pH 7 at 40^{o}C for 3 days. The culture filtrate from each flask was separated by filtration. In each flask, the protein concentration was measured twice as tube # 1 and tube # 2 and then, the means was calculated and protein concentration was estimated from standard curve of Bradford Protein Assay. The total protein was calculated using followed equation. Total protein = Protein concentration (mg/ml) x Volume (ml).

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Stains	Replication	Volume (ml)	Protein concentration							
				OD595			Protein conc.			
			Dilution	tube # 1	tube # 2	Mean	Prot. conc. (mg/ml)	Total prot. (mg)	Mean of Total prot. (mg)	SD of Total prot.
									Mean	SD
SS0109	1	50		0.045	0.034	0.039	0.004	0.179	0.207	0.034
	2	50		0.056	0.043	0.049	0.005	0.245		
	3	50		0.039	0.046	0.042	0.004	0.199		
SS0209	1	50		0.128	0.138	0.133	0.016	0.794	0.808	0.127
	2	50		0.150	0.161	0.155	0.019	0.942		
	3	50		0.115	0.119	0.117	0.014	0.689		

Table 9 Protease concentration of fungi isolated from fertilized hay (continuous)

The fungi strains SS0109-SS0509 were cultured in 3-separeted flasks on CHM media, pH 7 at 40^{o}C for 3 days. The culture filtrate from each flask was separated by filtration. In each flask, the protein concentration was measured twice as tube # 1 and tube# 2 and then, the means was calculated and protein concentration was estimated from standard curve of Bradford Protein Assay. The total protein was calculated using followed equation. Total protein = Protein concentration (mg/ml) x Volume (ml).

Stains	Replication	Volume (ml)	Protein concentration							
			Dilution	OD595			Protein conc.			
				tube # 1	tube # 2	Mean	Prot. conc. (mg/ml)	Total prot. (mg)	Mean of Total prot. (mg)	SD of Total prot.
									Mean	SD
SS0309	1	50		0.124	0.128	0.126	0.015	0.748	0.741	0.046
	2	50		0.129	0.134	0.1315	0.016	0.784		
	3	50		0.114	0.121	0.1175	0.014	0.692		
SS0409	1	50		0.146	0.139	0.1425	0.017	0.857	0.850	0.063
	2	50		0.153	0.148	0.1505	0.018	0.909		
	3	50		0.135	0.128	0.1315	0.016	0.784		
SS0509	1	50		0.124	0.119	0.1215	0.014	0.718	0.739	0.021
	2	50		0.120	0.129	0.1245	0.015	0.738		
	3	50		0.129	0.127	0.128	0.015	0.761		

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Table 10 Protease activity of fungi isolated from fertilized hay

The fungi strains SS0109-SS0509 were cultured in 3-separeted flasks on CHM media, pH 7 at 40^{o}C for 3 days. The culture filtrate from each flask was separated by filtration. In each flask, the protease activity was measured twice as tube # 1 and tube# 2 and then, the means was calculated and the amount of amino acid was estimated from standard curve of Bradford Protein Assay. Enzyme unit, total enzyme and specific activity were calculated using followed equation. Enzyme unit = 3.5(ml) x X (mg) / 0.5(ml). Total enzyme = Enzyme unit x Volume (ml) and Specific activity = Total enzyme/Total protein.

Protease activity											
Strains	OD595			Amount of amino acid (mg/ml)	Enzyme unit (unit)	Total enzyme (unit)	Mean of Total enzyme (unit)	SD of Total enzyme	Specific activity (unit/mg)	Mean of Specific activity (unit/mg)	SD of Specific activity (unit/mg)
	tube # 1	tube # 2	Mean								
							Mean of	SD		Mean	SD
SS0109	0.010	0.017	0.013	0.000	$1.11x10^{-3}$	$5.53 x10^{-2}$	$2.63 x10^{-1}$	0.188	$3.09x10^{-1}$	1.20	0.775
	0.019	0.024	0.021	0.001	$8.47 x10^{-3}$	$4.24 x10^{-1}$			1.73		
	0.020	0.018	0.019	0.001	$6.17 x10^{-3}$	$3.09 x10^{-1}$			1.55		
SS0209	0.109	0.111	0.110	0.013	$9.00 x10^{-2}$	4.50	4.48	0.048	5.67	5.64	0.932
	0.108	0.109	0.108	0.013	$8.86 x10^{-2}$	4.43			4.70		
	0.110	0.111	0.110	0.013	$9.04 x10^{-2}$	4.52			6.57		

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Table 10 Protease activity of fungi isolated from fertilized hay (continuous)

The fungi strains SS0109-SS0509 were cultured in 3-separeted flasks on CHM media, pH 7 at 40^{o}C for 3 days. The culture filtrate from each flask was separated by filtration. In each flask, the protease activity was measured twice as tube # 1 and tube# 2 and then, the means was calculated and the amount of amino acid was estimated from standard curve of Bradford Protein Assay. Enzyme unit, total enzyme and specific activity were calculated using followed equation. Enzyme unit = 3.5(ml) x X (mg) / 0.5(ml). Total enzyme = Enzyme unit x Volume (ml) and Specific activity = Total enzyme/Total protein.

Protease activity											
Strains	OD595			Amount of amino acid (mg/ml)	Enzyme unit (unit)	Total enzyme (unit)	Mean of Total enzyme (unit)	SD of Total enzyme	Specific activity (unit/mg)	Mean of Specific activity (unit/mg)	SD of Specific activity (unit/mg)
	tube # 1	tube # 2	Mean								
SS0309	0.122	0.124	0.123	0.015	1.02×10^{-1}	5.10	5.10	0.069	6.82	6.89	0.345
	0.129	0.120	0.124	0.015	1.03×10^{-1}	5.17			6.59		
	0.119	0.124	0.121	0.014	1.01×10^{-1}	5.03			7.27		
SS0409	0.111	0.114	0.112	0.013	9.23×10^{-2}	4.61	4.79	0.153	5.39	5.66	0.476
	0.117	0.120	0.118	0.014	9.78×10^{-2}	4.89			5.38		
	0.120	0.116	0.118	0.014	9.74×10^{-2}	4.87			6.21		
SS0509	0.140	0.136	0.138	0.017	1.16×10^{-1}	5.79	5.56	0.211	8.06	7.53	0.460
	0.13	0.128	0.129	0.015	1.07×10^{-1}	5.37			7.28		
	0.135	0.129	0.132	0.016	1.10×10^{-1}	5.51			7.24		

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Table 11 The effect of different culture media on protease production of *Aspergillus fumigatus* SS0509

The fungus strain SS0509 was cultured in 3-separeted flasks on Basal medium, CHM, YHM and PHM, pH 7 at 40°C for 3 days. The culture filtrate from each flask was separated by filtration. In each flask, the protein concentration was measured twice as tube # 1 and tube# 2 and then, the means was calculated and protein concentration was estimated from standard curve of Bradford Protein Assay. The total protein was calculated using followed equation. Total protein = Protein concentration (mg/ml) x Volume (ml).

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Media	Replication	Volume (ml)	Protein concentration							
			Dilution	OD595			Protein conc.			
				tube # 1	tube # 2	Mean	Prot. conc. (mg/ml)	Total prot. (mg)	Mean of Total prot. (mg)	SD of Total prot.
									Mean	SD
Basal medium	1	50		0.054	0.055	0.054	0.006	0.278	0.221	0.069
	2	50		0.032	0.036	0.034	0.003	0.143		
	3	50		0.045	0.053	0.049	0.005	0.241		
CHM	1	50		0.11	0.108	0.109	0.013	0.636	0.642	0.009
	2	50		0.111	0.112	0.111	0.013	0.653		
	3	50		0.112	0.106	0.109	0.013	0.636		

Table 11 The effect of different culture media on protease production of *Aspergillus fumigatus* SS0509 (continuous)

The fungus strain SS0509 was cultured in 3-separeted flasks on Basal medium, CHM, YHM and PHM, pH 7 at 40°C for 3 days. The culture filtrate from each flask was separated by filtration. In each flask, the protein concentration was measured twice as tube # 1 and tube# 2 and then, the means was calculated and protein concentration was estimated from standard curve of Bradford Protein Assay. The total protein was calculated using followed equation. Total protein = Protein concentration (mg/ml) x Volume (ml).

Media	Replication	Volume (ml)	Protein concentration							
			Dilution	OD595			Protein conc.			
				tube # 1	tube # 2	Mean	Prot. conc. (mg/ml)	Total prot. (mg)	Mean of Total prot. (mg)	SD of Total prot.
									Mean	SD
YHM	1	50		0.179	0.178	0.178	0.022	1.093	1.005	0.077
	2	50		0.158	0.160	0.159	0.019	0.965		
	3	50		0.158	0.157	0.157	0.019	0.955		
PHM	1	50		0.175	0.172	0.173	0.021	1.061	1.053	0.019
	2	50		0.175	0.174	0.174	0.021	1.067		
	3	50		0.168	0.170	0.169	0.021	1.031		

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Table 12 The effect of different culture media on protease activity of *Aspergillus fumigatus* SS0509

The fungus strain SS0509 was cultured in 3-separeted flasks on Basal medium, CHM, YHM and PHM, pH 7 at 40^{o}C for 3 days. The culture filtrate from each flask was separated by filtration. In each flask, the protease activity was measured twice as tube # 1 and tube# 2 and then, the means was calculated and the amount of amino acid was estimated from standard curve of Bradford Protease Assay. Enzyme unit, total enzyme and specific activity were calculated using followed equation. Enzyme unit = 3.5(ml) x X (mg) / 0.5(ml). Total enzyme = Enzyme unit x Volume (ml) and Specific activity = Total enzyme/Total protein.

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Protease activity											
Media	OD595			Amount of amino acid (mg/ml)	Enzyme unit (umole/min/ml)	Total enzyme (unit)	Mean of Total enzyme (unit)	SD of Total enzyme	Specific activity (unit/m)	Mean of Specific activity (unit/mg)	SD of Specific activity (unit/mg)
	tube # 1	tube # 2	Mean								
							Mean	SD		Mean	SD
Basal medium	0.001	0.001	0.001	0.000	0.00	0.00	0.00	0	0.00	0.00	0
	0.009	0.007	0.008	0.000	0.00	0.00			0.00		
	0.008	0.006	0.007	0.000	0.00	0.00			0.00		
CHM	0.108	0.105	0.106	0.012	8.68 $x10^{-2}$	4.34	4.29	0.046	6.82	6.69	0.163
	0.104	0.105	0.104	0.012	8.49 $x10^{-2}$	4.25			6.51		
	0.106	0.105	0.105	0.012	8.58 $x10^{-2}$	4.29			6.75		

Table 12 The effect of different culture media on protease activity of *Aspergillus fumigatus* SS0509 (continuous)

The fungus strain SS0509 was cultured in 3-separeted flasks on Basal medium, CHM, YHM and PHM, pH 7 at 40^{o}C for 3 days. The culture filtrate from each flask was separated by filtration. In each flask, the protease activity was measured twice as tube # 1 and tube# 2 and then, the means was calculated and the amount of amino acid was estimated from standard curve of Bradford Protein Assay. Enzyme unit, total enzyme and specific activity were calculated using followed equation. Enzyme unit = 3.5(ml) x X (mg) / 0.5(ml). Total enzyme = Enzyme unit x Volume (ml) and Specific activity = Total enzyme/Total protein.

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Protease activity											
Media	OD595			Amount of amino acid	Enzyme unit	Total enzyme	Mean of	SD of	Specific activity	Mean of	SD of
	tube # 1	tube # 2	Mean	(mg/ml)	(umole/min/ml)	(unit)	Total enzyme (unit)	Total enzyme	(unit/mg)	Specific activity (unit/mg)	Specific activity (unit/mg)
							Mean	SD		Mean	SD
YHM	0.177	0.175	0.176	0.022	1.51×10^{-1}	7.54	7.060	0.552	6.89	7.04	0.368
	0.167	0.170	0.1685	0.021	1.44×10^{-1}	7.19			7.45		
	0.15	0.155	0.1525	0.018	1.29×10^{-1}	6.46			6.76		
PHM	0.173	0.17	0.1715	0.021	1.47×10^{-1}	7.33	7.03	0.339	6.91	6.68	0.225
	0.167	0.166	0.1665	0.020	1.42×10^{-1}	7.10			6.65		
	0.154	0.16	0.157	0.019	1.33×10^{-1}	6.66			6.46		

Table 13 The effect of different pH on protease production of *Aspergillus fumigatus* SS0509

The fungus strain SS0509 was cultured in 3-separeted flasks on YHM, pH 5-10 at 40^{o}C for 3 days. The culture filtrate from each flask was separated by filtration. In each flask, the protein concentration was measured twice as tube # 1 and tube# 2 and then, the means was calculated and protein concentration was estimated from standard curve of Bradford Protein Assay. The total protein was calculated using followed equation. Total protein = Protein concentration (mg/ml) x Volume (ml).

pH	Replication	Volume (ml)	Protein concentration							
			Dilution	OD595			Protein conc.			
				tube # 1	tube # 2	Mean	Prot. conc. (mg/ml)	Total prot. (mg)	Mean of Total prot. (mg)	SD of Total prot.
									Mean	SD
5	1	40		0.240	0.236	0.238	0.031	1.246	1.094	0.188
	2	40		0.222	0.218	0.220	0.029	1.151		
	3	40		0.170	0.168	0.169	0.022	0.883		
6	1	40		0.240	0.236	0.238	0.031	1.246	1.094	0.188
	2	40		0.222	0.218	0.220	0.029	1.151		
	3	40		0.170	0.168	0.169	0.022	0.883		
7	1	40		0.240	0.236	0.238	0.031	1.246	1.094	0.188
	2	40		0.222	0.218	0.220	0.029	1.151		
	3	40		0.170	0.168	0.169	0.022	0.883		

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Table 13 The effect of different pH on protease production of *Aspergillus fumigatus* SS0509 (continuous)

The fungus strain SS0509 was cultured in 3-separeted flasks on YHM, pH 5-10 at 40^{o}C for 3 days. The culture filtrate from each flask was separated by filtration. In each flask, the protein concentration was measured twice as tube # 1 and tube# 2 and then, the means was calculated and protein concentration was estimated from standard curve of Bradford Protein Assay. The total protein was calculated using followed equation. Total protein = Protein concentration (mg/ml) x Volume (ml).

pH	Replication	Volume (ml)	Protein concentration							
			Dilution	OD595			Protein conc.			
				tube # 1	tube # 2	Mean	Prot. conc. (mg/ml)	Total prot. (mg)	Mean of Total prot. (mg)	SD of Total prot.
									Mean	SD
8	1	40		0.240	0.236	0.238	0.031	1.246	1.094	0.188
	2	40		0.222	0.218	0.220	0.029	1.151		
	3	40		0.170	0.168	0.169	0.022	0.883		
9	1	40		0.240	0.236	0.238	0.031	1.246	1.094	0.188
	2	40		0.222	0.218	0.220	0.029	1.151		
	3	40		0.170	0.168	0.169	0.022	0.883		
10	1	40		0.240	0.236	0.238	0.031	1.246	1.094	0.188
	2	40		0.222	0.218	0.220	0.029	1.151		
	3	40		0.170	0.168	0.169	0.022	0.883		

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Table 14 The effect of different pH on protease activity of *Aspergillus fumigatus* SS0509

The fungus strain SS0509 was cultured in 3-separeted flasks on YHM, pH 5-10 at 40°C for 3 days. The culture filtrate from each flask was separated by filtration. In each flask, the protease activity was measured twice as tube # 1 and tube# 2 and then, the means was calculated and the amount of amino acid was estimated from standard curve of Bradford Protein Assay. Enzyme unit, total enzyme and specific activity were calculated using followed equation. Enzyme unit = 3.5(ml) x X (mg) / 0.5(ml). Total enzyme = Enzyme unit x Volume (ml) and Specific activity = Total enzyme/Total protein.

Protease activity											
pH	OD595			Amount of amino acid (mg/ml)	Enzyme unit (umole/min/ml)	Total enzyme (unit)	Mean of Total enzyme (unit)	SD of Total enzyme	Specific activity (unit/mg)	Mean of Specific activity (unit/mg)	SD of Specific activity (unit/mg)
	tube # 1	tube # 2	Mean								
							Mean	SD		Mean	SD
5	0.009	0.01	0.009	0.000	0.00	0.00	0.00	0	0.00	0.00	0
	0.06	0.048	0.054	0.000	0.00	0.00			0.00		
	0.058	0.05	0.054	0.000	0.00	0.00			0.00		
6	0.238	0.266	0.252	0.032	2.21×10^{-1}	8.83	6.87	1.971	7.09	6.20	0.796
	0.201	0.198	0.199	0.025	1.72×10^{-1}	6.90			5.99		
	0.14	0.15	0.145	0.017	1.22×10^{-1}	4.89			5.54		
7	0.237	0.23	0.233	0.029	2.04×10^{-1}	8.15	7.69	0.750	6.54	7.21	1.721
	0.185	0.210	0.197	0.024	1.71×10^{-1}	6.82			5.93		
	0.230	0.234	0.232	0.029	2.02×10^{-1}	8.09			9.17		

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Table 14 The effect of different pH on protease activity of *Aspergillus fumigatus* (continuous)

The fungus strain SS0509 was cultured in 3-separeted flasks on YHM, pH 5-10 at 40^{o}C for 3 days. The culture filtrate from each flask was separated by filtration. In each flask, the protease activity was measured twice as tube # 1 and tube# 2 and then, the means was calculated and the amount of amino acid was estimated from standard curve of Bradford Protein Assay. Enzyme unit, total enzyme and specific activity were calculated using followed equation. Enzyme unit = 3.5(ml) x X (mg) / 0.5(ml). Total enzyme = Enzyme unit x Volume (ml) and Specific activity = Total enzyme/Total protein.

Protease activity											
pH	OD595			Amount of amino acid (mg/ml)	Enzyme unit (umole/min/ml)	Total enzyme (unit)	Mean of Total enzyme (unit)	SD of Total enzyme	Specific activity (unit/mg)	Mean of Specific activity (unit/mg)	SD of Specific activity (unit/mg)
	tube # 1	tube # 2	Mean								
							Mean	SD		Mean	SD
8	0.239	0.234	0.236	0.030	2.07×10^{-1}	8.26	7.74	0.754	6.63	7.26	1.68
	0.20	0.198	0.199	0.025	1.72×10^{-1}	6.88			5.97		
	0.229	0.235	0.232	0.029	2.02×10^{-1}	8.09			9.17		
9	0.223	0.225	0.224	0.028	1.95×10^{-1}	7.80	6.98	1.014	6.26	6.50	1.613
	0.170	0.172	0.171	0.021	1.46×10^{-1}	5.85			5.08		
	0.219	0.202	0.210	0.026	1.83×10^{-1}	7.30			8.27		
10	0.203	0.216	0.209	0.026	1.82×10^{-1}	7.27	4.91	2.045	5.83	4.42	1.258
	0.120	0.118	0.119	0.014	9.83×10^{-2}	3.93			3.41		
	0.109	0.108	0.108	0.013	8.86×10^{-2}	3.54			4.01		

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Table 15 The effect of different temperature on protease production of *Aspergillus fumigatus* SS0509

The fungus strain SS0509 was cultured in 3-separeted flasks on YHM, pH 8 at 35-50°C for 3 days. The culture filtrate from each flask was separated by filtration. In each flask, the protein concentration was measured twice as tube # 1 and tube# 2 and then, the means was calculated and protein concentration was estimated from standard curve of Bradford Protein Assay. The total protein was calculated using followed equation. Total protein = Protein concentration (mg/ml) x Volume (ml).

Temp	Replication	Volume (ml)	Protein concentration							
			Dilution	OD595			Protein conc.			
				tube # 1	tube # 2	Mean	Prot. conc. (mg/ml)	Total prot. (mg)	Mean of Total prot. (mg)	SD of Total prot.
35	1	50		0.187	0.184	0.1855	0.228	11.395	10.518	0.924
	2	50		0.174	0.173	0.1735	0.212	10.605		
	3	50		0.153	0.162	0.1575	0.191	9.553		
40	1	50		0.185	0.19	0.1875	0.231	11.526	10.759	0.828
	2	50		0.160	0.165	0.1625	0.198	9.882		
	3	50		0.175	0.18	0.1775	0.217	10.868		
45	1	50		0.234	0.231	0.2325	0.290	14.487	14.783	0.600
	2	50		0.25	0.245	0.2475	0.309	15.474		
	3	50		0.234	0.228	0.231	0.288	14.388		
50	1	50		0.248	0.247	0.2475	0.309	15.474	15.265	0.479
	2	50		0.232	0.240	0.236	0.294	14.717		
	3	50		0.249	0.250	0.2495	0.312	15.605		

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Table 16 The effect of different temperature on protease activity of *Aspergillus fumigatus* SS0509

The fungus strain SS0509 was cultured in 3-separeted flasks on YHM, pH 8 at 35-50^{o}C for 3 days. The culture filtrate from each flask was separated by filtration. In each flask, the protease activity was measured twice as tube # 1 and tube# 2 and then, the means was calculated and the amount of amino acid was estimated from standard curve of Bradford Protein Assay. Enzyme unit, total enzyme and specific activity were calculated using followed equation. Enzyme unit = 3.5(ml) x X (mg) / 0.5(ml). Total enzyme = Enzyme unit x Volume (ml) and Specific activity = Total enzyme/Total protein.

Protease activity											
Temp	OD595			Amount of amino acid (mg/ml)	Enzyme unit (umole/min/ml)	Total enzyme (unit)	Mean of Total enzyme (unit)	SD of Total enzyme	Specific activity (unit/mg)	Mean of Specific activity (unit/mg)	SD of Specific activity (unit/mg)
	tube # 1	tube # 2	Mean								
35	0.105	0.106	0.105	0.123	8.58×10^{-1}	4.29×10^{1}	4.32×10^{1}	0.352	3.77	4.13	0.403
	0.105	0.107	0.106	0.123	8.63×10^{-1}	4.32×10^{1}			4.07		
	0.108	0.106	0.107	0.125	8.72×10^{-1}	4.36×10^{1}			4.57		
40	0.180	0.184	0.182	0.223	1.56	7.82×10^{1}	7.37×10^{1}	4.881	6.78	6.85	0.075
	0.158	0.164	0.161	0.196	1.37	6.85×10^{1}			6.93		
	0.170	0.178	0.174	0.213	1.49	7.45×10^{1}			6.85		
45	0.156	0.16	0.158	0.192	1.34	6.71×10^{1}	6.74×10^{1}	0.352	4.63	4.56	0.189
	0.154	0.163	0.158	0.192	1.35	6.73×10^{1}			4.35		
	0.154	0.165	0.159	0.194	1.36	6.78×10^{1}			4.71		
50	0.124	0.123	0.123	0.146	1.02	5.12×10^{1}	5.05×10^{1}	0.691	3.33	3.31	0.074
	0.120	0.121	0.120	0.142	9.97×10^{-1}	4.98×10^{1}			3.39		
	0.120	0.124	0.122	0.144	1.01	5.05×10^{1}			3.24		

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Table 17 The effect of pH on the protease concentration of the *Aspergillus fumigatus* SS0509 culture filtrate

The fungus strain SS0509 was cultured in 3-separeted flasks on YHM, pH 8 at 40^{o}C for 3 days. The culture filtrate from each flask was separated by filtration. In each flask, the protein concentration was measured twice as tube # 1 and tube# 2, pH 5-10 and then, the means was calculated and protein concentration was estimated from standard curve of Bradford Protein Assay. The total protein was calculated using followed equation. Total protein = Protein concentration (mg/ml) x Volume (ml).

pH	Replication	Volume (ml)	Protein concentration							
			Dilution	OD595			Protein conc.			
				tube # 1	tube # 2	Mean	Prot. conc. (mg/ml)	Total prot. (mg)	Mean of Total prot. (mg)	SD of Total prot.
									Mean	SD
5	1	50		0.153	0.152	0.153	0.018	0.922	1.115	0.315
	2	50		0.155	0.157	0.156	0.019	0.945		
	3	50		0.234	0.240	0.237	0.030	1.478		
6	1	50		0.163	0.160	0.162	0.020	0.982	1.802	0.721
	2	50		0.364	0.370	0.367	0.047	2.334		
	3	50		0.340	0.320	0.330	0.042	2.090		
7	1	50		0.196	0.195	0.196	0.024	1.205	1.027	0.188
	2	50		0.172	0.170	0.171	0.021	1.044		
	3	50		0.140	0.137	0.139	0.017	0.830		

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Table 17 The effect of pH on the protease concentration of the *Aspergillus fumigatus* SS0509 culture filtrate (continuous)

The fungus strain SS0509 was cultured in 3-separeted flasks on YHM, pH 8 at 40°C for 3 days. The culture filtrate from each flask was separated by filtration. In each flask, the protein concentration was measured twice as tube # 1 and tube# 2, pH 5-10 and then, the means was calculated and protein concentration was estimated from standard curve of Bradford Protein Assay. The total protein was calculated using followed equation. Total protein = Protein concentration (mg/ml) x Volume (ml).

pH	Replication	Volume (ml)	Protein concentration							
			Dilution	OD595			Protein conc.			
				tube # 1	tube # 2	Mean	Prot. conc. (mg/ml)	Total prot. (mg)	Mean of Total prot. (mg)	SD of Total prot.
									Mean	SD
8	1	50		0.242	0.243	0.243	0.030	1.514	1.738	0.632
	2	50		0.200	0.204	0.202	0.025	1.248		
	3	50		0.390	0.380	0.385	0.049	2.452		
9	1	50		0.211	0.212	0.212	0.026	1.311	1.569	0.263
	2	50		0.250	0.249	0.250	0.031	1.561		
	3	50		0.296	0.287	0.292	0.037	1.837		
10	1	50		0.260	0.256	0.258	0.032	1.616	1.723	0.093
	2	50		0.284	0.285	0.285	0.036	1.791		
	3	50		0.281	0.279	0.280	0.035	1.761		

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Table 18 The effect of pH on the protease activity of the *Aspergillus fumigatus* SS0509 culture filtrate

The fungus strain SS0509 was cultured in 3-separeted flasks on YHM, pH 8 at 40°C for 3 days. The culture filtrate from each flask was separated by filtration. In each flask, the protease activity was measured twice as tube # 1 and tube# 2, pH 5-10 and then, the means was calculated and the amount of amino acid was estimated from standard curve of Bradford Protein Assay. Enzyme unit, total enzyme and specific activity were calculated using followed equation. Enzyme unit = 3.5(ml) x X (mg) / 0.5(ml). Total enzyme = Enzyme unit x Volume (ml) and Specific activity = Total enzyme/Total protein.

Protease activity											
pH	OD595			Amount of amino acid	Enzyme unit	Total enzyme	Mean of	SD of	Specific activity	Mean of	SD of
	tube # 1	tube # 2	Mean	(mg/ml)	(umole/min/ml)	(unit)	Total enzyme (unit)	Total enzyme	(unit/mg)	Specific activity (unit/mg)	Specific activity (unit/mg)
							Mean	SD		Mean	SD
	0.024	0.025	0.025	0.002	0.00	0.00			0.00		
5	0.034	0.04	0.037	0.003	0.00	0.00	0.00	0	0.00	0.00	0
	0.026	0.025	0.026	0.002	0.00	0.00			0.00		
	0.13	0.14	0.135	0.016	1.13×10^{-1}	5.65			5.76		
6	0.266	0.271	0.269	0.034	2.36×10^{-1}	1.18	9.76	3.563	5.06	5.49	0.381
	0.25	0.289	0.270	0.034	2.37×10^{-1}	1.18			5.67		
	0.158	0.153	0.156	0.019	1.32×10^{-1}	6.59			5.47		
7	0.132	0.130	0.131	0.016	1.09×10^{-1}	5.47	5.70	0.808	5.24	5.59	0.423
	0.123	0.12	0.122	0.014	1.01×10^{-1}	5.03			6.06		

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Table 18 The effect of pH on the protease activity of the *Aspergillus fumigatus* SS0509 culture filtrate (continuous)

The fungus strain SS0509 was cultured in 3-separeted flasks on YHM, pH 8 at 40^{o}C for 3 days. The culture filtrate from each flask was separated by filtration. In each flask, the protease activity was measured twice as tube # 1 and tube # 2, pH 5-10 and then, the means was calculated and the amount of amino acid was estimated from standard curve of Bradford Protein Assay. Enzyme unit, total enzyme and specific activity were calculated using followed equation. Enzyme unit = 3.5(ml) x X (mg) / 0.5(ml). Total enzyme = Enzyme unit x Volume (ml) and Specific activity = Total enzyme/Total protein.

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Protease activity											
pH	OD595			Amount of amino acid (mg/ml)	Enzyme unit (umole/min/ml)	Total enzyme (unit)	Mean of Total enzyme (unit)	SD of Total enzyme	Specific activity (unit/mg)	Mean of Specific activity (unit/mg)	SD of Specific activity (unit/mg)
	tube # 1	tube # 2	Mean								
							Mean	SD		Mean	SD
8	0.205	0.201	0.203	0.025	1.76 x10^{-1}	8.78	1.10	3.983	5.80	6.34	0.536
	0.199	0.198	0.199	0.025	1.72 x10^{-1}	8.58			6.87		
	0.349	0.352	0.351	0.045	3.12 x10^{-1}	11.56			6.35		
9	0.177	0.184	0.181	0.022	1.55 x10^{-1}	7.76	9.00	1.084	5.91	5.77	0.486
	0.224	0.219	0.222	0.028	1.93 x10^{-1}	9.63			6.17		
	0.219	0.223	0.221	0.027	1.92 x10^{-1}	9.61			5.23		
10	0.236	0.234	0.235	0.029	2.05 x10^{-1}	11.03	11.01	0.336	6.34	5.91	0.444
	0.226	0.223	0.225	0.028	1.95 x10^{-1}	9.77			5.46		
	0.239	0.238	0.239	0.030	2.08 x10^{-1}	11.04			5.91		

Table 19 The effect of temperature on the protease concentration of the *Aspergillus fumigatus* SS0509 culture filtrate

The fungus strain SS0509 was cultured in 3-separeted flasks on YHM, pH 8 at 40^{o}C for 3 days. The culture filtrate from each flask was separated by filtration. In each flask, the protein concentration was measured twice as tube # 1 and tube# 2, at 35-70^{o}C and then, the means was calculated and protein concentration was estimated from standard curve of Bradford Protein Assay. The total protein was calculated using followed equation. Total protein = Protein concentration (mg/ml) x Volume (ml).

temp	Replication	Volume (ml)	Protein concentration							
			Dilution	OD595			Protein conc.			
				tube # 1	tube # 2	Mean	Prot. conc. (mg/ml)	Total prot. (mg)	Mean of Total prot. (mg)	SD of Total prot.
35	1	40		0.238	0.273	0.2555	0.032	1.280	1.521	0.21644
	2	40		0.288	0.339	0.3135	0.040	1.585		
	3	40		0.373	0.297	0.335	0.042	1.698		
40	1	40		0.238	0.273	0.2555	0.032	1.280	1.521	0.21644
	2	40		0.288	0.339	0.3135	0.040	1.585		
	3	40		0.373	0.297	0.335	0.042	1.698		
45	1	40		0.238	0.273	0.2555	0.032	1.280	1.521	0.21644
	2	40		0.288	0.339	0.3135	0.040	1.585		
	3	40		0.373	0.297	0.335	0.042	1.698		
50	1	40		0.238	0.273	0.2555	0.032	1.280	1.521	0.21644
	2	40		0.288	0.339	0.3135	0.040	1.585		
	3	40		0.373	0.297	0.335	0.042	1.698		

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Table 19 The effect of temperature on the protease concentration of the *Aspergillus fumigatus* SS0509 culture filtrate (continuous)

The fungus strain SS0509 was cultured in 3-separeted flasks on YHM, pH 8 at 40^{o}C for 3 days. The culture filtrate from each flask was separated by filtration. In each flask, the protein concentration was measured twice as tube # 1 and tube# 2, at 35-70^{o}C and then, the means was calculated and protein concentration was estimated from standard curve of Bradford Protein Assay. The total protein was calculated using followed equation. Total protein = Protein concentration (mg/ml) x Volume (ml).

temp	Replication	Volume (ml)	Protein concentration							
			Dilution	OD595			Protein conc.			
				tube # 1	tube # 2	Mean	Prot. conc. (mg/ml)	Total prot. (mg)	Mean of Total prot. (mg)	SD of Total prot.
55	1	40		0.238	0.273	0.255	0.032	1.280		
	2	40		0.288	0.339	0.313	0.040	1.585	1.521	0.216
	3	40		0.373	0.297	0.335	0.042	1.698		
60	1	40		0.238	0.273	0.255	0.032	1.280		
	2	40		0.288	0.339	0.313	0.040	1.585		
	3	40		0.373	0.297	0.335	0.042	1.698	1.521	0.216
65	1	40		0.289	0.278	0.2835	0.035	1.427		
	2	40		0.254	0.298	0.276	0.034	1.387	1.512	0.181
	3	40		0.357	0.321	0.339	0.043	1.719		
70	1	40		0.289	0.278	0.283	0.035	1.427		
	2	40		0.254	0.298	0.276	0.034	1.387	1.512	0.181
	3	40		0.357	0.321	0.339	0.043	1.719		

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Table 20 The effect of temperature on the protease activity of the *Aspergillus fumigatus* SS0509 culture filtrate

The fungus strain SS0509 was cultured in 3-separeted flasks on YHM, pH 8 at 40^{o}C for 3 days. The culture filtrate from each flask was separated by filtration. In each flask, the protease activity was measured twice as tube # 1 and tube # 2, at 35-70^{o}C and then, the means was calculated and the amount of amino acid was estimated from standard curve of Bradford Protein Assay. Enzyme unit, total enzyme and specific activity were calculated using followed equation. Enzyme unit = 3.5(ml) x X (mg) / 0.5(ml). Total enzyme = Enzyme unit x Volume (ml) and Specific activity = Total enzyme/Total protein.

Protease activity											
Temp	OD595			Amount of amino acid (mg/ml)	Enzyme unit (umole/min/ml)	Total enzyme (unit)	Mean of Total enzyme (unit)	SD of Total enzyme	Specific activity (unit/mg)	Mean of Specific activity (unit/mg)	SD of Specific activity (unit/mg)
	tube # 1	tube # 2	Mean								
35	0.145	0.143	0.144	0.017	0.121	4.852	5.360	1.136	3.79	3.530	0.570
	0.143	0.129	0.136	0.016	0.114	4.557			2.87		
	0.196	0.190	0.193	0.024	0.166	6.657			3.92		
40	0.139	0.135	0.137	0.016	0.115	4.594	5.580	1.160	3.59	3.680	0.610
	0.201	0.196	0.199	0.025	0.172	6.860			4.33		
	0.157	0.155	0.156	0.019	0.132	5.294			3.12		
45	0.203	0.197	0.200	0.025	0.173	6.915	8.120	1.055	5.40	5.340	0.096
	0.200	0.289	0.245	0.031	0.214	8.555			5.40		
	0.259	0.248	0.254	0.032	0.222	8.886			5.23		
50	0.210	0.265	0.238	0.030	0.207	8.297	8.440	0.138	6.48	5.620	0.777
	0.250	0.240	0.245	0.031	0.214	8.573			5.41		
	0.235	0.248	0.242	0.030	0.211	8.444			4.97		

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Table 20 The effect of temperature on the protease activity of the *Aspergillus fumigatus* SS0509 culture filtrate (continuous)

The fungus strain SS0509 was cultured in 3-separeted flasks on YHM, pH 8 at 40^{o}C for 3 days. The culture filtrate from each flask was separated by filtration. In each flask, the protease activity was measured twice as tube # 1 and tube # 2, at 35-70^{o}C and then, the means was calculated and the amount of amino acid was estimated from standard curve of Bradford Protein Assay. Enzyme unit, total enzyme and specific activity were calculated using followed equation. Enzyme unit = 3.5(ml) x X (mg) / 0.5(ml). Total enzyme = Enzyme unit x Volume (ml) and Specific activity = Total enzyme/Total protein.

Protease activity											
Temp	OD595			Amount of amino acid (mg/ml)	Enzyme unit (umole/min/ml)	Total enzyme (unit)	Mean of Total enzyme (unit)	SD of Total enzyme	Specific activity (unit/mg)	Mean of Specific activity (unit/mg)	SD of Specific activity (unit/mg)
	tube # 1	tube # 2	Mean								
55	0.206	0.269	0.238	0.030	0.207	8.297	9.400	1.280	6.48	6.220	0.767
	0.296	0.315	0.306	0.039	0.270	10.802			6.81		
	0.268	0.250	0.259	0.032	0.227	9.089			5.35		
60	0.192	0.190	0.191	0.024	0.165	6.584	6.380	0.946	5.14	4.250	0.885
	0.157	0.158	0.158	0.019	0.134	5.349			3.37		
	0.200	0.216	0.208	0.026	0.180	7.210			4.25		
65	0.123	0.125	0.124	0.015	0.103	4.115	3.869	0.409	2.88	2.603	0.545
	0.121	0.126	0.124	0.015	0.102	4.097			2.95		
	0.100	0.109	0.105	0.012	0.085	3.397			1.98		
70	0	0	0	0	0	0	0	0	0	0	0
	0	0	0	0	0	0			0		
	0	0	0	0	0	0			0		

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Table 21 The effect of pH on the purified protease concentration of *Aspergillus fumigatus* SS0509

Fraction 19 of purified protease after gel filtration was collected. In each fraction, the protein concentration was measured twice as tube # 1 and tube# 2, pH 5-10 and then, the means was calculated and protein concentration was estimated from standard curve of Bradford Protein Assay. The total protein was calculated using followed equation. Total protein = Protein concentration (mg/ml) x Volume (ml).

pH	Replication	Volume (ml)	Protein concentration							
			Dilution	OD595			Protein conc.			
				tube # 1	tube # 2	Mean	Prot. conc. (mg/ml)	Total prot. (mg)	Mean of Total prot. (mg)	SD of Total prot.
									Mean	SD
5	1	1		0.159	0.160	0.160	0.019	0.019		
	2	1		0.158	0.158	0.158	0.019	0.019	0.019	0.000
	3	1		0.162	0.163	0.163	0.020	0.020		
6	1	1		0.159	0.160	0.160	0.019	0.019		
	2	1		0.158	0.158	0.158	0.019	0.019	0.019	0.000
	3	1		0.162	0.163	0.163	0.020	0.020		
7	1	1		0.159	0.160	0.160	0.019	0.019		
	2	1		0.158	0.158	0.158	0.019	0.019	0.019	0.000
	3	1		0.162	0.163	0.163	0.020	0.020		

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Table 21 The effect of pH on the purified protease concentration of *Aspergillus fumigatus* SS0509 (continuous)

Fraction 19 of purified protease after gel filtration was collected. In each fraction, the protein concentration was measured twice as tube # 1 and tube# 2, pH 5-10 and then, the means was calculated and protein concentration was estimated from standard curve of Bradford Protein Assay. The total protein was calculated using followed equation. Total protein = Protein concentration (mg/ml) x Volume (ml).

pH	Replication	Volume (ml)	Protein concentration							
			Dilution	OD595			Protein conc.			
				tube # 1	tube # 2	Mean	Prot. conc. (mg/ml)	Total prot. (mg)	Mean of Total prot. (mg)	SD of Total prot.
									Mean	SD
8	1	1		0.149	0.150	0.150	0.018	0.018		
	2	1		0.156	0.158	0.157	0.019	0.019	0.019	0.001
	3	1		0.165	0.164	0.165	0.020	0.020		
9	1	1		0.149	0.150	0.150	0.018	0.018		
	2	1		0.156	0.158	0.157	0.019	0.019	0.019	0.001
	3	1		0.165	0.164	0.165	0.020	0.020		
10	1	1		0.149	0.150	0.150	0.018	0.018		
	2	1		0.156	0.158	0.157	0.019	0.019	0.019	0.001
	3	1		0.165	0.164	0.165	0.020	0.020		

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Table 22 The effect of pH on the purified protease activity of *Aspergillus fumigatus* SS0509

Fraction 19 of purified protease after gel filtration was collected. In each fraction, the protein concentration was measured twice as tube # 1 and tube# 2, pH 5-10 and then, the means was calculated and the amount of amino acid was estimated from standard curve of Bradford Protein Assay. Enzyme unit, total enzyme and specific activity were calculated using followed equation. Enzyme unit = 3.5(ml) x X (mg) / 0.5(ml). Total enzyme = Enzyme unit x Volume (ml) and Specific activity = Total enzyme/Total protein.

Protease activity											
pH	OD595			Amount of amino acid (mg/ml)	Enzyme unit (umole/min/ml)	Total enzyme (unit)	Mean of Total enzyme (unit)	SD of Total enzyme	Specific activity (unit/mg)	Mean of Specific activity (unit/mg)	SD of Specific activity (unit/mg)
	tube # 1	tube # 2	Mean								
5	0.111	0.111	0.111	0.013	9.09×10^{-2}	9.09×10^{-2}	9.17×10^{-2}	0.003	4.69	4.72	0.226
	0.116	0.115	0.115	0.014	9.51×10^{-2}	9.51×10^{-2}			4.96		
	0.109	0.109	0.109	0.013	8.91×10^{-2}	8.91×10^{-2}			4.51		
6	0.140	0.129	0.134	0.016	1.13×10^{-1}	1.13×10^{-1}	1.05×10^{-1}	0.006	5.81	5.43	0.410
	0.130	0.123	0.126	0.015	1.05×10^{-1}	1.05×10^{-1}			5.49		
	0.119	0.120	0.119	0.014	9.87×10^{-2}	9.87×10^{-2}			5.00		
7	0.129	0.122	0.125	0.015	1.04×10^{-1}	1.04×10^{-1}	1.05×10^{-1}	0.105	5.38	5.42	0.309
	0.135	0.129	0.132	0.016	1.10×10^{-1}	1.10×10^{-1}			5.75		
	0.123	0.122	0.122	0.015	1.02×10^{-1}	1.02×10^{-1}			5.14		

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Table 22 The effect of pH on the purified protease activity of *Aspergillus fumigatus* SS0509 (continuous)

Fraction 19 of purified protease after gel filtration was collected. In each fraction, the protein concentration was measured twice as tube # 1 and tube# 2, pH 5-10 and then, the means was calculated and the amount of amino acid was estimated from standard curve of Bradford Protein Assay. Enzyme unit, total enzyme and specific activity were calculated using followed equation. Enzyme unit = 3.5(ml) x X (mg) / 0.5(ml). Total enzyme = Enzyme unit x Volume (ml) and Specific activity = Total enzyme/Total protein.

Protease activity											
pH	OD595			Amount of amino acid (mg/ml)	Enzyme unit (umole/min/ml)	Total enzyme (unit)	Mean of Total enzyme (unit)	SD of Total enzyme	Specific activity (unit/mg)	Mean of Specific activity (unit/mg)	SD of Specific activity (unit/mg)
	tube # 1	tube # 2	Mean								
							Mean	SD		Mean	SD
8	0.150	0.143	0.146	0.018	1.24×10^{-1}	1.24×10^{-1}			6.85		
	0.175	0.173	0.174	0.021	1.49×10^{-1}	1.49×10^{-1}	1.41×10^{-1}	0.015	7.82	7.41	0.503
	0.174	0.179	0.176	0.022	1.51×10^{-1}	1.51×10^{-1}			7.55		
9	0.140	0.142	0.141	0.017	1.19×10^{-1}	1.19×10^{-1}			6.57		
	0.130	0.130	0.13	0.015	1.08×10^{-1}	1.08×10^{-1}	1.19×10^{-1}	0.010	5.69	6.24	0.473
	0.152	0.153	0.152	0.018	1.29×10^{-1}	1.29×10^{-1}			6.45		
10	0.118	0.118	0.118	0.014	9.74×10^{-2}	9.74×10^{-2}			5.39		
	0.116	0.114	0.115	0.014	9.46×10^{-2}	9.46×10^{-2}	9.81×10^{-2}	0.003	4.97	5.16	0.215
	0.125	0.122	0.123	0.015	1.02×10^{-1}	1.02×10^{-1}			5.11		

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Table 23 The effect of temperature on the purified protease concentration of *Aspergillus fumigatus* SS0509

Fraction 19 of purified protease after gel filtration was collected. In each fraction, the protein concentration was measured twice as tube # 1 and tube# 2, at 35-70^{o}C and then, the means was calculated and protein concentration was estimated from standard curve of Bradford Protein Assay. The total protein was calculated using followed equation. Total protein = Protein concentration (mg/ml) x Volume (ml).

temp	Replication	Volume (ml)	Protein concentration							
			Dilution	OD595			Protein conc.			
				tube # 1	tube # 2	Mean	Prot. conc. (mg/ml)	Total prot. (mg)	Mean of Total prot. (mg)	SD of Total prot.
35	1	1		0.161	0.161	0.161	0.020	0.020	0.020	0.00058
	2	1		0.158	0.158	0.158	0.019	0.019		
	3	1		0.165	0.165	0.165	0.020	0.020		
40	1	1		0.161	0.161	0.161	0.020	0.020	0.020	0.00058
	2	1		0.158	0.158	0.158	0.019	0.019		
	3	1		0.165	0.165	0.165	0.020	0.020		
45	1	1		0.161	0.161	0.161	0.020	0.020	0.020	0.00058
	2	1		0.158	0.158	0.158	0.019	0.019		
	3	1		0.165	0.165	0.165	0.020	0.020		
50	1	1		0.161	0.161	0.161	0.020	0.020	0.020	0.00058
	2	1		0.158	0.158	0.158	0.019	0.019		
	3	1		0.165	0.165	0.165	0.020	0.020		

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Table 23 The effect of temperature on the purified protease concentration of *Aspergillus fumigatus* SS0509 (continuous)

Fraction 19 of purified protease after gel filtration was collected. In each fraction, the protein concentration was measured twice as tube # 1 and tube# 2, at 35-70^{o}C and then, the means was calculated and protein concentration was estimated from standard curve of Bradford Protein Assay. The total protein was calculated using followed equation. Total protein = Protein concentration (mg/ml) x Volume (ml).

temp	Replication	Volume (ml)	Protein concentration							
			Dilution	OD595			Protein conc.			
				tube # 1	tube # 2	Mean	Prot. conc. (mg/ml)	Total prot. (mg)	Mean of Total prot. (mg)	SD of Total prot.
55	1	1		0.130	0.130	0.130	0.015	0.015	0.018	0.00265
	2	1		0.158	0.158	0.158	0.019	0.019		
	3	1		0.165	0.165	0.165	0.020	0.020		
60	1	1		0.130	0.130	0.130	0.015	0.015	0.018	0.00265
	2	1		0.158	0.158	0.158	0.019	0.019		
	3	1		0.165	0.165	0.165	0.020	0.020		
65	1	1		0.130	0.130	0.130	0.015	0.015	0.018	0.00265
	2	1		0.158	0.158	0.158	0.019	0.019		
	3	1		0.165	0.165	0.165	0.020	0.020		
70	1	1		0.130	0.13	0.130	0.015	0.015	0.018	0.00265
	2	1		0.158	0.158	0.158	0.019	0.019		
	3	1		0.165	0.165	0.165	0.020	0.020		

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Table 24 The effect of temperature on the purified protease activity of *Aspergillus fumigatus* SS0509

Fraction 19 of purified protease after gel filtration was collected. In each fraction, the protein concentration was measured twice as tube # 1 and tube# 2, at 35-70^{o}C and then, the means was calculated and the amount of amino acid was estimated from standard curve of Bradford Protein Assay. Enzyme unit, total enzyme and specific activity were calculated using followed equation. Enzyme unit = 3.5(ml) x X (mg) / 0.5(ml). Total enzyme = Enzyme unit x Volume (ml) and Specific activity = Total enzyme/Total protein.

Protease activity											
temp	OD595			Amount of amino acid	Enzyme unit	Total enzyme	Mean of	SD of	Specific activity	Mean of	SD of
	tube # 1	tube # 2	Mean	(mg/ml)	(umole/min/ml)	(unit)	Total enzyme (unit)	Total enzyme	(unit/mg)	Specific activity (unit/mg)	Specific activity (unit/mg)
35	0.119	0.119	0.119	0.014	9.83×10^{-2}	0.098			4.91		
	0.116	0.115	0.115	0.014	9.51×10^{-2}	0.095	0.097	0.002	5.00	4.97	0.046
	0.12	0.121	0.120	0.014	9.97×10^{-2}	0.097			4.98		
40	0.126	0.126	0.126	0.015	1.05×10^{-1}	0.105			5.24		
	0.122	0.123	0.122	0.015	1.02×10^{-1}	0.010	0.103	0.001	5.34	5.26	6.88×10^{-2}
	0.126	0.125	0.125	0.015	1.04×10^{-1}	0.010			5.21		
45	0.146	0.146	0.146	0.018	1.23×10^{-1}	0.123			6.16		
	0.139	0.142	0.140	0.017	1.18×10^{-1}	0.118	0.120	0.002	6.21	6.12	1.13×10^{-1}
	0.145	0.14	0.142	0.017	1.20×10^{-1}	0.120			6.00		
50	0.14	0.139	0.142	0.017	1.19×10^{-1}	0.019			5.97		
	0.14	0.14	0.14	0.017	1.18×10^{-1}	0.018	0.019	0.000	6.19	6.03	1.41×10^{-1}
	0.140	0.142	0.141	0.017	1.19×10^{-1}	0.019			5.93		

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Table 24 The effect of temperature on the purified protease activity of *Aspergillus fumigatus* SS0509 (continuous)

Fraction 19 of purified protease after gel filtration was collected. In each fraction, the protein concentration was measured twice as tube # 1 and tube# 2, at 35-70^{o}C and then, the means was calculated and the amount of amino acid was estimated from standard curve of Bradford Protein Assay. Enzyme unit, total enzyme and specific activity were calculated using followed equation. Enzyme unit = 3.5(ml) x X (mg) / 0.5(ml). Total enzyme = Enzyme unit x Volume (ml) and Specific activity = Total enzyme/Total protein.

Protease activity											
temp	OD595			Amount of amino acid (mg/ml)	Enzyme unit (umole/min/ml)	Total enzyme (unit)	Mean of Total enzyme (unit)	SD of Total enzyme	Specific activity (unit/mg)	Mean of Specific activity (unit/mg)	SD of Specific activity (unit/mg)
	tube # 1	tube # 2	Mean								
55	0.157	0.143	0.150	0.018	1.27×10^{-1}	0.127	0.145	0.016	8.47	8.11	0.325
	0.175	0.173	0.174	0.021	1.49×10^{-1}	0.149			7.84		
	0.184	0.189	0.186	0.023	1.60×10^{-1}	0.160			8.02		
60	0.108	0.109	0.108	0.013	8.86×10^{-2}	0.088	0.099	0.011	5.91	5.55	0.343
	0.120	0.120	0.120	0.014	9.92×10^{-2}	0.092			5.22		
	0.132	0.133	0.132	0.016	1.11×10^{-1}	0.011			5.54		
65	0.084	0.084	0.084	0.000	0.00	0.000	0	0	0.00	0	0
	0.079	0.08	0.079	0.000	0.00	0.000			0.00		
	0.080	0.080	0.080	0.000	0.00	0.000			0.00		
70	0.050	0.050	0.050	0.000	0.00	0.000	0	0	0.00	0	0
	0.050	0.060	0.055	0.000	0.00	0.000			0.00		
	0.060	0.050	0.055	0.000	0.00	0.000			0.00		

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Table 25 Effect of inhibitors on the purified protease concentration

Fraction 19 of purified protease after gel filtration was collected. In each fraction, the protein concentration was measured twice as tube # 1 and tube# 2, incubated with inhibitors and then, the means was calculated and protein concentration was estimated from standard curve of Bradford Protein Assay. The total protein was calculated using followed equation. Total protein = Protein concentration (mg/ml) x Volume (ml).

Inhibitors	Replication	Volume (ml)	Protein concentration							
			Dilution	OD595			Protein conc.			
				tube # 1	tube # 2	Mean	Prot. conc. (mg/ml)	Total prot. (mg)	Mean of Total prot. (mg)	SD of Total prot.
EDTA	1	1		0.110	0.117	0.113	0.013	0.013		
	2	1		0.113	0.112	0.112	0.013	0.013	0.013	0.00017
	3	1		0.118	0.112	0.115	0.014	0.014		
PMSF	1	1		0.110	0.117	0.113	0.013	0.013		
	2	1		0.113	0.112	0.112	0.013	0.013	0.013	0.00017
	3	1		0.118	0.112	0.115	0.014	0.014		
TLCK	1	1		0.110	0.117	0.113	0.013	0.013		
	2	1		0.113	0.112	0.112	0.013	0.013	0.013	0.00017
	3	1		0.118	0.112	0.115	0.014	0.014		
TPCK	1	1		0.110	0.117	0.113	0.013	0.013		
	2	1		0.113	0.112	0.112	0.013	0.013	0.013	0.00017
	3	1		0.118	0.112	0.115	0.014	0.014		

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Table 25 Effect of inhibitors on the purified protease concentration (continuous)

Fraction 19 of purified protease after gel filtration was collected. In each fraction, the protein concentration was measured twice as tube # 1 and tube# 2, incubated with inhibitors and then, the means was calculated and protein concentration was estimated from standard curve of Bradford Protein Assay. The total protein was calculated using followed equation. Total protein = Protein concentration (mg/ml) x Volume (ml).

Inhibitors	Replication	Volume (ml)	Protein concentration							
			Dilution	OD595			Protein conc.			
				tube # 1	tube # 2	Mean	Prot. conc. (mg/ml)	Total prot. (mg)	Mean of Total prot. (mg)	SD of Total prot.
Iodoacetic	1	1		0.11	0.117	0.113	0.013	0.013		
	2	1		0.113	0.112	0.112	0.013	0.013	0.013	0.00017
	3	1		0.118	0.112	0.115	0.014	0.014		
Pepstatin A	1	1		0.110	0.117	0.113	0.013	0.013		
	2	1		0.113	0.112	0.112	0.013	0.013	0.013	0.00017
	3	1		0.118	0.112	0.115	0.014	0.014		
Control	1	1		0.110	0.117	0.113	0.013	0.013		
	2	1		0.113	0.112	0.112	0.013	0.013	0.013	0.00017
	3	1		0.118	0.112	0.115	0.014	0.014		

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Table 26 Effect of inhibitors on the purified protease activity

Fraction 19 of purified protease after gel filtration was collected. In each fraction, the protein concentration was measured twice as tube # 1 and tube# 2, incubated with inhibitors and then, the means was calculated and the amount of amino acid was estimated from standard curve of Bradford Protein Assay. Enzyme unit, total enzyme and specific activity were calculated using followed equation. Enzyme unit = 3.5(ml) x X (mg) / 0.5(ml). Total enzyme = Enzyme unit x Volume (ml) and Specific activity = Total enzyme/Total protein.

Protease activity											
Inhibitors	OD595			Amount of amino acid (mg/ml)	Enzyme unit (umole/min/m)	Total enzyme (unit)	Mean of Total enzyme (unit)	SD of Total enzyme	Specific activity (unit/mg)	Mean of Specific activity (unit/mg)	SD of Specific activity (unit/mg)
	tube # 1	tube # 2	Mean								
	0.229	0.221	0.225	0.028	0.196	0.019			0.147		
EDTA	0.228	0.23	0.229	0.029	0.200	0.020	0.197	0.002	0.151	0.148	0.357
	0.228	0.220	0.224	0.028	0.195	0.019			0.144		
	0	0	0	0.000	0.000	0.000			0.000		
	0	0	0	0.000	0.000	0.000	0.00	0	0.000	0.00	0.00
PMSF	0	0	0	0.000	0.000	0.000			0.000		
	0.23	0.222	0.226	0.028	0.197	0.019			0.148		
	0.223	0.223	0.223	0.028	0.194	0.019	0.197	0.003	0.147	0.148	0.005
TLCK	0.23	0.23	0.23	0.029	0.201	0.200			0.148		
	0.214	0.219	0.216	0.027	0.188	0.188			0.141		
	0.22	0.218	0.219	0.027	0.190	0.190	0.189	0.001	0.144	0.014	0.023
TPCK	0.220	0.215	0.217	0.027	0.189	0.189			0.140		

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Table 26 Effect of inhibitors on the purified protease activity (continuous)

Fraction 19 of purified protease after gel filtration was collected. In each fraction, the protein concentration was measured twice as tube # 1 and tube# 2, incubated with inhibitors and then, the means was calculated and the amount of amino acid was estimated from standard curve of Bradford Protein Assay. Enzyme unit, total enzyme and specific activity were calculated using followed equation. Enzyme unit = 3.5(ml) x X (mg) / 0.5(ml). Total enzyme = Enzyme unit x Volume (ml) and Specific activity = Total enzyme/Total protein.

Protease activity											
Inhibitors	OD595			Amount of amino acid (mg/ml)	Enzyme unit (umole/min/m)	Total enzyme (unit)	Mean of Total enzyme (unit)	SD of Total enzyme	Specific activity (unit/mg)	Mean of Specific activity (unit/mg)	SD of Specific activity (unit/mg)
	tube # 1	tube # 2	Mean								
Iodoacetic A	0.23	0.22	0.225	0.028	0.196	0.196			0.147		
	0.228	0.23	0.229	0.029	0.200	0.200	0.198	0.001	0.151	0.014	0.271
	0.224	0.23	0.227	0.028	0.198	0.019			0.146		
Pepstatin A	0.229	0.23	0.229	0.029	0.200	0.200			0.150		
	0.226	0.227	0.226	0.028	0.197	0.019	0.197	0.002	0.150	0.014	0.327
	0.226	0.222	0.224	0.028	0.195	0.019			0.144		
control	0.224	0.226	0.225	0.028	0.196	0.019			0.147		
	0.231	0.225	0.228	0.028	0.199	0.019	0.197	0.001	0.151	0.014	0.231
	0.229	0.225	0.227	0.028	0.198	0.019			0.146		

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Table 27 Storage stability of purify protease from *Aspergillus fumigatus* SS0509

Fraction 19 of purified protease after gel filtration was collected. The purified protease was incubated at 55^{o}C for 0- 90 minutes. In each fraction, the protein concentration was measured twice as tube # 1 and tube# 2 and then, the means was calculated and protein concentration was estimated from standard curve of Bradford Protein Assay. The total protein was calculated using followed equation. Total protein = Protein concentration (mg/ml) x Volume (ml).

time	Replication	Volume (ml)	Protein concentration							
			Dilution	OD595			Protein conc.			
				tube # 1	tube # 2	Mean	Prot. conc. (mg/ml)	Total prot. (mg)	Mean of Total prot. (mg)	SD of Total prot.
									Mean	SD
0	1	1		0.161	0.161	0.161	0.020	0.020	0.020	0.001
	2	1		0.158	0.158	0.158	0.019	0.019		
	3	1		0.165	0.165	0.165	0.020	0.020		
15	1	1		0.161	0.161	0.161	0.020	0.020	0.020	0.001
	2	1		0.158	0.158	0.158	0.019	0.019		
	3	1		0.165	0.165	0.165	0.020	0.020		
30	1	1		0.161	0.161	0.161	0.020	0.020	0.020	0.001
	2	1		0.158	0.158	0.158	0.019	0.019		
	3	1		0.165	0.165	0.165	0.020	0.020		

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Table 27 Storage stability of purify protease from *Aspergillus fumigatus* SS0509 (continuous)

Fraction 19 of purified protease after gel filtration was collected. The purified protease was incubated at 55°C for 0- 90 minutes. In each fraction, the protein concentration was measured twice as tube # 1 and tube# 2 and then, the means was calculated and protein concentration was estimated from standard curve of Bradford Protein Assay. The total protein was calculated using followed equation. Total protein = Protein concentration (mg/ml) x Volume (ml).

time	Replication	Volume (ml)	Protein concentration							
			Dilution	OD595			Protein conc.			
				tube # 1	tube # 2	Mean	Prot. conc. (mg/ml)	Total prot. (mg)	Mean of Total prot. (mg)	SD of Total prot.
									Mean	SD
45	1	1		0.161	0.161	0.161	0.020	0.020	0.020	0.001
	2	1		0.158	0.158	0.158	0.019	0.019		
	3	1		0.165	0.165	0.165	0.020	0.020		
60	1	1		0.161	0.161	0.161	0.020	0.020	0.020	0.001
	2	1		0.158	0.158	0.158	0.019	0.019		
	3	1		0.165	0.165	0.165	0.020	0.020		
90	1	1		0.161	0.161	0.161	0.020	0.020	0.020	0.001
	2	1		0.158	0.158	0.158	0.019	0.019		
	3	1		0.165	0.165	0.165	0.020	0.020		

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Table 28 Storage stability of purify protease from *Aspergillus fumigatus* SS0509

Fraction 19 of purified protease after gel filtration was collected. The purified protease was incubated at 55°C for 0- 90 minutes. In each fraction, the protein concentration was measured twice as tube # 1 and tube# 2 and then, the means was calculated and the amount of amino acid was estimated from standard curve of Bradford Protein Assay. Enzyme unit, total enzyme and specific activity were calculated using followed equation. Enzyme unit = 3.5(ml) x X (mg) / 0.5(ml). Total enzyme = Enzyme unit x Volume (ml) and Specific activity = Total enzyme/Total protein.

Protease activity											
time	OD595			Amount of amino acid	Enzyme unit	Total enzyme	Mean of	SD of	Specific activity	Mean of	SD of
	tube # 1	tube # 2	Mean	(mg/ml)	(umole/min/ml)	(unit)	Total enzyme (unit)	Total enzyme	(unit/mg)	Specific activity (unit/mg)	Specific activity (unit/mg)
0	0.032	0.021	0.026	0.000	0.000	0.000	0.000	0.000	0.00	0.000	0.000
	0.022	0.030	0.026	0.000	0.000	0.000			0.00		
	0.041	0.024	0.032	0.000	0.000	0.000			0.00		
15	0.168	0.167	0.167	0.020	0.014	0.014	0.014	0.006	7.15	7.37	0.230
	0.160	0.168	0.164	0.020	0.014	0.014			7.35		
	0.174	0.181	0.177	0.022	0.015	0.015			7.61		
30	0.182	0.179	0.180	0.022	0.015	0.015	0.015	0.001	7.75	7.92	0.210
	0.180	0.181	0.180	0.022	0.015	0.015			8.15		
	0.184	0.182	0.183	0.022	0.015	0.015			7.86		

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Table 28 Storage stability of purify protease from *Aspergillus fumigatus* SS0509 (continuous)

Fraction 19 of purified protease after gel filtration was collected. The purified protease was incubated at 55°C for 0- 90 minutes. In each fraction, the protein concentration was measured twice as tube # 1 and tube# 2 and then, the means was calculated and the amount of amino acid was estimated from standard curve of Bradford Protein Assay. Enzyme unit, total enzyme and specific activity were calculated using followed equation. Enzyme unit = 3.5(ml) x X (mg) / 0.5(ml). Total enzyme = Enzyme unit x Volume (ml) and Specific activity = Total enzyme/Total protein.

Protease activity											
time	OD595			Amount of amino acid (mg/ml)	Enzyme unit (umole/min/ml)	Total enzyme (unit)	Mean of Total enzyme (unit)	SD of Total enzyme	Specific activity (unit/mg)	Mean of Specific activity (unit/mg)	SD of Specific activity (unit/mg)
	tube # 1	tube # 2	Mean								
45	0.175	0.185	0.180	0.022	0.015	0.015			7.72		
	0.178	0.180	0.179	0.022	0.015	0.015	0.015	0.001	8.08	7.80	0.256
	0.178	0.176	0.177	0.022	0.015	0.015			7.58		
60	0.177	0.175	0.176	0.022	0.015	0.015			7.54		
	0.178	0.175	0.176	0.022	0.015	0.015	0.015	0.001	7.96	7.73	0.214
	0.182	0.176	0.179	0.022	0.015	0.015			7.68		
90	0.154	0.155	0.154	0.019	0.015	0.013			6.55		
	0.145	0.145	0.145	0.017	0.012	0.012	0.012	0.005	6.43	6.55	0.115
	0.158	0.156	0.157	0.019	0.013	0.013			6.66		

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Academic Presentation

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