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PURIFICATION AND CHARACTERIZATION OF XYLANASE FROM ENDOPHYTIC FUNGUS *Alternaria alternata* ISOLATE PTRa9

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จุลินทรีย์เป็นแหล่งสร้างไซลาเนสที่สำคัญ และมีความสำคัญทางการเกษตรและอุตสาหกรรม ในงานวิจัยนี้ได้มุ่งเน้นการศึกษาไซลาเนสสร้างโดยราเอนโดไฟต์ ทดสอบราเอนโดไฟต์จำนวน 54 ไอโซเลต ที่แยกราเอนโดไฟต์จากใบพืชเปล้าใหญ่ ปาล์ม และไม้โกงกาง พบว่ามีราเพียง 31 ไอโซเลตที่สร้างบริเวณใสรอบโคโลนีที่เจริญบนอาหารเลี้ยงเชื้อที่มีไซแลนเป็นองค์ประกอบ ซึ่งแสดงความสามารถสร้างไซลาเนสได้ ในการคัดเลือกขั้นปฐมภูมิ และตรวจพบว่าราไอโซเลต PTRa9 มีค่ากิจกรรมของไซลาเนสสูงสุดในการคัดเลือกขั้นทุติยภูมิ สภาวะที่ราเอนโดไฟต์ PTRa9 มีกิจกรรมไซลาเนสสูงสุดเมื่อใช้รำข้าวที่ความเข้มข้น 2 เปอร์เซ็นต์เป็นแหล่งคาร์บอน แอมโมเนียมซัลเฟตที่ความเข้มข้น 0.1 เปอร์เซ็นต์เป็นแหล่งไนโตรเจน บ่มที่ระยะเวลา 4 วัน ทำไซลาเนสให้บริสุทธิ์ประกอบด้วยการตกตะกอนโปรตีนด้วยเกลือแอมโมเนียมซัลเฟต โครมาโทกราฟีแบบแลกเปลี่ยนประจุ และโครมาโทกราฟีแบบแยกตามขนาดโมเลกุล พบว่ามีค่ากิจกรรมแบบจำเพาะเพิ่มสูงขึ้นถึง 161.1 ยูนิต/มิลลิกรัม โปรตีน มีความบริสุทธิ์เพิ่มขึ้น 60.8 เท่า ความเสถียรที่อุณหภูมิ 40 องศาเซลเซียสเป็นเวลา 20 นาที ที่ช่วงค่าความเป็นกรดด่างตั้งแต่ 3 ถึง 11 เป็นระยะเวลา 60 นาที ให้กิจกรรมไซลาเนสโดยเทียบเคียงมีค่าเท่ากับ 131.27 เปอร์เซ็นต์และมากกว่า 100 เปอร์เซ็นต์ของชุดควบคุมตามลำดับ ปรอทมีฤทธิ์ยับยั้งการทำงานของไซลาเนสได้สูง ขณะที่แมกนีเซียม แมงกานีส คอปเปอร์ สังกะสี เหล็ก และ อีดีทีเอ ยับยั้งการทำงานของไซลาเนสได้ปานกลาง ค่า K_m มีค่าเท่ากับ 2.369 มิลลิกรัม/มิลลิลิตร และ V_{max} มีค่าเท่ากับ 2.142 ไมโครโมล/นาที/มิลลิกรัมโปรตีน ไซลาเนสบริสุทธิ์ของราเอนโดไฟต์ไอโซเลต PTRa9 มีน้ำหนักโมเลกุลเท่ากับ 54.8 กิโลดาลตัน

สาขาวิชา เทคโนโลยีชีวภาพ ลายมือชื่อนิสิต .....

ปีการศึกษา 2553 ลายมือชื่อ อ.ที่ปรึกษาวิทยานิพนธ์หลัก .....

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NICHAWEE WIPUSAREE: PURIFICATION AND CHARACTERIZATION OF XYLANASE FROM ENDOPHYTIC FUNGUS *Alternaria alternate* ISOATE PTRa9. ADVISOR: APHICHART KARNCHANATAT, Ph.D., CO-ADVISOR: ASSOC. PROF. PRAKITSIN SIHANONTH, Ph.D., ASST. PROF. JITTRA PIAPUKIEW, Ph.D., 79 pp.

Microorganisms were the major sources of xylanase production due to there were important in agriculture and industrial. The aim of this research was to study the xylanase production from endophytic fungi. Totally 54 endophytes isolated from *Corton oblongifolius* Roxb., Palm and mangrove leaves were tested for xylanase activity. There were only 31 endophytes isolated produce clear zone around colony in selective xylan agar medium which indicated xylanase-producing in primary screening. Endophyte isolate PTRa9 yield the highest xylanase-production in secondary screening. The optimal conditions that PTRa9 produced highest xylanase activity when 2% rice bran and 0.1% $(NH_4)_2SO_4$ as carbon and grew in the medium supplemented with nitrogen source, respectively for 4 days. Xylanase purification was done by ammonium sulfate precipitation, ion exchange chromatography and gel filtration chromatography which resulted in an apparent increase in specific activity as 161.1 U/mg protein and 60.8 times of purity. Xylanase characterization includes thermal stability, pH stability, metal ions, kinetic analysis and molecular weight were studied and the result showed that at 40^{o}C for 20 min, at pH range 3 to 11 for 60 min had relative xylanase activity of 131.27 and higher than 100 %, respectively. PTRa9 xylanase was strongly inhibited by Hg^{2+}, and was moderately inhibited by Mg^{2+}, Mn^{2+}, Cu^{2+}, Zn^{2+}, Fe^{2+} and EDTA. For kinetic analysis, it had K_m of 2.369 mg/ml and V_{max} of 2.142 μl/min/mg protein. Molecular weight of Xylanase of PTRa9 was of 54.8 kDa.

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CONTENTS

	Page
ABSTRACT IN THAI…….....	iv
ABSTRACT IN ENGLISH……..…...	v
ACKNOWLEDGEMENTS..……..….	vi
CONTENTS……...……...	vii
LIST OF TABLES……..…….....	x
LIST OF FIGURES……..…...	xi
LIST OF ABBREVIATIONS…….……	xiv
CHAPTER	
I INTRODUCTION…….	1
II LITERATURE REVIEWS……..…….	5
2.1 Xylans: an overview.……..……	5
2.2 Xylanases…….....……..……	6
2.3 Xylanase production……..……	8
2.4 Purification and characterization of xylanase....……	13
2.5 Biotechnological potentials of xylanase……...……	15
2.5.1 Pulp and paper bleaching industry…….	15
2.5.2 Animal feed supplement…….……...	17
2.5.3 Baking industry…….…….……	18
2.5.4 Beer making……...……...…….……	18
2.5.5 Treating plant wastes …….……	18
2.6 Endophytes ……...……	18
2.6.1 Historical perspective…….	18
2.6.1.1 Definition…….....….	18
2.6.1.2 Origin and evolution…….....….	19
2.6.2 Endophytic fungi as producers of xylanase..…….	21

CHAPTER	Page
III EXPERIMENTAL……..…...……..…	22
3.1 Chemical materials…….…...……..	22
3.2 Equipment…….…….…...……..	23
3.3 Tested Endophytic fungi ……..…….…...……..	24
3.3.1 Isolation of endophytic fungi from *Croton oblongifolius* leaves……..	24
3.3.2 Endophytic fungi from culture collection…….	24
3.4 Screening of endophytic fungi for extracellular xylanase production…….……..	24
3.5 Assay for xylanase activity...…….…...……..	25
3.6 Identification of endophytic fungi…….…...……..	25
3.7 Xylanase production…….....…….…...……..	26
3.8 Determination protein content ……...…….…...……	26
3.9 Xylanase purification…….....…….…...……..	27
3.9.1 $(NH_4)_2SO_4$ Precipitation……..……..…….…..	27
3.9.2 DEAE- cellulose ion exchange chromatography..…...……	27
3.9.3 Superdex-75 gel filtration chromatography.....……	27
3.10 Determination enzyme purity by native-PAGE and xylanase activity staining…...….....…….…...……..	28
3.10.1 Non-denaturating gel electrophoresis……	28
3.10.2 Coomassie blue staining of native SDS-PAGE ……	28
3.10.3 Staining for xylanase activity.....…….……	28
3.11 Determination molecular weight of SDS PAGE ……..	29
3.12 Thermostability on the xylanase activity……...……..	29
3.13 pH stability on the xylanase activity…….…..……..	29
3.14 Effect of metal ions on the xylanase activity…..……..	30
3.15 Determination of kinetic parameters……...……..	30

CHAPTER	Page
IV RESULT AND DISCUSSION …….……..……..	31
4.1 Isolation and screening of xylanase producing from endophytic fungi…….……...	31
4.2 Identification of endophytic fungi…..…….…...……..	32
4.3 Production of xylanase ……...……	34
4.3.1 Effects of various carbon source on xylanase production….....	34
4.3.2 Effects of various nitrogen source on xylanase production…..	36
4.4 Xylanse purification…...……...…….....	37
4.5 Determination of enzyme purity and protein pattern by native-PAGE……...…….	40
4.6 Determination molecular weight of SDS-PAGE……...	40
4.7 Thermostability on the xylanase activity……....……...	41
4.8 pH stability on the xylanase activity……..…..……..	44
4.9 Effect of metals and reagents…….….	45
4.10 Determination kinetic parameters……	46
V CONCLUSION…….……...……..	47
REFERENCES…….	48
APPENDICES……..	65
Appendix A…….	66
Appendix B…….	69
Appendix C…….	73
Appendix D…….	77
BIOGRAPHY……...	79

LIST OF TABLES

Table		Page
4.1	Enrichment summary for the xylanase from *Alternaria alternate* isolated PTRa9…….	40
4.2	The effect of divalent cation salts and the chelating agent EDTA on the xylanase activity of the enriched xylanase fraction from *Alternaria alternata* isolated PTRa9…….	45
1A	Various carbon sources in modified basal medium…..…….……..	67
2A	Various nitrogen sources in modified basal medium…….…….……..	68
1C	Preparation of standard curve of xylose……..…..…..…….……..	73
2C	Preparation of standard curve of BSA……...……..…..…….……..	75

LIST OF FIGURES

Figure **Page**

2.1 (A) Xylanolytic enzymes hydrolyzing the backbone of xylans. (B) β-D-xylosidases convert xylo-oligosaccharide into xylose monomers…… 7

2.2 Lignin associated xylan fraction removal from pulp structure by the action of xylanase.……...…….. 16

4.1 Representative plate showing the growth of one of the selected xylanase-positive endophytic fungi (isolate PTRa9) on 1% birch wood xylan……. 32

4.2 Morphology character of xylanse-positive endophytic fungi isolated PTRa9 showing colony growth on PDA plate as (A) top view (B) bottom view…….. 33

4.3 Scanning electron micrograph of *Alternaria alternata* isolated PTRa9. (A); bar 10 μm, (B); bar 5 μm, and (C); bar 1 μm……... 33

4.4 Time course of xylanase production by endophytic fungus *Alternaria alternata* isolated PTRa9 grown on various 1% carbon source. Values represent the average of three replicates ± SE. Chaff (●); baggasse (○); rice bran (▼); rice straw (Δ); and sawdust (■)……. 34

4.5 Effect of rice bran concentration on xylanase activity of endophytic fungus *Alternaria alternata* isolated PTRa9. Values represent the average of three replicates ± SE. 0.5% (●); 1.0% (o); and 2.0% (▼)…. 35

4.6 Effect of nitrogen source and on xylanase activity of endophytic fungus *Alternaria alternata* isolated PTRa9**.** Values represent the average of three replicates ± SE. 0.1% (white); 0.2% (gray); and 0.5% (black)…… 36

Figure **Page**

4.7 Profile of the enrichment of the *Alternaria alternata* isolated PTRa9 extracellular xylanase extract by; (A) DEAE-cellulose ion-exchange chromatography of the ammonium sulfate cut fraction (400 mg protein) eluted in 50 mM Tris-HCl (pH 7.0) with a 0 - 1 M NaCl linear gradient; and (B) Superdex-75 gel chromatography of the post-DEAE-cellulose xylanase fraction (50 mg) eluted in 100 mM NaCl / 50 mM Tris-HCl (pH 7.0). For panels A; absorbance at 280 nm (○), xylanase activity (●) and panels B; absorbance at 280 nm (●), xylanase activity (○)……. 39

4.8 (A) Coomassie blue stained native-PAGE analysis of the *Alternaria alternata* isolated PTRa9 xylanase fractions from each step of the enrichment and stained for protein by coomassie blue (Lanes 1 - 4) or for xylanase enzyme activiy (Lane 5). Lane 1, crude enzyme (20 μg of protein); Lane 2, ammonium sulfate cut fraction (20 μg of protein); Lane 3, post-DEAE-cellulose xylanase fraction (15 μg of protein); Lanes 4 & 5, enriched xylanase fraction (post-Superdex-75) (10 μg of protein). (B) Reducing SDS-PAGE analysis, after coomassie blue staining, of the enriched xylanase fraction (post-Superdex-75) from *Alternaria alternata* isolated PTRa9: Lane 1, Low molecular weight protein markers; Lane 2, enriched xylanase fraction (5 μg of protein).. 41

4.9 Thermostability on xylanase activity from *Alternaria alternata* isolated PTRa9 (A and B) was assayed in 50 mM Tris-HCl (pH 7.0) at (□); 30 ^{o}C, (■); 40 ^{o}C, (▼); 50 ^{o}C and (●); 60 ^{o}C. For both panels A and B the data are shown as the mean of three replicates ± SE. Means with a different lower case letter above them are significantly different ($p<0.05$).....……. 43

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Figure **Page**

4.10 The pH stability on the activity of the enriched xylanase fraction from *Alternaria alternata* isolated PTRa9. The pH stability on xylanase activity was evaluated in (all 20 mM) (⋯); glycine-HCl buffer for pH 2-4, (—); sodium acetate buffer for 4-6, (−••); potassium phosphate buffer for 6-8, (− −); tris-HCl buffer for 8-10 and (•••); glycine-NaOH buffer for 10-12 for time (●); 30 min, (o); 60 min and (▼); 90 min. The data are shown as the mean of three replicates ± SE…….. 44

4.11 Lineweaver-Burk plot of the enriched xylanase fraction from *Alternaria alternata* isolated PTRa9. The xylanase fraction was incubated with different concentrations of birch wood xylan (1.25-20 mg) as substrate. Data are represent as mean of three replicates ± SE.. 46

1C Standard curve of xylose…….…….. 74

2C Standard curve of protein.....…….. 76

LIST OF ABBREVIATIONS

%	percentage
°C	degree celsius
μg	microgram
μl	microlitre
A	absorbance
BLAST	basic local alignment search tool
BSA	bovine serum albumin
cm	centimeter
Da	Dalton
EDTA	ethylenediamine tetraacetic acid
g	gram
hr	hour
IC_{50}	The half maximal inhibitory
kDa	kilodaton
K_m	Michaelis-Menten constant
l	litre
M	molar
mA	milliampere
mg	milligram
min	minute
ml	milliliter
mM	millimolar
MW	molecular weight
N	normal
nm	nanometer
NaCl	sodium chloride
PAGE	polyacrylamide gel electrophoresis
rpm	revolution per minute
SDS	sodium dodecyl sulfate

SEM	standard error of the mean
TEMED	*N*,*N*,*N'*,*N'*-tetramethyl ethylenediamine
TCA	trichloroacetic acid
Tris	Tris (hydroxymethyl)aminomethane
U	Unit activity
V_{max}	maximum velocity
W/V	weight by volume

CHAPTER l

INTRODUCTION

Enzymes are distinct biological polymers that catalyze the chemical reactions and convert substrates to particular products. They are specific in function and speed up reactions by providing alternative pathways of lower activation energy without being consumed. These are the fundamental elements for biochemical processes and utilized in a number of food processing industries (Haq *et al*., 2006). The manipulation of biotechnological techniques have played an important role in the recent advances occurred in baking industry. Thailand is an agricultural country; resultantly agro-industrial wastes and by-products are in abundance here. Rice bran wheat bran, corn cobs, sugar cane bagasse, etc. are some of the prominent waste materials from the allied food and energy industries. These waste materials, if not handled properly, certainly are source of environmental pollution. Producers and stack holders are taking interest to utilize even a bit of resources to cope with the economic cost of finished products. The processing units generating agro-waste materials/by-products are struggling hard for their conversion into value added products. The agricultural waste materials if manipulated properly can play a significant role in the economic uplift of a state. There is an increasing gist to utilize such neglected materials in the production of enzymes which can be employed further processing (Mohammadi *et al*., 2006; Okafor *et al*., 2007).

Xylan is the second most abundant biopolymer after cellulose and the major hemicellulosic polysaccharide found in the plant cell wall (Timell, 1967). It has a complex structure consisting of β-1,4-linked xylose residues in the backbone to which short side chains of O-acetyl, α-L-arabinofuranosyl, D-α-glucuronic and phenolic acid residues are attached (Coughlan and Hazlewood, 1993). This biopolymer constitutes one third of all renewable organic carbon sources on earth (Poorna and Prema, 2007). Considerable amount of xylan was found in solid agricultural and agro-industrial residues, as well as in effluents released during wood processing, which, due to frequent inappropriate discard, cause great damage to the ecosystem (Biely, 1985; Prade, 1995). Hydrolysis of xylan was an important step towards the proper utilization of

lignocellulosic material in nature (Poorna and Prema, 2007). Chemical hydrolysis of lignocelluloses results in harzardous byproducts, forcing the use of microbial enzymes which were specific in action for xylan hydrolysis, and are an environmentally friendly option (Biely, 1985). Due to structural heterogeneity of xylan, complete degradation of this biopolymer requires synergistic action of different xylanolytic enzymes such as endo-xylanase, β-xylosidase, α-glucuronidase, α-arabinofuranosidase and esterase (Poorna and Prema, 2007). Among these, one of the most important was endo-1,4-β-xylanase (1,4-β-D-xylan xylohydrolase, EC 3.2.1.8), which is also known as xylanase and it initiates the degradation of xylan into xylose and xylooligosaccharides of different sizes (Collin *et al*., 2005).

There were different types of xylanases which varying in substrate specificities, primary sequences, folds and physicochemical properties (Wong *et al*., 1988; Collins *et al*., 2005). These were produced by a number of bacteria and fungi (Kulkarni *et al*., 1999; Subramaniyan and Prema, 2002). Filamentous fungi have been reported to be interesting and good producers of xylanases from industrial point of view due to extracellular release of the enzymes, higher yield compared to yeast and bacteria and also the production of several auxiliary enzymes that are necessary for debranching of substituted xylans (Haltrich *et al*., 1996). However, fungal xylanases were generally associated with concurrent production of cellulases (Steiner *et al*., 1987). Traditionally, the application of xylanases in conjunction with cellulocytic enzymes has been mainly considered for the bioconversion of lignocellulosic materials, especially residues and wastes produced by agriculture and forestry to produce highervalue products such as ethanol fuel and other chemicals (Biely, 1985; Mandels, 1985). Other potential applications of crude xylanase preparations containing cellulases, β-glucanases, or pectinases include bread making, fruit juice extraction, beverage preparation, increasing digestibility of animal feed, converting lignocellulosic substances to feedstock and fiber separation (Beg *et al*., 2001; Subramaniyan and Prema, 2002), paper and pulp industries (Bajpai, 1997; Kenealy and Jeffries, 2003). However, in the paper and pulp industry, cellulase-free xylanases are required to avoid adverse effects of damaging the pulp fibres (Haltrich *et al*., 1996). Moreover, specific xylanases could be used in the prebleaching of craft pulps in order to reduce the amount of chlorine required to achieve target brightness (Viikari *et al*., 1994)

and consequently reduce the chloroorganics released in the effluent (Christakopoulos *et al*., 1996). Cellulase-free xylanases or xylanases containing negligible cellulose activity could be obtained by using suitable separation methods or using genetically engineered organisms to produce exclusively xylanase or by applying screening methods and selection of appropriate growth conditions (Balakrishnan *et al*., 1992).

Characterization of xylanolytic enzymes was important for their biotechnological applications. The cost of the enzyme was one of the main factors determining the economics of a process and this can be partially achieved by optimizing fermentation media (Shah and Madamwar, 2005). Several industrial processes coud be carried out using whole cells as sources of enzymes, but efficiency can be improved by using isolated and purified enzymes. Criteria for selection of a particular method of isolation and purification depend on the end use. A high state of purity is generally not required in food processing, detergent as well as paper and pulp industries, but it may be necessary to exclude certain contaminating enzymes (Price and Stevens, 1999). Recently, interest in microbial xylanases had been markedly increased due to their potential in biotechnological applications and attempts were being made to isolate new strains (Lee-Chiang *et al*., 2006; Schmeisser *et al*., 2007).

Endophyte was a term that describes as an organism living within a plant (Hawksworth *et al*., 1995). However, the actual meanings vary depending on researchers and had not been unified yet (Hawksworth et al., 1995). Although the existence of endophytes had been known for a long time (Lewis, 1924), many endophytic fungi had been isolated and studied only in the last two decades (Findlay *et al*., 1995; Hata and Futai, 1995; Hata and Futai, 1996; Koga *et al*., 1996; Hata *et al*., 1998). An endophyte was reported to produce some substances harmful to predators of the host plant (Bacon *et al*., 1977; Clay, 1989). Recently, *Acremonium* sp. isolated from *Taxus baccata* was found to produce leucinostatin A, which was active against breast cancer (Strobel, 1997). Some of the endophytes were also known to be producers of bioactive compounds (Findlay *et al*., 1995; Findlay *et al*., 1997; Ju *et al*., 1998; Lu *et al*., 2000; Rodrigues et al., 2000). However, very few microorganisms living in plants, e.g., the ericoid mycorrhizal fungus *Hymenoscyphus ericae* producing β-1,4-endoxylanase (Burkeand Cairney, 1997), were

reported to be xylanase producers even if one of the major components of the plant cell wall is xylan.

In this study, we focus on the inside part of plants as a new source for isolating microorganisms producing xylanase and describe the purification and characterization of a xylanase from endophytic fungi.

CHAPTER II

LITERATURE REVIEWS

2.1 Xylans: an overview

Xylan was the major type of hemicellulose and its hydrolysis depends on two classes of enzymes. The Endoxylanases (EC 3.2.1.8) cleave the xylan backbone into smaller parts like oligosaccharides that are further degraded to xylose by xylosidases (EC 3.2.1.37). During the last few decades, great interest had been developed in xylan and its hydrolytic enzymatic complex, for application in bread production, supplement in animal feed, preparation of drinks, textiles, bleaching of cellulose pulp, ethanol and xylitol production. Xylan was the second most abundant polysaccharide and major component in plant cell wall that consists of β-1,4-linked xylopyranosyl residues (Puls, 1997). The structure of xylans found in cell walls of plants can differ greatly depending on their origin and different structures attached to the xylan backbone. Although most of the xylans had branched structures, however some linear polysaccharides have been isolated (De Vries and Visser, 2001). Lignin is bound to xylans by an ester linkage to 4-*O*-methyl-D-glucuronic acid residues. These linkages in lignocellulose could be disrupted by using various pretreatment methods that expose most of the polysaccharide components to enzymatic hydrolysis (Chang *et al*., 1981). Selective hydrolysis of xylan has been observed when purified (Paice and Jurasek, 1984) and crude enzyme was applied in which cellulases were inhibited (Mora *et al.,* 1986).

In all of these cases, complete removal of the xylosyl residues from the fibers was not achieved. The residual xylosyl residues might be inaccessible to xylanolytic enzymes due to the presence of substituents; modification of fiber synthesis and occurrence of xylans enclosed by other polysaccharides. There were some suggestion that cellulose was protected from cellulases due to xylan and mannan (Sinner *et al*., 1979). When xylan or mannan was selectively removed from delignified fiber, the residual cellulose becomes accessible to cellulase hydrolysis. However, a similar prehydrolysis of cellulose or mannan did not improve accessibility of xylan to xylanases. Selective removal of xylans increases accessibility of other polysaccharides due to increased fiber porosity that is

positively correlated with cellulose hydrolysis in pretreated fibers (Grethlein, 1985; Wong *et al*., 1988).

In cereals, arabinoxylans are among the major non-starch polysaccharides. They constitute 4-8% of barley kernel and represent 25 and 70% of the cell wall polysaccharides of endosperm and aleurone layer, respectively. The arabinoxylans are partly water-soluble and result in highly viscous aqueous solution (Dervilly-Pinel *et al*., 2001). Cereal xylans contain large quantities of Larabinose and therefore, often referred as arabinoxylans whereas, hardwood xylans are often referred to as glucuronoxylans due to large amount of D-glucoronic acid attached to the backbone. Arabinose is connected to the backbone of xylan *via* α-1,2 or α-1,3 linkage either as single residues or as short side chains. The side chains also contain xylose α-1,2-linked to arabinose, and galactose, which can be either β-1,5-linked to arabinose or α-1,4-linked to xylose (De Vries and Visser, 2001).

2.2 Xylanases

Xylanases are genetically single chain glycoproteins, ranging from 6-80 kDa, active between pH 4.5-6.5, at 40-60 °C. Xylanases from different sources differ in their requirements for temperature, pH etc. for optimum functioning. The complete enzymatic hydrolysis of xylan into its constituent monosaccharides requires the synergistic action of a group of xylanolytic enzymes. This is due to the fact that xylans from different sources exhibit a significant variation in composition and structure (Hazlewood and Gilbert, 1993; Cesar and Mrsa, 1996; Latif *et al*., 2006). The most important enzyme is endo-1,4-xylanase (EC 3.2.1.8) that initiates the conversion of xylan into xylooligosaccharides. Xylosidase, debranching enzymes (L-arabinofuranosidase and glucuronidase) and esterases (acetyl xylan esterase, feruloyl esterase) allow the complete degradation of the xylooligosaccharides to their monomeric constituents, *shown* in *Figure 2.1* (Jeffries, 1996; Biely *et al*., 1997; Subramaniyan and Prema, 1998). The heterogeneity and complexity of xylan has resulted in an abundance of diverse xylanases with varying specificities, primary sequences and structures. Wong *et al.* (1988) classified xylanases into two groups on the basis of their physicochemical properties: (*i*) having low molecular mass (<30 kDa) and basic pI, and (*ii*) having high molecular mass (>30 kDa) and acidic pI. However, many xylanases, in particular fungal xylanases, cannot be

classified by this system. A more complete classification system had been introduced which allows the classification of not only xylanases, but also of glycosidases in general. This system was now become the standard means for the classification of these enzymes. It is based on primary structure comparison of the catalytic domains only and classifies the enzymes in families of related sequences (Henrissat and Coutinho, 2001). Different enzymes might be more effective in the hydrolysis of xylobiose, substituted xylo-oligosaccharides, xylosyl substituents or oligosaccharides containing xylosyl and other residues. The different forms may also had varying abilities to interact with xylanases in xylan hydrolysis (Reilly, 1981). Three apparent xylosidases had been classified as "exoxylanases" because they have detectable activity on xylan. Two of these enzymes apparently lack transferase activity and one enzyme causes configuration inversion (initially yield α-Dxylose during hydrolysis), which was the characteristic used to distinguish between β-glucosidases and exoglucanases (Reilly, 1981; Eriksson and Wood, 1985).

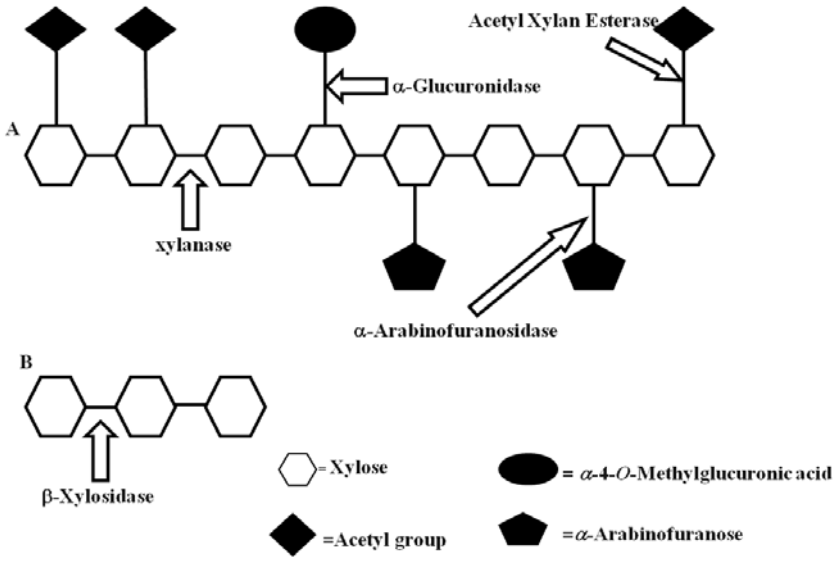


Figure 2.1 (A) Xylanolytic enzymes hydrolyzing the backbone of xylans. (B) β-D-xylosidases convert xylo-oligosaccharide into xylose monomers.

Furthermore, an exoglucanase from *Trichoderma viride* had been shown to attack xylan in an endwise fashion to initially yield xylobiose. Exoxylanases increase the rate of xylan hydrolysis by attacking large xylo-oligosaccharides, that were released by endoxylanases and were ineffectively hydrolyzed by β-xylosidases. This form of cooperation would not be expected to increase the extent of hydrolysis unless other factors are involved *e.g.* accessibility of xylosidic linkages in short and/or branched xylo-oligosaccharides, reduction of product inhibitions, or amounts of extracellular β -xylosidases (Shikata and Nisizawa, 1975). Xylans were usually cleaved at unsubstituted regions to yield mixtures of unsubstituted xylo-oligomers, as well as short and long chain substituted xylooligomers. Removal of the substituents groups by auxiliary enzymes creates new substrates for endoxylanase (EC 3.2.1.8) action (Si, 1997; Maheshwari *et al*., 2000).

2.3 Xylanase production

The various biotechnological techniques like submerged and solid state fermentation were employed for xylanase biosynthesis (Cai *et al*., 1998; Gawande and Kamat, 1999; Kansoh and Gammel, 2001). The submerged fermentation was the most beneficial as compared to other techniques due to more nutrients availability, sufficient oxygen supply and less time required for the fermentation (Hoq *et al*., 1994; Gomes *et al*., 1994; Veluz *et al*., 1999; Bim and Franco, 2000; Gouda, 2000). The production of microbial xylanases was preferred over plant and animal sources because of their availability, structural stability and easy genetic manipulation (Bilgrami and Pandy, 1992).

Over the years, a number of organisms including the strains of *Penicillium* spp (Fadel and Fouda, 1993; Gasper *et al*., 1997), *Trichoderma reesei* (Liu *et al*., 1999), *Aspergillus nidulans* (Pinaga *et al*., 1994; Ganga *et al*., 1998), *Aspergillus kawachii* (Ito *et al*., 2000), *Streptomyces* (Patel *et al*., 1994; Kansoh and Gammel, 2001) and *Bacilus pumilus* (Rashid, 1999) were being manipulted for xylanase biosynthesis. However, *A. niger* is described as the most potent organism for xylanase biosynthesis (Wang *et al*., 1998; Chen *et al*., 1999; Wu *et al*., 2000; Haq *et al*., 2002b). *Aspergillus niger* has been investigated for xylanase synthesis and Gawande and Kamat (1999) reported maximum xylanase activity (26.7 IU/ml) after 48 hours of incubation whereas, Chen *et al*. (1999)

reported maximum enzyme recovery (357.2 U/ml) at 28-32 °C after 60 hrs of incubation. Palma *et al*. (1996) and Kohli *et al*. (2001) observed maximum enzyme production after 96 hrs. However, in another study conducted by Ismail (1996), *Aspergillus niger* A-20 produced highly active enzyme system in shake flask culture when the fermentation was carried out for 5 days.

Agricultural waste materials/by products like wheat bran, corn cobs, sugar cane bagasse , rice husk, rice straw and oat straw have been used by many scientists for xylanase synthesis (Siedenberg *et al*., 1998; Christov *et al*., 1999; Gawande and Kamat, 1999; Haq *et al*., 2002a). When sugarcane bagasse was used as the subsratse for the crude enzyme production of highest yields of (0.26 g/g) reducing sugar (Sawarachorn, 1999). During the investigations on biosynthesis of xylanase from *Aspergillus niger* using pure xylan polymer, wheat bran, sugar cane bagasse and rice straw, it was observed that wheat bran gave maximum activity of xylanase as compared to other substrates (Ferriera *et al*., 1999; Park *et al*., 2002; Haq *et al*., 2002a). Grajek and Gervais, (1987) produced D-xylanase by thermophillic fungi using different culture methods. Seven strains of fungi were examined for their ability to produce xylanase in liquid and solid state fermentation. It was found that the best organisms for xylanase synthesis were *Humicola lanuginosa* and *Sporotrichum thermophile.*

Mutant strains of *Aspergillus niger* NCIM 1207 when tested for xylanase, CMCase and β-glucosidase production, it secreted low levels of all enzymes in the culture broth. However, mutants UV-10 and UVIII-39 showed almost two times enhanced productivity of xylanase over the wild strain in shake flask culture (Gokhale *et al*., 1988). Biswas *et al*. (1990) produced xylanase from *Aspergillus ochraceus* employing both fermentation methods i.e. liquid broth and solid state fermentation. The enzyme was purified by using ammonium sulphate precipitation and gel filtration. The optimum pH for the enzyme activity was found to be 6.0. Chen *et al*. (1990) screened a strain of *Aspergillus niger* C-2 from the soil and treated with UV and EMS to obtain mutant colonies and the conditions for submerged fermentation were studied. The produced enzyme had weak thermalstability and when incubated at 55 °C for one hour, it lost 60% of its stability.

Higher levels of xylanase and β-glucosidase had been obtained when *Aspergillus niger* NCIM 1207 was cultivated by submerged fermentation. Ammonium sulphate, ammonium-di-hydrogen-orthophosphate and corn-steep liquor were found to be the best carbon source for the enzymes production (Gokhale *et al*., 1991). In another study, Haq *et al*. (1993) obtained higher yields of xylanase and cellulase when *Aspergillus niger* was cultured on wheat bran. Culture conditions parameters for optimization enzyme synthesis rate such as diluents and nitrogen sources for recovery maximum cellulase and xylanase. Costa *et al*. (1994) investigated *Aspergillus niger* CCMI 850 for the production of xylanolytic enzymes in batch cultures with 4% xylan as carbon source. A maximum activity of 65 IU/ml of β-xylanase was observed during the experiment. Likewise, Siedenberg *et al*. (1997) studied xylanase production from *Aspergillus niger* awamori on synthetic medium and examinated the role of stirred tank and airlift tower top reactors, stirrer speed and phosphate concentration for xylanase biosynthesis. The highest xylanase activity was obtained at intermediate stirrer speed and low phosphate concentration. Veluz *et al*. (1999) screened 67 *Rhizopus sp*. strains on the capability xylanase productivity in solid state and liquid fermentation. The highest xylanase activity of 516 IU/ml for liquid culture was exhibited by *Rhizopus peka* strain in Philippine. For solid state fermentation, the highest activity of 7802 IU/ml was achieved by *Rhizopus sp*. MKU 32 in Thailand. The xylanase could be synthesized by *Trichoderma reesei* by providing proper fermentation conditions. The rate of synthesis depends on the nature of carbon source, its concentration and carbon to nitrogen ratio. It could be enhanced by reducing the carbon source and increasing C/N ratio (Liu *et al*., 1999).

Couri *et al*. (2000) synthesized a mixture of polygalacturonase, cellulase, xylanase and protease by using *Aspergillus niger* 3T5B8 when grown on various agricultural waste materials in solid state fermentation. Wheat bran as carbon source resulted in highest concentrations of mixture of enzymes (xylanase 30.62 U/ml). Haq *et al*. (2002a) conducted studies to investigate the impact of carbon and nitrogen sources and their concentrations on the synthesis of xylanase by mutant strain of *Aspergillus niger* GCBMX-45. The fungus produced maximum xylanase (2350 U/g) when 2% starch was used as carbon source whereas, 0.2% ammonium sulphate resulted in 2480 U/g xylanase activity. Xiong *et al*. (2005) studied the effect of L-arabinose-rich plant

hydrolysate for the synthesis of xylanase by *Trichoderma reesei* C-30. The researchers reported higher activities of xylanase in cultures containing oat husk and sugar beet pulp hydrolysate than on lactose. The xylanase activity was 9 times higher with supplement oat husk (510 IU/ml) than in lactose (60 IU/ml). In the case of batch cultivations on sugar beet pulp hydrolysate and lactose even higher xylanase activity (630IU/ml) were obtained.

Gokhale *et al*. (1986) obtained higher yields of the enzyme when *Aspergillus niger* NCIM 1207 was grown on either xylan (3%) or wheat bran (4%). The optimum pH and temperature for β-xylosidase were 4.5 and 65 ^{o}C respectively. Bailey *et al*. (1992) used *Aspergillus fumigatus* and *Aspergillus oryzae* for xylanase production using the xylan as carbon source. *Aspergillus fumigatus* produced higher levels of xylanase on insoluble xylan than on soluble oligosaccharides due to the presence of inhibitors. It produced high levels of xylanase at pH below 3.0. In case of birch wood xylan, *Aspergillus oryzae* produced less xylanase activities as compared to *Aspergillus fumigatus*. Cai *et al*. (1997) studied the potential of *Aspergillus niger* A3 for the production of xylanase in solid state fermentation. At the initial pH 4.6, temperature 28 °C, 1.0 ml spore suspension inoculum, ratio of wheat bran to bagasse 1: 1.5 and fermentation for 72 hours; optimum activity of xylanase (5147IU/g) was observed. Later, Ilieva and companions (1995) investigated the biosynthesis of xylanase by *Aspergillus awamori* K-1 through submerged and solid state fermentation. Wheat straw and mixture of milled maize stems and wheat bran (2: 1) showed highest xylanase activities at 30 °C and pH 3.5-4.0. The xylanase activity in culture filtrates for measuries reducing sugars by Somogyi Nelson method was 100 IU/ml. Marquez *et al.* (1999) investigated *Aspergillus flavus* for culture optimal conditions to synthesize xylanase and β-xylosidase. Highest xylanase activity (190 IU/ml) and β-xylosidase (35 IU/ml) were achieved when the culture medium was supplemented with 3% (w/v) corn cob as the carbon source. The enzyme showed maximum activity at pH levels between 5.5-6.

In another study, Chen *et al*. (1999) investigated 150 fungal strains for xylanase synthesis. Eight strains produced mainly xylanase with activity more than 100 IU/ml. The fungus strain No 49 produced the highest activities of xylanase and was identified as *Aspergillus niger*. The most suitable medium for the xylanase synthesis was NaNO3 1%

and wheat bran 1% that was prepared in Mandel's nutritional solution without NH_4SO_4 and Urea. After incubation by shake flask fermentation, the maximum activity (357.2 IU/ml) was obtained at 28-32 °C and 60 hours of incubation period. The optimum pH for the xylanase activity was 4.6 and the enzyme was able to retain its stability between the pH values 3-11. Gawande and Kamat (1999) separated xylanase using affinity precipitation and a commercial enteric polymer Eudragit S100 from crude culture filtrates of *Aspergillus* sp 5 and *Aspergillus* sp 44. The yields after precipitation were 85.3 and 82.7% and the purification folds in specific activity were 10.8 and 4.08 for *Aspergillus* sp 5 and *Aspergillus* sp 44, respectively. The analysis Zymograms revealed the recovery of three and two forms of xylanases from *Aspergillus* sp 5 and *Aspergillus* sp 44, respectively. Later, Gawande and Kamat (1999) studied *Aspergillus terreus* and *Aspergillus niger* for the production of xylanolytic enzymes. It was found that the both strains produced valuable amounts of xylanase with almost undetectable activities of cellulase under solid state fermentation conditions. The medium of *Aspergillus terreus* consisted wheat bran moistened with Mandel's and Stenberg mineral solution containing 0.1 % tryptone at 35 °C. *Aspergillus terreus* and *Aspergillus niger* produced of xylanase 68.9 IU/ml and 74.5 IU/ml, respectively.

During another research work, Gouda (2000) evaluated *Aspergillus tamarii* for the xylanase biosynthesis in both solid state and submerged fermentation methods. The fungus produced the maximum xylanase activity of 124.83U/g under solid state condition when corncobs were used as carbon source. In case of solid state fermentation, the optimum temperature was 35 °C while 30 °C was found to be the best maximum enzyme recovery during submerged fermentation. The cellulase free, endo 1,4-β-xylanase production was carried out at 50 °C and pH 8.5 by *Thermoactinomyces thalophilus*. The maximum xylanase activity was achieved in the fermentation medium using birch wood xylan as carbon source after 96 hours of growth (Kohli *et al*., 2001). Kansoh and Gammal (2001) isolated 24 strains of *Streptomyces* from soil and evaluated their ability to produce xylanase. Among all the isolates, *Streptomyces lividans* was found the most potent to produce xylanase. The optimum temperature and time of incubation for xylanase activity were at 30 °C for 5 days. Park *et al*. (2002) optimized conditions in solid state fermentation for xylanase synthesis. The activity of xylanase obtained after 5 days of

fermentation was 50171 IU/ml. Similarly, Haq *et al*. (2002a) used the mutant strain *Aspergillus niger* GCBMX-45 for enhanced xylanase production. The organism was found to produce high activities of xylanase (1845U/g). Later, it was selected for optimization of conditions for solid state fermentation. Ten gram wheat bran with 1: 1 dilution ratio was found to be the best for optimum xylanase synthesis. The xylanase production obtained from the mutant strain was 1.36 folds higher than the parental *Aspergillus niger*. Senthilkumar *et al*. (2005) used *Aspergillus fischeri* to produce alkali-stable xylanase at pH 9.0 using wheat bran as carbon source in solid state fermentation. The workers reported 1.9 fold boost in the enzyme synthesis (1024 U/g) when the medium contained (in g/l) $NaNO_2$ 7.0, K_2HPO_4 1.0, $MgSO_4$ 0.5 and yeast extract 5.0, in contrast to the initial level (540 U/g) at 72 hours of incubation.

2.4 Purification and characterization of xylanase

During enzyme production, in addition to the required enzyme, growth medium may have some undesirable metabolites of the micro-organisms which lead to lower enzyme activity. Purified enzymes exhibit higher activity, lesser risk of harmful substances and better application for the specific product. Moreover, characterization of enzyme is vital to achieve better performance in a particular application; as it provides information regarding suitable conditions for enzyme functioning.

Kavita *et al*. (2002) observed higher yields of xylanase (40 IU/ml) when grew *A. nidulans* KK-99 on a basal medium supplemented with wheat bran (2% w/v) and KNO_3 (at 0.15% N) at pH 10.0 and 37 °C. The enzyme was alkaline, thermostable and the optimum activity of partially purified xylanase was found at pH 8.0 and 55 °C temperature. It retained more than 80% of its activity at 55 °C over a broad pH range of 4.0-9.5. In another study, Taneja *et al*. (2002) used *A. nidulans* KK-99 to produce xylanase and reported an activity of 40IU/ml when incubated in basal medium containing 2% wheat bran (w/v) and KNO_3 (0.15% N) at pH 10.0 and 37 °C. The partially purified enzyme showed maximum activity at 55 °C and pH 8.0. The xylanase was active in a wide range of pH i.e. 4.0-9.5 for 1 hour at 55°C and retained almost 80% of its activity. Later, Anthony *et al*. (2005) used *A. niger* BRFM281 in shake flask fermentation for overproduction of XynB and reported a yield of 900 mg/L. The recombinant enzyme was

purified to 1.5-fold by immobilized metal affinity chromatography with enzyme recovery of 71%. During characterization of the
enzyme, it was found that it had molecular weight 23 kDa, optimum pH 5.5 and optimum temperature 50 °C. The enzyme showed stability over a pH range of 4.0 to 7.0 and temperature up to 50 °C.

The thermophilic fungus *Humicola grisea* var. *thermoidea* had potential to produce several extracellular enzymes (Chaves *et al*., 1989; Peralta *et al*., 1990; Tosil *et al*., 1993; Zimmermann *et al*., 1990). Monti *et al*. (2003) used this fungus to produce two forms of extracellular xylanase. After the production, a fraction of crude enzyme was purified by electroelution method. The molecular mass of the purified enzyme was found to be 61.8 kDa. An alkaline xylanase was purified from crude xylanase fermentation broth extracted in aqueous two phase system (ATPS) composed of 16% polyethylene glycol (PEG 6000) and 6.0% phosphate salt. A purification factor 57 and 41% yield of the enzyme activity were calculated for the system containing 16% PEG 6000, 8% K_2HPO_4 and 12% NaCl (Duarte *et al*., 1999). Eudragit *et al*. (1999) separated xylanase from *Aspergillus* spp. 5 and *Aspergillus* spp 44 crude culture filtrates by using affinity precipitation. The enzyme yield was 85.3 and 82.7% by *Aspergillus* sp 5 and *Aspergillus* sp 44, respectively. Carmona *et al*. (1998) described that when different steps of purification were carried out, total protein contents decreased with each purification step while the specific activity in response to each purification step exhibited an increase and the specific activity in the crude extract was 33.79 U/mg. In a research, crude filtrate secreted by *Aspergillus sp.* was purified by ammonium sulfate precipitation, it improved the purification yield, specific activity and purification fold as 62%, 51.89 and 2.15, respectively (Gawande and Kamat, 1999). Goulart *et al*. (2005) cultivated *Rhizopus stolonifer* on wheat bran to produce cellulase freee xylanase. The purified xylanase exhibited optimum pH and temperature as 6.0 and 45 °C respectively. Huang, (1991) reported the pH 5.5 and temperature 60 °C as optimum for highest xylanase activities Carmona *et al.* (1998) reported that when xylanase was purified by gel filtration using sephadex G-75, it yielded total protein, specific activity and purification fold as 2.0 mg, 635 and 18.8, respectively. Coelho and Carmona (2003) stated that xylanase exhibited high thermal stability in the pH range 4.5 to 10.5. Earlier Christakopoulos *et al*. (1996)

demonstrated that xylanase II from the fungus *Fusarium oxysporum* F3 was stable at a temperature 44-55 °C.

Damasco *et al*. (2000) produced cellulase free xylanase from *Thermomyces lanuginosus* in shake cultures by using corn cobs as carbon source and found that crude xylanase exhibited appreciable thermostability, retaining almost 50% of activity during 24 hours of incubation at 50 ^{o}C and about 50% of activity was present at 60 ^{o}C even after 4 hours of incubation. Damasco *et al*. (2000) reported that xyalanase was active in the broad range of pH and temperature, however the optimum pH and temperature were found as 6.0 and 75 ^{o}C respectively. Carmona *et al*. (1998) described the temperature 55 ^{o}C for the optimum activity of xylanase from *A. versicolor* and 70 ^{o}C from *Aspergillus niger*. However according to Uhlig, (1998), temperature range of 50-55 ^{o}C seems the best for optimum xylanase activity. The optimum pH for xylanase activity described in literature is in the range of 4.0-5.5, however some bacterial xylanases exhibit the optimal activity at pH range between 6.0 to 7.0. Coral *et al*. (2002) determined the molecular weight of xylanase produced by an *Aspergillus niger* strain was 36 kDa. These results are also supported by the findings of Kulkarni *et al*. (1999); according to them, xylanase from the microbial origin are single sub unit proteins with the molecular mass ranging from 8-145 kDa. Camacho and Aguillar (2003) estimated the molecular weight of xylanase from *Aspergillus sp* was 22 kDa. A study conducted by Sardar *et al*. (2000) indicated that when the purified xylanase was subjected to SDS-PAGE, the xylanase molecular weight was found to be 24 kDa.

2.5 Biotechnological potentials of xylanases

Xylanases have high industrial potential and used in various processes. The different areas of application of xylanases are summarized as follows.

2.5.1 Pulp and paper bleaching industry

In classical process, chemical bleaching processes use a large amount of chlorine and chlorine dioxide. Organic chlorine compounds formed during the bleaching of chemical pulp, these compounds arise mainly from the reactions between residual lignin present in wood fibres, causing the brown colour of unbleached pulp, and the chlorine used for bleaching. Organic chlorine compounds are well known to generate toxic, mutagenic, highly persistent chlorinated organic by-products which eventually pollute

water bodies (Viikari *et al*., 1994). For the same amount of active chlorine used, chlorine dioxide causes the formation of adsorbable organic halogen (AOX) in an amount of only one fifth of that caused by chlorine gas (Germgfird and Larsson, 1983).

Today, the pulp and paper industry had been forced to consider any new technique available for the reduction of chlorine consumption, or for the increase of brightness of totally chlorine-free pulps. xylanases can be used to replace chlorine for specific modifications of pulp for the development of clear environmentally, friendly, economically attractive technology, and can decrease the amount of bleaching chemicals required to attain a given brightness in subsequent chemical bleaching stage (Zhao *et al*., 2006; Savitha *et al*., 2007; Viikari *et al*., 1986). Delignification in biobleaching process: xylanase attacks hemicellulose (xylan) and removal the lignin-associated xylans by an ester linkage to 4-O-methyl-D-glucuronic acid with minimal damage from pulp as shown in Figure 2.2. Cellulase-free xylanase or xylanase with low cellulase activity have been regarded as environment-friendly alternatives for effective bleaching of paper pulp without employing toxic chlorine compounds (Srinivasan *et al*., 1999). Cellulase-free xylanase selectively hydrolyse xylan without degrading cellulose of pulp fibres.

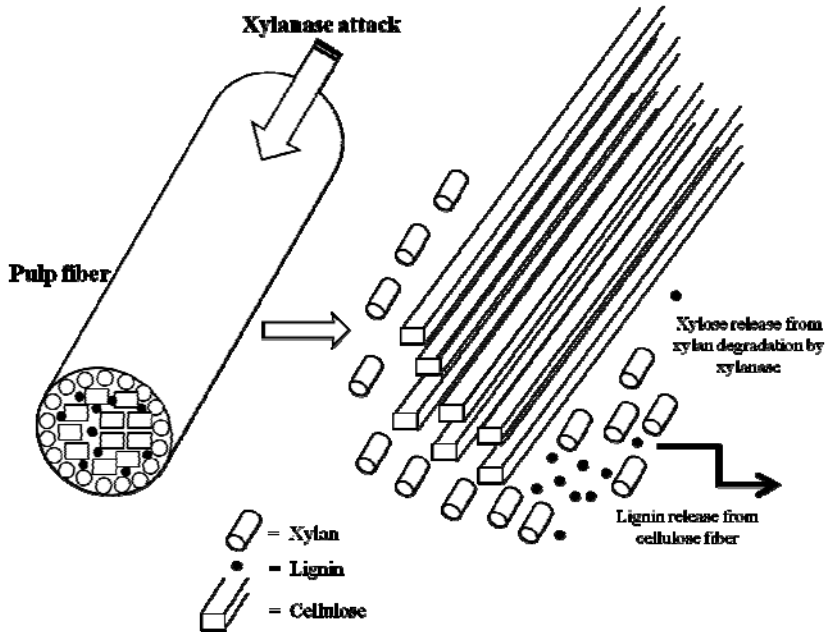


Figure 2.2 Lignin associated xylan fraction removal from pulp structure by xylanase action.

2.5.2 Animal feed supplement

Although cereals like wheat, barley, triticale, rye and oats are commonly used as animal feed, they contain relatively high proportion of anti-nutritive carbohydrates like *beta*-glucon, pentosans and arabinoxylan which are collectively known as non-starch polysaccharides (NSPs) (Friesen *et al*., 1992). The content of NSPs in the diet was inversely related to the apparent metabolizable energy (AME) of wheat (Annison and Choct, 1991). A lot of feed additives are being currently used and new concepts are continuously developed. Enzymes as additives to animal feed have had a great impact on the livestock industry. As reported by Amare Gessese (1998), xylanases, proteases, phytases and amylases are found important in such industry. The use of xylanase in poultry feeds has predominantly been related to the hydrolysis of fiber or NSP fractions in cereal grains. These NSPs could not digested by the endogenous poultry enzymes and can have anti-nutritive effects (Friesen *et al*., 1992). The intestinal viscosity caused by water soluble NSPs dramatically reduces the nutritive value of the feed (Annisoon and Choct, 1991). Feed additive that contain xylanase help to such anti-nutritive factor or NSPs thus liberating the nutrients for easier digestion, absorption and utilization (Zhang *et al*., 1996).

Endo-β-D-xylanase (xylanase) is added to feeds to catalyze depolymerization of this polysaccharide. McCleary (2004) demonstrated that endo-cleavage by xylanase of just one bond per thousand in the arabinoxylan back bone can significantly remove viscosity properties. Xylanase also changes the hemicellulose to sugars so that nutrients formerly trapped with in the cell walls are released. This means the chickens get sufficient energy from less food (Zhang *et al*., 1996). Xylanase could also improve the quality of the environment by reducing the out put of excreta and pollutants, such as phosphate and nitrogen, including ammonia (Petterson and Aman, 1989). In addition, chickens eggs become cleaner because the excrement in the laying area is drier (Classen and Bedford, 1991). Although xylanases have proven to be highly beneficial, the use of xylanase in animal fed is still in its infancy. Most of the problems should be solved before their full potential is realized. As explained in Amare Gessese (1998) supplementation of xylan-rich feed with xylanase doesn’t completely eliminate the stickiness of the litter, which may show that no complete depolymerization takes place.

Therefore searching xylanases having better efficiency under the condition of the animal gut should be of an interest.

2.5.3 Baking industry

Xylanases were introduced to the bakery industry in the 1970s and they are currently used frequently in combination with amylases, lipases and various oxido-reductases (Elliot, 1996). Xylanase enhanced the dough and bread quality leading to improved dough flexibility, machinability and stability and a larger loaf (10%) volume as well as an improved crumb structure(Collins *et al*., 2006). Flour generally consists of approximately 80% starch and 12% proteins with arabinoxylan content varying from 2-3% in wheat flour and this small amount is an extremely important functional ingredient as it can bind almost 10 times its own weight with water, accounting for almost 30% of the water binding capacity of wheat flour (Elliot, 1996). The exact mechanism of the functionality of xylanases in bread making is not yet fully elucidated, but currently believed that the redistribution of water from the arabinoxylan in the flour to the starch and gluten phases is important (Collins *et al*., 2006).

2.5.4 Beer making

Xylanase had the ability to break the hemicelluloses down in to sugars. This indicates extraction of more fermentable sugars from barley for making beer. It also helps processing the spent barley for animal feed and in addition, added xylanase can reduce the viscosity of the brewing liquid improving its filterability (Mohagheghi, 1986).

2.5.5 Treating plant wastes

Annually a large amount of xylan containing waste is released in the form of industrial, agricultural and municipal wastes. Treating plant waste by xylanase dissolves the xylan and reduces the amount of organic wastes that should be disposed in to the land fill. As reported by Amare Gessese (1998), this treatment might not be efficient in treating the plant waste as it is composed of complexed polysaccharides. Therefore the use of other plant polymer degrading enzymes together with xylanase might involve in efficient hydrolysis of lignocellulosic wastes.

2.6 Endophytes

2.6.1 Historical perspective

2.6.1.1 Definition

The term “endophyte”, originally introduced by De Bary in 1866 (Rodrigues, 1996), referred to any organisms occurring within plant tissues, distinct from the epiphytes that live on plant surfaces (“endo-” means inside; “phyte” is derived from the Greek word *phyto*, which means plant). Since the discovery of endophytic fungus in darnel (*Lolium temulentum*) in Germany, in 1904 (Tan and Zou, 2001), various investigators have defined endophytes in different ways. Carroll (1986) defined endophytes as “mutualists, those fungi that colonize aerial parts of living plant tissues and do not cause symptoms of disease”. Petrini (1991) proposed an expansion of definition to include “all organisms inhabiting plant organs that at some time in their life, can colonize internal plant tissues without causing apparent harm to the host”. Wilson (1995) pointed out that “endophytes are fungi or bacteria which, for all or part of their life cycle, invade the tissues of living plants and cause unapparent and asymptomatic infections entirely within plant tissues, but cause no symptoms of disease”. Bacon and White (2000) give an inclusive and widely accepted definition of endophytes-“microbes that colonize living, internal tissues of plants without causing any immediate, overt negative effects”, which includes virtually any organism residing inside a plant host (Zhang *et al*., 2006b).

2.6.1.2 Origin and evolution

Plant-fungus association has a long evolutionary history. These specific relationships were referred to as mutualisms or symbiosis (Bacon and Hill, 1996). Evidence of plant-associated microorganisms found in the fossilized tissues of stems and leaves has revealed that the symbiosis of endophyte-host most probably dates back to the emergence of vascular plants (Strobel, 2003; Zhang *et al*., 2006). Some phytopathogens in the environment are related to endophytes and have an endophytic origin (Carroll, 1988). However, direct interactions between endophytes and pathogens are complex, diverse, and sensitive to host-specific leaf chemistry (Arnold *et al*., 2003). Ganley *et al*. (2004) found that endophytic fungi are actually most closely related to, but distinct from parasites. In certain environments, some microbes appear actively to penetrate plant tissues through invading openings or wounds, as well as proactively using hydrolytic enzymes such as cellulase and pectinase (Zhang *et al*., 2006). Majewska-Sawkaa and Nakashima (2004) found young embryos did not contain mycelium, but as they matured, endophytic *Neotyphodium lolii* was transmitted to the embryo exclusively via sporophytic

maternal tissue. Schulz *et al*. (2002) hypothesized that the interaction of fungal endophyte-plant host is characterized by a finely tuned equilibrium between fungal virulence and plant defence. Many innocuous fungal endophytes were quiescent phytopathogens which may cause infectious symptoms when the host plant is aged or stressed (Tan and Zou, 2001). However, endophytes in contrast to known pathogens, generally have far greater phenotypic plasticity and thus more options than pathogens: infection, local but also extensive colonization, latency, virulence, pathogenicity and/or saprophytism. This phenotypic plasticity is a motor of evolution (Schulz and Boyle, 2005).

Fungal endophytes had evolved two transmission modes: vertical and horizontal transmission. The former transmits the systemic fungus from plant to offspring via host seeds, and the latter operates by sexual or asexual spore transfer (Saikkonen *et al*., 2004). During the long co-evolution of endophytes and their host plants, endophytes have adapted themselves to their special microenvironments by gene mutation, including uptake of some plant DNA into their own genomes (Germaine *et al*., 2004). This might lead to the ability of certain endophytes to biosynthesize some phytochemicals originally associated with the host plants (Stierle *et al*., 1993; Zhang *et al*., 2006b). Endophytic microbes also improved the resistance of the hosts to adversity by secreting a variety of bioactive secondary metabolites (Tan and Zou, 2001; Schulz *et al*.; 2002; Strobel *et al*., 2004; Zhang *et al*., 2006b). For example, herbivores reduced the exposed biomass of non-host plants relative to the endophyte hosts, which can drive plant-microbe dynamics and modify plant community structures. In hereditary symbioses, genomes of both partners are co-inherited. Therefore, these symbionts are linked directly to evolutionary changes in their host populations (Clay et al., 2005; Zhang et al., 2006b). Prior studies indicated that interspecific hybridization promoted genetic variation, and was common during the evolution of endophyte colonization of grasses (Gentile et al., 2005). However, Brem and Leuchtmann (2003) reported that genetically differentiated and host-adapted races of the fungal endophyte *Epichloe bromicola* presumably emerged after host shifts and they might have evolved through host-mediated reproductive isolation toward independent species. The evolved relationships between endophytes and host plants are

complicated. Many problems regarding the evolutionary origin and speciation remain to be answered.

2.6.2 Endophytic fungi as producers of xylanase

One hundred and sixty-nine endophytic fungi were isolated from 14 plants in Hokkaido. Among them, 155 fungi (91.7%) were found to produce xylanase. The best xylanase producer among them showed high activity of 0.191 U/ml when they were cultivated at 27 °C for 5 days (Suto *et al*., 2002). It was noteworthy that xylanase producers were found in endophytes in large numbers. This result suggested that the invasion and proliferation of xylanase producers were advantageous to host plants. The inside part of plants is a novel and good source for isolating xylanase producers in comparison with soil. (Nielsen and Sorensen, 1997). Choi *et al*., (2005) studied about that twenty-one endophytic fungi isolates from *Brucea javanica* plant were tested for their ability to produce extracellular and intracellular xylanase appeared that fifteen of the endophytes tested produced extracellular xylanase, but only one had a strong reaction, six had medium reactions and eight had weak reactions furthermore, eight strains were intracellular producers of xylanase. *Colletotrichum* species B10810 and L27103, *Phoma* species (B25603) and the xylariaceous taxa (B25605) produced higher amounts of extracellular or intracellular xylanase than the other strains.

CHAPTER III

EXPERIMENTAL

3.1. Chemical materials

Acetic acid (Merck Ag Darmstadt, Germany)

Acrylamind (Plusone Pharmacia Biotech, Sweden)

Agar (Sigma, USA)

Ammonium Chloride (Sigma, USA)

Ammonium Nitrate (Sigma, USA)

Ammonium Persulfate (Sigma, USA)

Ammonium Sulfate (Sigma, USA)

Angiotensin Converting Enzyme from rabbit lung (Sigma,U.S.A)

Bagasse

Birch wood xylan (Sigma, USA)

Bis-acrylamide (Promega, USA)

Bovine serum albumin (Sigma, USA)

Bromophenol Blue (USB, USA)

Calcium Chloride (Sigma, USA)

Captopril (Fluka, China)

Chaff

Congo red (Sigma, USA)

Coomassie Brilliant Blue G-250 (USB, USA)

Corn steep

Corton oblongifolius Roxb. (Plao-yai) leaves

Dextrose (Sigma, USA)

Di- Ammonium Hydrogen Phosphate (Sigma, USA)

Di- Potassiumhydrogen phosphate (Merck Ag Darmstadt, Germany)

Endophytic fungi

Ethylenediaminetetraacetic acid, EDTA (Sigma, USA)

Ethanol (Merck Ag Darmstadt, Germany)

Ethyl acetate (Ajax Finechem, New Zealand)

Hydrochloric acid (J.T. Baker, USA)

Magnesium Sulfate (Sigma,U.S.A)

Peptone (Sigma, USA)

Potassium dihydrogen phosphate (Merck Ag Darmstadt, Germany)

Potato

Rice bran

Rice straw

Sawdust

Sodium azide (Merck Ag Darmstadt, Germany)

Sodium chloride (Merck Ag Darmstadt, Germany)

Sodium hydroxide (Merck Ag Darmstadt, Germany)

Soybean (Sigma, USA)

Standard Molecular Weight Marker (Sigma, U.S.A)

Tetramethylethylenediamine, TEMED (Plusone Pharmacia Biotech, Sweden)

Tris (USB, U.S.A)

Urea (Sigma, USA)

Yeast (Sigma, USA)

3.2. Equipment

Autoclave (Taladlab, Thailand)

Auto pipette (Pipetman, Gilson, France)

Dialysis bag (Snake Skin Dialysis Tubing, Pierce, U.S.A)

Electrophoresis unit (Hoefer mini VE, Amersham Pharmacia Biotech, Sweden)

Freeze dryer (Labconco, U.S.A)

High Speed Refrigerated Centrifuge (Kubota 6500, Japan) formance Liquid

Hot plate stirrer (HL instrument, Thailand)

Laminar Flow (Safety Lab, Asian Chemical and Engineering Co., Ltd., Thailand)

LC/MS/MS mass spectrometry

Micro-centrifuge (Tomy MTX-150)

Orbital Shaker (OS-10 Biosan, Latvia)

pH meter (Mettler Toledo, U.S.A)

Pipette tips (Bioline, U.S.A)

Spectrophotometer (Synergy HT Biotek, USA)

Speed vacuum centrifuge (Heto-Holten, Denmark)

Ultrasonic (leaner D200, D.S.C)

Vortex mixer (Vortex-Genie2, Scientific Industries, U.S.A)

Water Bath (NTT-1200 Tokyo kikakikai, Japan)

96-well microtiter plate (greiner, USA)

3.3 Tested Endophytic fungi

3.3.1 Isolation of endophytic fungi from *Croton oblongifolius* leaves

Endophytic fungi were isolated by using modified Petrini's method (Petrini, 1986). The healthy leaves of *Croton oblongifolius* were rinsed with running tap water, and dried in the laminar air flow Plant leaves were cut into small pieces size 5 × 5 mm. The specimen were the surface sterilized by immersing the cut pieces sequentially into 95% (v/v) ethanol for 1 min, 12% (w/v) sodium hypochlorite for 5 min and then 95% (v/v) ethanol for 30 second. Finally, they were immersed in sterilized water twice, and dried with sterile tissue paper and placed on to potato dextrose agar (PDA) plates. Plates were incubated at room temperature and examined for interral fungal. Fungal endophytes were subcultured to new PDA medium plates, and incubated for 7 - 14 days at room temperature until obtained pure culture. The pure culture was checked under light microscope. Fungal isolates with a different morphology were collected for further study.

3.3.2 Endophytic fungi from culture collection

Twenty- two fungal endophytes isolated from mangrove leaves and six fungal endophytes isolated from palm leaves were obtained from culture collection of Department of Microbiology, Faculty of Science, Chulalongkorn University, Thailand.

3.4 Screening of endophytic fungi for extracellular xylanase production

All endophytic fungal isolates were grown on PDA plates for 7 days. Mycelial agar disc size 0.5 mm in diameter were out by sterilized cork borer at the edge of growing and inoculated on selective xylan-agar plate by birchwood xylan. The plates were incubated colony for 5 days, at room temperature and flooded with 2% aqueous congo red. The solution allowed sitting for 15 minutes. The stain was renoved from the agar surface and washed with distilled water and the then were flooded with 1 M NaCl to

destain for 15 minutes. The NaCl solution was then removed. Xylan degradation around the colonies which indicated xylanase activity showed as a yellow-opaque area against a red color for the undegraded xylan. In order to select the best xylanase producer, strains with xylanase activity on the plates were cultured in 100 ml of basal medium (peptone 1% (w/v), KH_2PO_4 0.15% (w/v), $NaNO_3$ 0.2% (w/v), NaCl 0.05% (w/v), $MgSO_4$ 0.05% (w/v), $CaCl_2$ 0.025% (w/v), $FeSO_4$ 0.0001% (w/v), $ZnSO_4$ 0.0001% (w/v), $CuSO_4$ 0.0001% (w/v) and birchwood xylan 1% (w/v)) at pH 7.0. Each flask was inoculated with three 0.5 cm-diameter agar pieces and shaked on a rotary shaker at speed 150 rpm at 30 ^oC. Then, mycelia were removed by filtered through filter paper and the filtrates were tested for xylanase activity.

3.5 Assay for xylanase activity

Xylanase activity was measured by following Saha method (2002) by using 0.5 ml of 1% (w/v) solution of oat spelt xylan incubated with 0.5 ml of an appropriately diluted enzyme in 20 mM acetate buffer (pH 5.0) for 30 min at 50 ºC The released reducing sugars were assayed using the DNS method (Miller, 1959). One unit of xylanase activity was defined as the amount of the enzyme that liberated 1 µmol of xylose equivalents per minute under the assay conditions. The experiments were done with three replicates.

3.6 Identification of endophytic fungi

The endophytic fungal strain which showed highest xylanase activity was identified to species by morphological structure and molecular analysis. Morphological structure was examined for macroscopic and microscopic characters under light microscope. Molecular analysis was based upon the DNA sequence similarity of the internal transcribed spacer (ITS) regions of the rDNA, comparing this isolate to those in the NCBI GenBank database. Genomic DNA was prepared from fresh mycelial cultures of the selected endophytic fungal isolate and extracted with cetyltrimethylammonium bromide (CTAB), as described in Zhou *et al*. (1999). PCR amplification of the internal transcribed spacer (ITS) was performed in a total volume of 35 µl which was comprised of approx. 100 ng genomic DNA, 1 × PCR master Mix (Fermentas, Califonia, USA), and nM of each of the ITS1F and ITS4 primers. The amplification was performed in a thermocycler with a PCR profile of 94 ^oC for 5 min, followed by 38 cycles of 94 ^oC for 1

min, 51 ^{o}C for 1 min and 72 ^{o}C for 1 min, plus a final extension of 72 ^{o}C for 5 min. The PCR reactions were purified using the NucleoSpin® (Macherey-Nagel Inc., Easton, USA) and were direct sequenced on both the leading and lagging strands (using the ITSF1 and ITS4 primers, respectively) commercially by Macrogen (Seoul, Korea). The complete consensus sequence was then used to BLASTn search the NCBI GenBank database using the default settings, with the top 100 highest sequence similarity hits being recorded and compared. Species annotation of the deposited ITS sequences in the GenBank database were taken on trust and used to convert the molecular operational taxonomic unit (MOTU) designation of the fungal isolate to a likely species designation where the % sequence similarity was high enough (>97%).

3.7 Xylanase production

The selected endophytic fungal isolate was cultivated in a modified basal medium, as described in section 3.5, and tested the xylanase activity. Carbon and nitrogen sources effect on the extracellular xylanase (activity) production were investigated. In this work, various kinds of agricultural residues were tested to xylanase production by cultures for a 20 day. Various carbon sources such as chaff, bagasse, rice bran, rice straw and sawdust were replaced birchwood xylan supplemented in the basal medium (section 3.4) with similar concentrations. Various organic nitrogen sources such as soybean powder, yeast extract, corn steep liquor, peptone and various inorganic nitrogen sources such as urea, ammonium sulfate, ammonium persulfate, ammonium hydrogen phosphate and ammonium chloride were replaced ammonium nitrate into basal medium. Note that this was performed as a univariate analysis and not a multivariate, and so any potential interaction between these components is not ascertained. Various concentrations of each selected carbon sources (0.5, 1 and 2% (w/v)), and nitrogen sources (0.1, 0.2 and 0.5% (w/v)) were also tested. All experiments were done in triplicate and calculated mean ± SE.

3.8 Determination protein content

Protein contents were determined by the Bradford assay (Bradford, 1976), using 5, 10, 15 and 20 µg/ml of bovine serum albumin (BSA) as the standard to construct the calibration curve. For each serial two-fold dilution of the sample in deionized water, 50 µl aliquots were transferred into each of three wells of a microtiter plate and 50 µl of

Bradford's reagent (100 ml contains: 10 mg Coomassie Brilliant Blue G-250 and 10 ml of 85% (v/v) phosphoric acid, dissolved in 95% (v/v) ethanol) was added to each well. The plate was shaken (Biosan, OS-10, Latvia) for 5 min and then left for 10 min before reading the absorbance at 595 nm using an ELISA plate reader (Biotek Synergy HT, Biotek instrument, USA). The obtained OD was converted to the protein concentration using the linear equation computed from the standard curve. During the column chromatographic separations, the elution peak profiles of proteins were determined by measuring the absorbance at 280 nm.

3.9 Xylanase purification

All the procedures as follow were performed at 4 ^{o}C, unless otherwise stated.

3.9.1. $(NH_4)_2SO_4$ Precipitation

To 5 liters of culture supernatant, $(NH_4)_2SO_4$ was slowly added with stirring to a final 80% saturation and then left to stand overnight at 4 ^{o}C. The precipitate was collected by centrifugation at 10,000 × g for 30 min (Beckman Coulter, USA), and dissolved in distilled water, dialyzed in dialyzed bag (3,500 MWCO) with 3 changes of 5 L distilled water at 4 ^{o}C and then concentrated by lyophilization (Labconco, USA) which is referred to hereafter as the "ammonium sulfate cut fraction".

3.9.2. DEAE-cellulose ion exchange chromatography

DEAE-cellulose ion exchange chromatography was performed with a 1.6 cm × 15 cm column using an automatic liquid chromatography system (AKTA prime, Amersham Pharmacia Biotech, Sweden). The column was equilibrated with 5 column-volumes of 50 mM Tris-HCl (pH 7.0). Thereafter, 5 ml samples (400 mg protein) of the ammonium sulfate cut fraction were injected into the column and eluted with the same buffer at a flow rate of 1.0 ml/min, collecting 10 ml fractions before a linear 0 - 1.0 M NaCl gradient in the same buffer was applied over the next 55 fractions. The eluted fractions were monitored for protein content with a UV detector at 280 nm and for xylanase activity as described in section 3.4. The fractions containing xylanase activity from the column were pooled, dialyzed against 3 changes of 5 L of distilled water and concentrated, and is referred to as the "post-DEAE-cellulose xylanase fraction".

3.9.3. Superdex-75 gel filtration chromatography

The post-DEAE-cellulose xylanase fraction was then further enriched by preparative Superdex-75 column (1.6 cm × 60 cm) chromatography. The column was equilibrated with two column-volumes of 100 mM NaCl / 50 mM Tris-HCl (pH 7.0), and then 2 ml of the post-DEAE-cellulose xylanase fraction solution (50 mg protein) was injected and eluted in the same buffer at a flow rate of 0.5 ml/min and collecting 5 ml fractions. Fractions were monitored for protein with a UV detector at 280 nm and for xylanase activity as described in section 2.3. The xylanase active fractions were pooled, dialyzed against 3 changes of 5 L of distilled water and concentrated, and is referred to as the “enriched xylanase fraction”.

3.10 Determination enzyme purity by native-PAGE and xylanase activity staining

The enzyme from each step of purification was analyzed by its native protein pattern and its purity according to the method of Bollag *et al*. (1996). Electrophoresis conditions, protein and activity staining are described below.

3.10.1. Non-denaturating gel electrophoresis

Native PAGE was performed with 10% and 5% (w/v) acrylamide separating and stacking gels, respectively, with 100 mM Tris-glycine (pH 8.3) as the electrode buffer. Electrophoresis was run at a constant current of 20 mA per slab at room temperature in a Mini-Gel Electrophoresis unit (Hoefer model miniVE, Pharmacia Biotech, UK). After electrophoresis, proteins in the gel were visualized by Coomassie blue R-250 (Sigma) staining and activity staining.

3.10.2. Coomassie blue staining of native SDS-PAGE

Native (section 3.10.1.) and reducing SDS-PAGE (section 3.11) gels were stained by immersion in 0.1% (w/v) Coomassie blue R-250 in1 0% (v/v) acetic acid / 45% (v/v) methanol for 45 min. Destaining was performed by immersing the gel in 10% (v/v) acetic acid / 45% (v/v) methanol, with several changes of this destaining solution until the background was clear.

3.10.3. Staining for xylanase activity

After native-PAGE resolution the gel was directly immersed in 1% (w/v) birchwood xylan / 50 mM Tris-HCl (pH 7.0) at room temperature for 30 min. The Gel was then transferred to 0.1% solution of Congo red (Sigma) and incubated at 25oC with constant shaking for 10 min. The Gel was destained by washing with 1M sodium

chloride. The activity band was clearly visible as yellowish clearances against a deep red background by the end of 10 min of destaining (Karnchanatat *et al*., 2008).

3.11 Determination molecular weight of SDS PAGE

Discontinuous reducing 0.1% (w/v) SDS-PAGE was performed according to the procedure of Laemmli (1970) using 15% and 5% (w/v) acrylamide resolving and stacking gels, respectively. Samples were treated with reducing (2-mercaptoethanol containing) sample buffer and boiled for 5 min prior to application to the gel. Electrophoresis was run at a constant current of 20 mA per slab at room temperature in a Mini-Gel Electrophoresis unit. High and low molecular weight standards were coresolved on each gel and used to determine the subunit molecular weight of the enriched xylanase enzyme. After electrophoresis, proteins in the gel were visualized by staining with Coomassie blue R-250 as described in section 3.10.2.

3.12 Thermostability on the xylanase activity

Thermostability on the xylanase activity of the enriched xylanase fraction (post-superdex-75) was determined by incubating the enriched xylanse fraction in 20 mM sodium acetate buffer pH 5.0 at various temperatures ((-20) - 90 ^{o}C at 10 ^{o}C intervals) for 30 min. In addition, the xylanase was investigated by preincubating the enriched xylanse fraction at various temperatures (30 - 60 ^{o}C in 10 ^{o}C intervals) in the same buffer for the indicated fixed time intervals (10 - 120 min), cooling to 4 ^{o}C and then assaying the residual xylanase activity as described above.

3.13 pH stability on the xylanase activity

Incubating the enriched xylanase fraction in buffers of broadly similar salinity levels, but varying in pH from 2 to 14, was used to assess the pretreatment pH stability of the xylanase. The buffers used were (all 20 mM) glycine-HCl (pH 2 to 4), sodium acetate (pH 4 to 6), potassium phosphate (pH 6 to 8), Tris-HCl (pH 8 to 10) and glycine-NaOH (pH 10 to 12). The enriched xylanase fraction was mixed in each of the different pH-buffer compositions, plus the control (20 mM sodium acetate buffer pH 5.0). The xylanase-buffer mixtures were left for 30, 60 and 90 min at room temperature and then adjusted back to 20 mM sodium acetate buffer pH 5.0 and assayed for xylanase activity as in section 3.4. The control incubation was set at 100% activity and the activity of the

samples from the different pH buffers were expressed relative to that of the control (100% activity).

3.14 Effect of metal ions on the xylanase activity

The effect of preculture with different divalent metal cation salts (mostly chloride anions but also two sulfate anions) and the chelating agent ethylenediamine tetraacetic acid (EDTA), on the xylanase activity of the enriched xylanase fraction was evaluated. The enriched xylanase fraction was incubated for 30 min with one of Ca^{2+}, Fe^{2+}, Hg^{2+}, Mg^{2+}, Mn^{2+} (all as chlorides), Cu^{2+} or Zn^{2+} (as sulfates) or EDTA, at one of three concentrations (1, 5 and 10 mM) with continuous shaking. The residual xylanase activity was then evaluated (section 2.4), and from this the relative xylanase activity (%) was calculated taking the residual xylanase activity found in the control samples (without the addition of metal salts or EDTA) as 100%.

3.15 Determination of kinetic parameters

The Michaelis constant (K_m) and maximum velocity (V_{max}) values of the enriched xylanase fraction were determined by measuring the rate of birchwood xylan hydrolysis under standard assay conditions. The reaction mixture was 20 mM sodium acetate buffer pH 5.0 with the birchwood xylan substrate at concentrations ranging from 1.0-20 mg. The values for K_m and V_{max} were then determined from the Lineweaver-Burk plot.

CHAPTER IV

RESULT AND DISCUSSION

4.1. Isolation and screening of xylanase producing from endophytic fungi

Fifty-four tested endophytic fungal species were screened for extracellular xylanase production by using birch wood xylan agar plates (section 3.3). The basal medium containing birch wood xylan was used for screening xylanolytic activity was reported by several workers (Bhalla and Joshi, 1993; Kvesitadze *et al*., 1999; Abdel-Sater and El-Said, 2001). Even though a clearing zone was obtained after 5 days of incubation the clarity of the clearing zone increased when the medium was stained with 0.1% congo red, followed by washing with 1 M NaCl. Pajnu *et al*. (1989) demonstrated the carboxymethyl cellulase activity with 0.1% congo red. According to Capalash *et al*. (1990) congo red plate assay (Teather and Wood, 1982) was the most widely used method for the screening of hemicellulase activities. The detection of clearing zone with 0.1% congo red in *Streptomyces sp.* was done by Techapun *et al*. (2001). Bhalerao *et al*. (1990) detected the xylanase positive colonies by flooding with 0.1% congo red and then followed by washing with 1M NaCl. Among of thirty one active isolates, one isolate (PTRa9) produced a much larger clearing zone was observed than the others (Figure 4.1 and data not shown). Nevertheless, to ensure that the highest xylanse activity was selected, all thirty one of these isolates were cultured in a liquid culture in the basal medium (section 3.4) and subjected to quantitative xylanse activity analysis in the culture media. Frown on study endophytic from isolated PTRa9 (*C. oblongifolius*) was found to produce the highest extracellular xylanase (2.56 U/ml). This isolate was then furthermore evaluation on the factors influencing the xylanase production and enzyme activity and kinetics and identified.

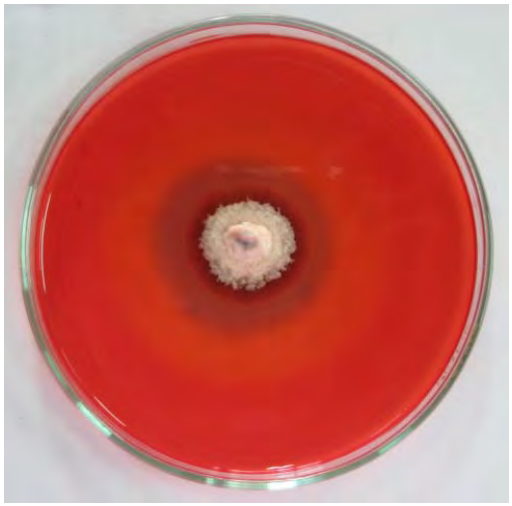


Figure 4.1 Representative plate showing the growth of one of the selected xylanase-positive endophytic fungi (isolate PTRa9) on 1% birch wood xylan.

4.2. Identification of endophytic fungi

The endophyte isolated PTRa9, which showed the highest xylanase activity production, was identified to species level based on morphological structure and molecular analysis. With respect to morphological identification, the isolate showed showing green-black colony on PDA medium (Figure 4.2A and B). Scanning electron micrograph (SEM) showed the macrocondia (Figure 4.3A to C). The identification of isolate was confirmed by molecular technique using the DNA sequence of the rDNA ITS region. The BLASTn search revealed highly similarity (>97% identity) ITS sequences but these were all from *Alternaria alternata* isolates, with the highest sequence identity being to *Alternaria alternata* isolated VC38 (GQ916545.1) 99% sequence identity.

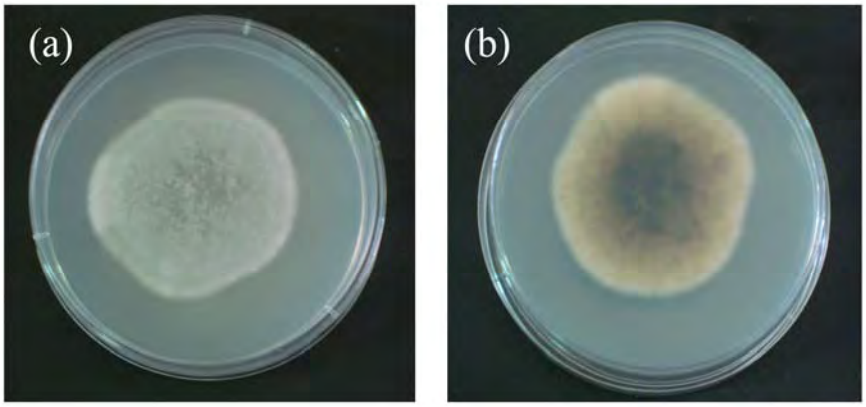


Figure 4.2 Morphology character of xylanse-positive endophytic fungi isolated PTRa9 showing colony growth on PDA plate as (A) top view (B) bottom view.

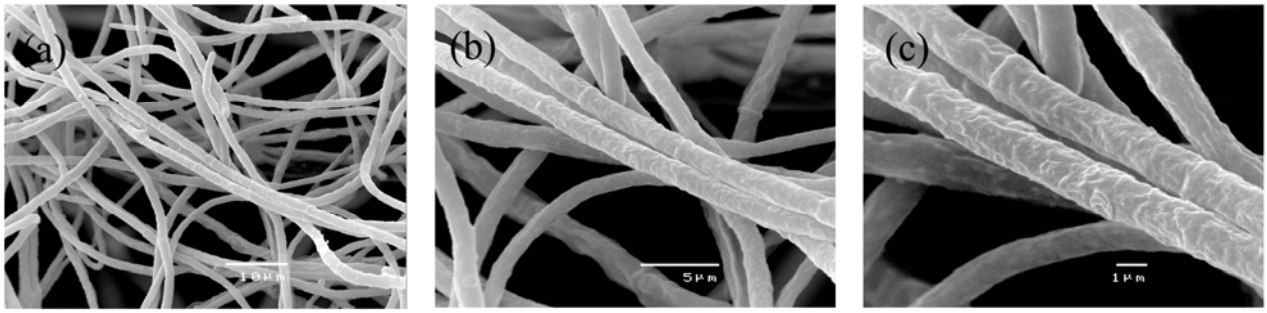


Figure 4.3 Scanning electron micrograph of *Alternaria alternata* isolated PTRa9. (A); bar 10 μm, (B); bar 5 μm, and (C); bar 1 μm

Molecular analysis had been successfully used for identifying endophytic fungi and several recent studies have shown that genetic methods exhibit high sensitivity and specificity (Promputtha *et al*., 2005; Sette *et al*., 2006; Tedersoo *et al*., 2006; Morakotkarn *et al*., 2007). Most of the endophytic fungi are detected and identified by 18S rDNA sequence comparisons or internal transcribed spacer (ITS1 and ITS2) and 5.8S rDNA sequence examinations (Harney *et al*., 1997; Guo *et al*., 2000; Lacap *et al*., 2003). Moreover, random amplified polymorphic DNA (RAPD) techniques can be employed for detecting genetic diversity of endophytic fungi from medicinal plants and

for pre-selection of these isolates for bioactive screening program (Tejesvi *et al*., 2007). Some endophytic fungi may be lost during isolation processes, however, molecular techniques can be used to alleviate the requirement of cultivation to measure diversity of fungi in natural environment (Guo, 1999).

4.3. Production of xylanase

4.3.1. Effect of various carbon source on xylanase production

A number of different carbon sources were tested in growth experiments for ability to promote the development of fungal mycelium and stimulate the secretion of xylanolytic enzyme. Rice bran was the most effective inducer of xylanase activity (1.70 U/ml) of the carbon sources tested (Figure 4.4). The highest level of total xylanase activity (1.95 U/ml) was produced in 2% (w/v) of rice bran after 4 days growth (Figure 4.5).

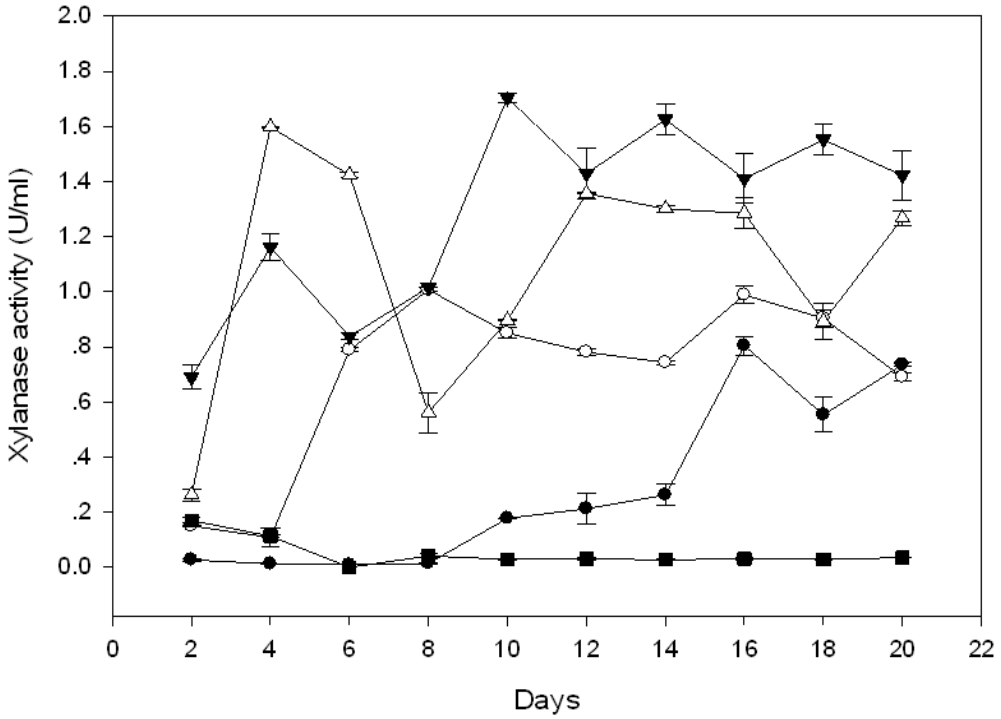


Figure 4.4 Time course of xylanase production by endophytic fungus *Alternaria alternata* isolated PTRa9 grown on various 1% carbon source. Values represent the average of three replicates ± SE. Chaff (●); baggasse (○); rice bran (▼); rice straw (Δ); and sawdust (■).

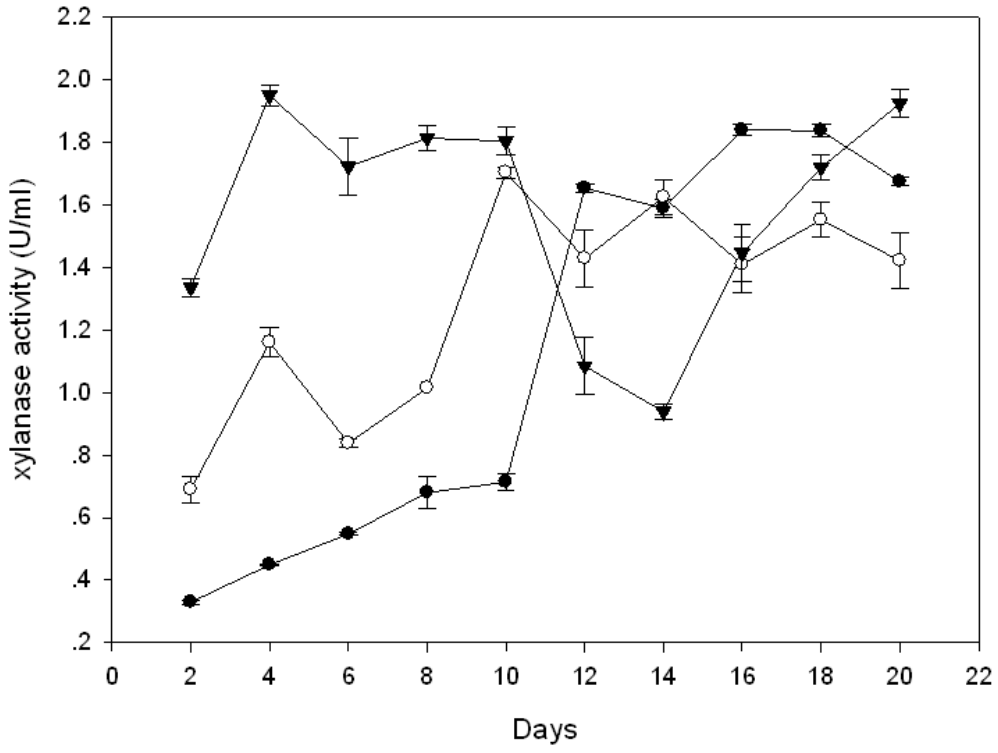


Figure 4.5 Effect of rice bran concentration on xylanase activity of endophytic fungus *Alternaria alternata* isolated PTRa9. Values represent the average of three replicates ± SE. 0.5% (●); 1.0% (o); and 2.0% (▼).

Mean values in Figure 4.4 and 4.5 indicated a relative capability of five carbon sources i.e. chaff, baggasse, rice bran, rice straw, and sawdust at different concentrations to synthesize xylanase by *Alternaria alternata* isolated PTRa9. It is obvious that rice bran gave the maximum high xylanase yield and followed by the others. The graphical depiction also elaborated that 2.0% concentration was considered best for all of the carbon sources as they exhibited maximum enzyme activity at this level. Moreover, when the concentration of mentioned carbon sources was increased above 2.0%, the enzyme activity decreased because higher levels of substrates caused problem of agitation resulting in reduced accessibility of fungus to the substrates and hence less enzymatic activity. Rice bran produced maximum enzyme activities; one possible reason could be that it is diversified in its composition and contains most of the minerals. Therefore, it is concluded that rice bran at concentration of 2.0% can be used to get higher yield of

enzyme. The findings are also in close conformity with the findings of Coelho and Carmona (2003), they reported wheat bran as good substrate for xylanase production. Many researchers have also observed that wheat bran gives maximum activities of xylanase as compared to other substrates (Chen *et al*., 1999; Ferriera *et al*., 1999; Park *et al*., 2002; Haq *et al*., 2002).

4.3.2. Effect of various nitrogen source on xylanase production

The effect of nitrogen source supplementation on the production of xylanase by selected endophytic fungi was also examined. The results obtained using various nitrogen sources are shown in Figure 4.6. Among the nitrogen sources tested, $(NH_4)_2SO_4$ was found to enhance the production of xylanase at 0.1% (w/v) by about 1.95 U/ml in basal medium at 4 days.

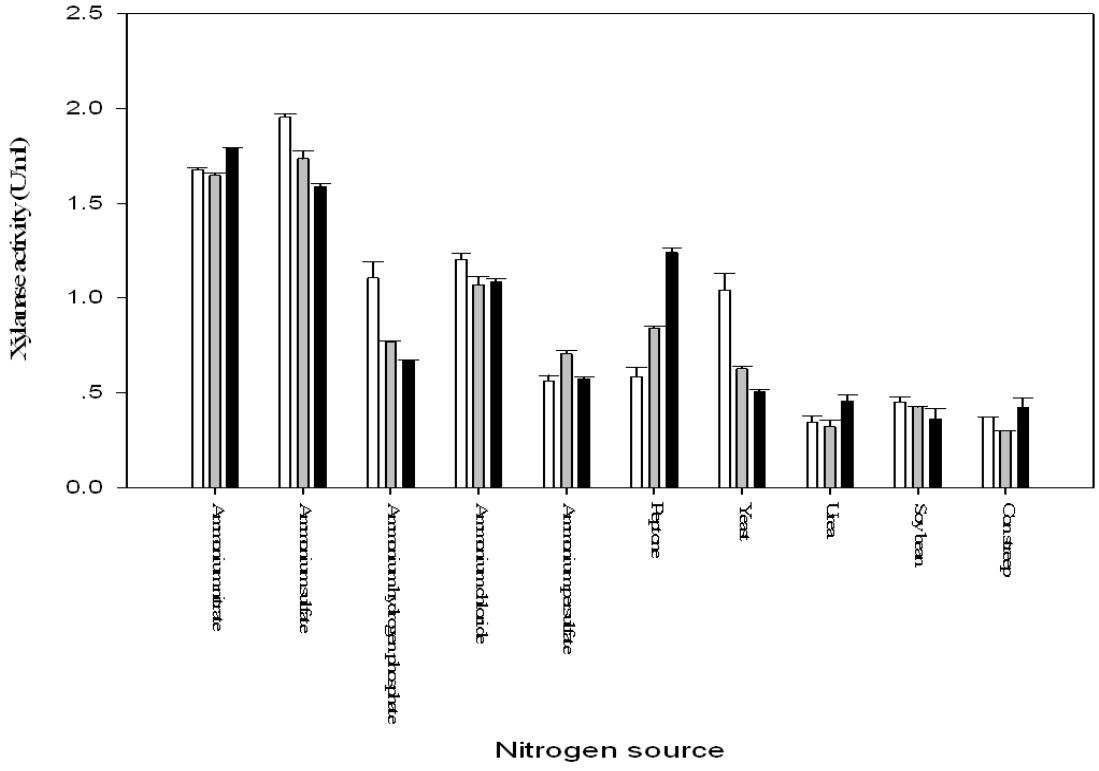


Figure 4.6 Effect of nitrogen source and on xylanase activity of endophytic fungus *Alternaria alternata* isolated PTRa9**.** Values represent the average of three replicates ± SE. 0.1% (white); 0.2% (gray); and 0.5% (black).

Nitrogen source used in the production medium is one of the major factors affecting enzyme production and level. When selecting inorganic nitrogen sources for inclusion in growth medium, similar studies in the literature were taken into account and as a result $(NH_4)_2SO_4$ gave better results (Figure 4.6). In a study carried out with *T. harzianum*, $NaNO_3$ and peptone were the nitrogen sources in production medium (Abdel-Sater and El-Said, 2001), whilst NH_4NO_3 was used in a study with *Schizophyllum commune*, and $(NH_4)_2HPO_4$ was found suitable in another study with *Thermomyces lanuginosus* RT9 (Haltrich *et al*., 1993; Hoq *et al*.,1994). In summary, it can be concluded that xylanase activity of *A. alternata* is maximum with rice bran as sole carbon source. Ammonium sulphate was found to be the most appropriate inorganic nitrogen source for production.

4.4. Xylanse purification

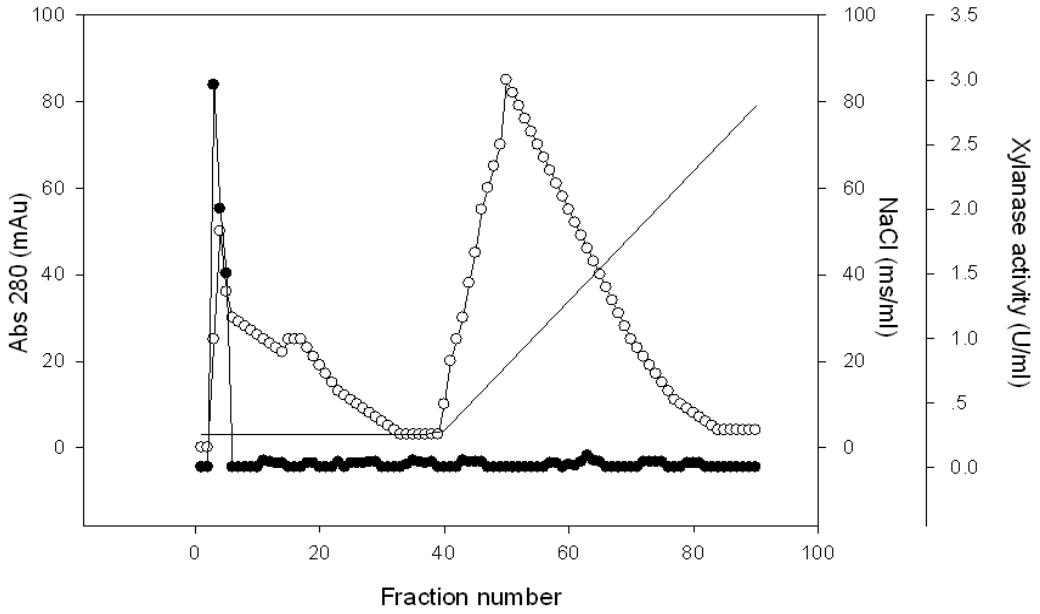
The development of techniques and methods for the separation and purification of proteins has been an essential pre-requisite for many of the recent advancement in bioscience and biotechnology research. The global aim of a protein purification process is not only the removal of unwanted contamination, but also the concentration of the desired protein and its transfer to an environment where it is stable and in a form ready for the intended application (Queiroz *et al*., 2001). The principle properties of the enzymes that can be exploited in separation methods are size, charge, solubility and the possession of specific binding sites (Queiroz *et al*., 2001; and Amersham Pharmacia Biotechnol, 1999). Most purification protocols require more than one step to achieve the desired level of product purity. Hence, the key to successful and efficient protein purification is to select the most appropriate techniques, optimize their performance to suit the requirements and combine them in a logical way to maximize yield and minimize the number of step required (Amersham Pharmacia Biotechnol, 1999).

At the end of the cultivation period, mycelia were removed by filtration through Whatman 3M chromatography paper. An extracellular xylanase was purified to homogeneity from the culture filtrate of *Alternaria alternata* isolated PTRa9 grown on rice bran growth medium. xylanase was successfully purified through ammonium sulfate precipitation, DEAE-cellulose, and Superdex-75 column chromatography. Upon

fractionation of xylanase active fraction with ammonium sulfate approximately 19.8% (Table 4.1) of the activity was obtained in the fraction saturated with 80% ammonium sulfate. After ion-exchange chromatography the enrich xylanse fraction was found (Figure 4.7). The major active peak was unadsorbed onto the DEAE-cellulose column (fractions 3-5), whereas the unenrich xylanse fraction (fractions 40-75) was adsorbed on the resin and eluted with most of the loading protein in the wash with starting buffer. Thus, the elution pattern showed a single xylanse activity peak which was harvested and pooled. Compared to the ammonium sulfate cut fraction, the post-DEAE-cellulose xylanse fraction showed a 87% reduction in the total protein content for only a loss of 70% xylanse activity (Table 4.1), but the preparation was still not homogenous (Figure 4.6).

Thus, the post-DEAE-cellulose xylanse fraction (section 3.9.2) was further fractionated using Superdex-75 gel column chromatography (section 3.9.3), where a sharp peak was eluted free of most of the other xylanase activity negative proteins (Figure 4.7B). Compared to the post-DEAE-cellulose xylanase fraction, although the post-Superdex-75 fraction (enriched xylanse fraction) showed a 98.2% reduction in the total protein content this was achieved at the cost of a 77.6% loss of xylanse activity, (Table 4.1). Overall, a 60.8-fold enrichment for a 1.3 % yield was obtined after the three enrichmnt stages, compared to the crude culture filtrate (Table 4.1). The enriched xylanase fraction (post-Superdex-75; section 3.9.3.), with a specific activity of 161.1 U/mg of protein (Table 4.1) and was enrinched to or near to apparent homogeneity (Figure 4.8), was used for all further enzyme characterization. The xylanase activity in the gel filtration was distributed across most of the elution profile. This is probably due to the expression of a wide spectrum of the xylanases by filamentous fungi as observed with *Aspergillus niger* (Berrin *et al*., 2000). A combination of this broad elution of xylanase and loss in specific activity, possibly due to removal of some stabilizing elements in the culture supernatant on purification of the enzyme, may be largely accountable for the low yields of activity in the selected distinct peak. Despite the low yields, the isolation procedure provided pure xylanase that allowed characterization of the protein and preliminary studies on the amino acid sequence. The findings of the present study regarding purification of xylanase are in corroboration with the results of Carmona *et al*.

(1998), they described that when different steps of purification were carried out, the total protein contents decreased with each step while the specific activity increased. Carmona *et al.* (1998) calculated specific activity in the crude xylanase extract as 33.79 IU/mg that is in conformity with the present case i.e. 41.85 IU/mg.



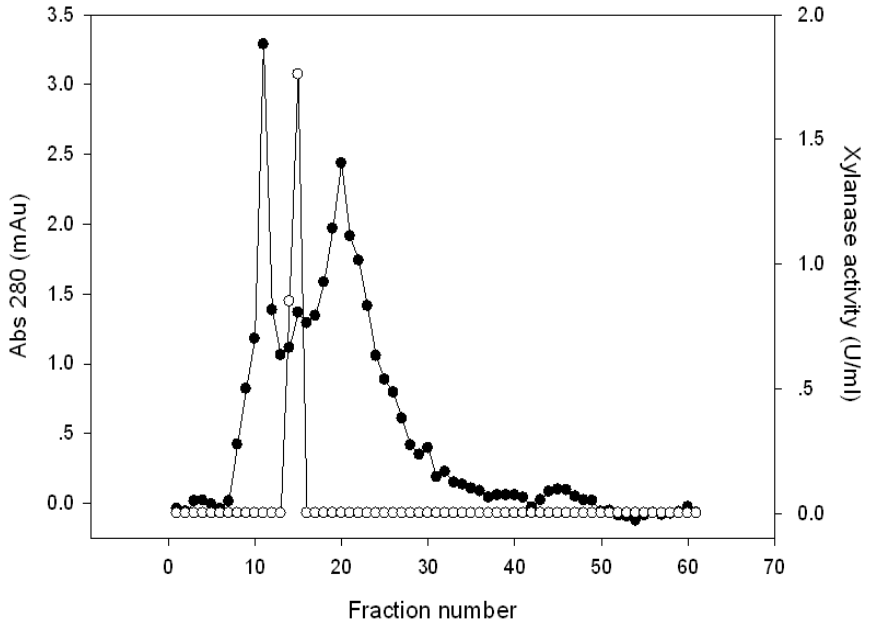


Figure 4.7 Profile of the enrichment of the *Alternaria alternata* isolated PTRa9 extracellular xylanase extract by; (A) DEAE-cellulose ion-exchange chromatography of the ammonium sulfate cut fraction (400 mg protein) eluted in 50 mM Tris-HCl (pH 7.0) with a 0 - 1 M NaCl linear gradient; and (B) Superdex-75 gel chromatography of the post-DEAE-cellulose xylanase fraction (50 mg) eluted in 100 mM NaCl / 50 mM Tris-HCl (pH 7.0). For panels A; absorbance at 280 nm (○), xylanase activity (●) and panels B; absorbance at 280 nm (●), xylanase activity (○).

Table 4.1 Enrichment summary for the xylanase from *Alternaria alternata* isolated PTRa9.

Purification step	Total protein (mg)	Total activity (U)	Specific activity (U/mg)	Yield (%)	Purification (fold)
Culture filtrate	765.2	2027.9	2.7	100.0	1.0
80% $(NH_4)_2SO_4$ cut	86.2	402.1	4.7	19.8	1.8
DEAE-cellulose	11.1	118.3	10.7	5.8	4.0
Sephadex-75	0.2	26.4	161.1	1.3	60.8

4.5. Determination of enzyme purity and protein pattern on native-PAGE

The xylanase from each step of enrichment was analyzed for purity and protein pattern by native-PAGE, with protein and enzyme activity staining (Figure 4.8A). Whilst the post-DEAE-cellulose xylanase fraction still showed multiple components, the enriched xylanase fraction (post-Superdex-75 xylanase fraction) showed a single protein band on native-PAGE. On the same gel, a rapid visualization of the enzyme acting on 1% birch wood xylan was permitted by subsequent staining with congo red. A single band was observed at the same migration distance, indicating that the purified xylanase from Sephadex-75 column was the pure enzyme.

4.6. Determination molecular weight of SDS-PAGE

Discontinuous reducing SDS-PAGE, a relatively sensitive technique for xylanase separation, revealed a single strong band with an apparent molecular weight of 54.8 kDa after Coomassie blue R250 staining (Figure 4.8B). This supports enrichment to near homogeneity and suggests that the purified xylanase could be a monomeric protein, or at

least if a multimeric one that dissociates into subunits under these enrichment conditions, that this 54.8 kDa subunit has xylanase activity alone.

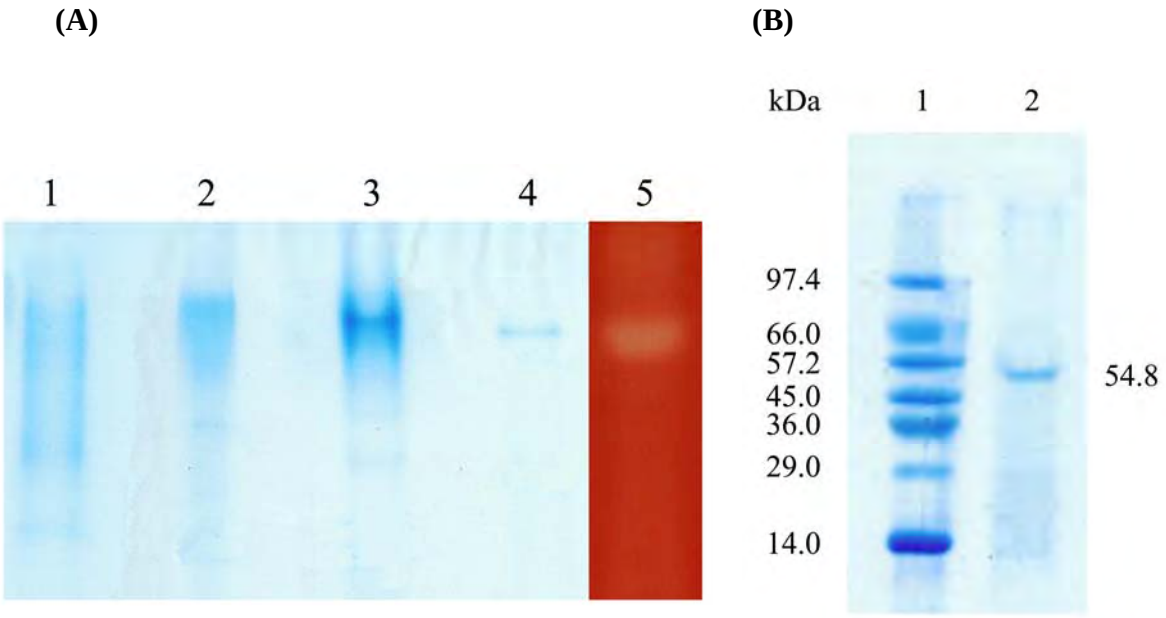


Figure 4.8 (A) Coomassie blue stained native-PAGE analysis of the *Alternaria alternata* isolated PTRa9 xylanase fractions from each step of the enrichment and stained for protein by coomassie blue (Lanes 1 - 4) or for xylanase enzyme activiy (Lane 5). Lane 1, crude enzyme (20 μg of protein); Lane 2, ammonium sulfate cut fraction (20 μg of protein); Lane 3, post-DEAE-cellulose xylanase fraction (15 μg of protein); Lanes 4 & 5, enriched xylanase fraction (post-Superdex-75) (10 μg of protein). (B) Reducing SDS-PAGE analysis, after coomassie blue staining, of the enriched xylanase fraction (post-Superdex-75) from *Alternaria alternata* isolated PTRa9: Lane 1, Low molecular weight protein markers; Lane 2, enriched xylanase fraction (5 μg of protein).

4.7. Thermostability on the xylanase activity

The Figure 4.9 (A) depicts the effect of different temperatures on relative activity of xylanase. It is obvious that when enzyme assay was performed at various temperatures, the xylanase activity increased with rise in temperature up to 40 °C and exhibited maximum activity, which was similar to the xylanase from *A. ficuum* AF-98

acted stably at 45 ^{o}C (Fengxia *et al*., 2008) or xylanase from *T. reesei* also acted stably at 45 ^{o}C (Tenkanen *et al*., 1992). However, further increase in temperature caused a decrease in activity. Minimum activity was observed at 90°C, the highest temperature studied in the present study. The results of current work are in close conformity to the findings of Kavita *et al*. (2002), they reported that the purified xylanase from the fungus *A. nidulans KK-99* exhibited highest activity at 55 °C. Likewise, Carmona *et al*. (1998), and Uhlig, (1998), calculated highest activity of xylanase at 55°C however, Damasco *et al*. (2000) reported 75 °C as thermostability. In addition, the enzyme stability was determined by maintaining the enzyme at various temperatures of 30, 40, 50, and 60 ^{o}C for 10-120 minutes in 20 mM sodium acetate buffer pH 5.0. The enzyme retained more than 120% of its original activity at 40 ^{o}C for 10-120 minutes. The enzyme has showed good stability up to 20 minutes at 40 ^{o}C as shown in Figure 4.9 (B)

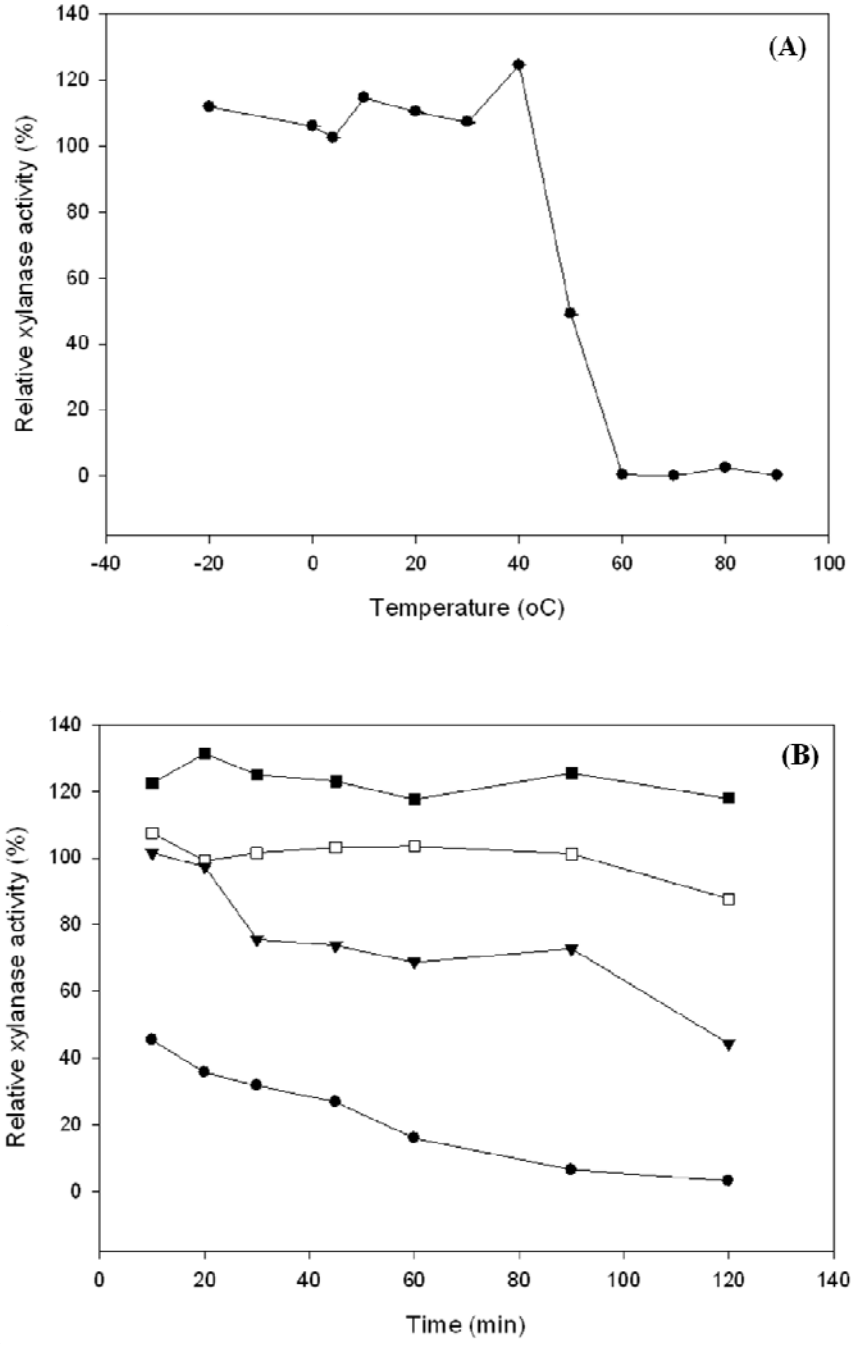


Figure 4.9 Thermostability on xylanase activity from *Alternaria alternata* isolated PTRa9 (A and B) was assayed in 50 mM Tris-HCl (pH 7.0) at (□); 30 ^{o}C, (■); 40 ^{o}C, (▼); 50 ^{o}C and (●); 60 ^{o}C. For both panels A and B the data are shown as the mean of three replicates $\pm$ SE. Means with a different lower case letter above them are significantly different ($p<0.05$).

4.8. pH stability on the xylanase activity

The pH stability for xylanase activity was 5.0 at room temperature for 60 min, the relative xylanase activity was 112.82%, it was maintained over 90% at pH 3.0 - 11.0 for 60 min and it suddenly decreased at pH 2 and 12 for whole times. (Fig. 4.10) The results showed that xylanase isolated from fungus PTRa9 identified from *Alternaria alternata* isolated PTRa9 showed high activity under rather high acidic or alkali conditions. A considerable stability at acid-alkaline pH values makes it potentially effective for use in industry. The results of current work are corroborated with the findings of Kavita *et al*. (2002) that maximum xylanase activity is in the pH range 4.0 to 9.5. Likewise, Uhlig (1998) calculated maximum xylanase activity at pH levels 6.0 to 7.0. The findings are also in agreement with Huang, (1991) that pH 5.5 and temperature 60 °C, as optimum for highest xylanase activities. Goulart *et al*. (2005), cultivated *R. stolonifer* on wheat bran to produce cellulase free xylanase. The purified xylanase exhibited pH stability and temperature as 6.0 and 45 °C respectively.

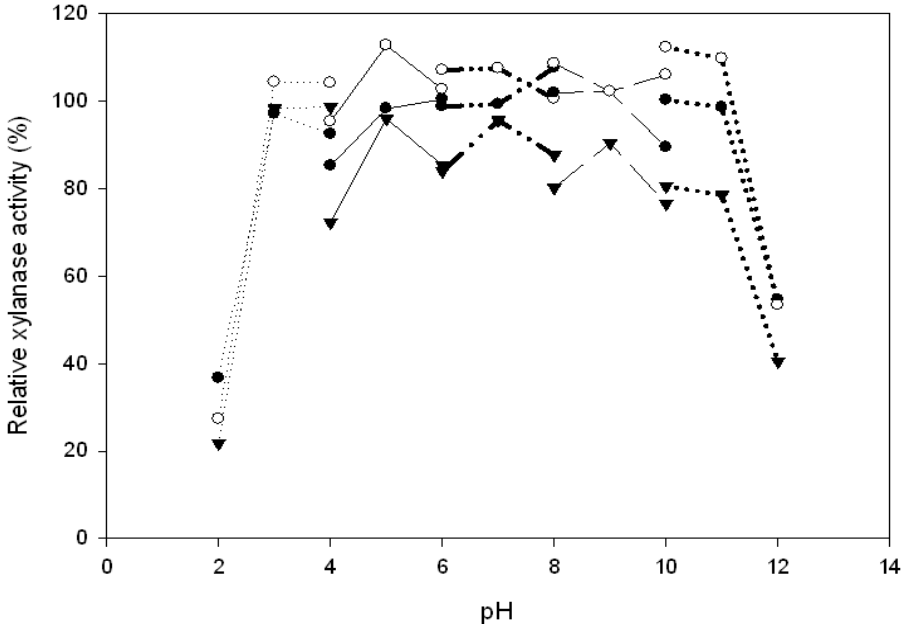


Figure 4.10 The pH stability on the activity of the enriched xylanase fraction from *Alternaria alternata* isolated PTRa9. The pH stability on xylanase activity was evaluated in (all 20 mM) (⋯); glycine-HCl buffer for pH 2-4, (—); sodium acetate buffer for 4-6, (−••); potassium phosphate buffer for 6-8, (− −); tris-HCl buffer for 8-10 and (•••);

glycine-NaOH buffer for 10-12 for time (●); 30 min, (o); 60 min and (▼); 90 min. The data are shown as the mean of three replicates ± SE.

4.9. Effect of metals and reagents

Xylanase activity was strongly inhibited by Hg^{2+} which might be due to its interaction with sulfhydryl groups present on the enzyme. EDTA was also found to be inhibitory causing 57% inhibition of enzyme activity (Table 4.2). Some other metal ions like Mg^{2+}, Mn^{2+}, Ca^{2+}, Cu^{2+}, Fe^{2+}, and Zn^{2+} decreased the enzyme activity but to a very less extent. The inhibition of the enzyme activity by Hg^{2+} ions may be due to its interaction with sulphydril groups, suggesting that there is an important cysteine residue in or close to the active site of the enzyme. Hg^{2+} has earlier been reported to completely inhibit the activity of xylanase from different sources (Bataillon *et al*., 2000; Khandeparker and Bhosle, 2000; Khanna and Gauri, 1993; Qureshy, 2002).

Table 4.2 The effect of divalent cation salts and the chelating agent EDTA on the xylanase activity of the enriched xylanase fraction from *Alternaria alternata* isolated PTRa9.

Cation salt	**Percentage relative xylanase activity[a] at various cation salt concentration**		
	1 mM	5 mM	10 mM
Control[b]	100.00	100.00	100.00
$MgCl_2$	89.79	75.53	64.68
$MnCl_2$	79.41	78.65	73.65
$CuSO_4$	65.70	22.82	19.45
$CaCl_2$	86.38	90.72	100.23
$ZnSO_4$	94.72	84.60	57.85
$FeCl_2$	66.70	55.73	54.95
$HgCl_2$	50.34	20.97	6.78
EDTA	57.00	46.57	43.33

[a]The percentage relative activity was determined by measuring the xylanase activity at 30 min at 50 ^{o}C in 20 mM sodium acetate buffer pH 5.0 after pre-incubation at 30 ^{o}C for 30 min with the indicated reagents and concentrations, [b]using the activity seen in the absence of such reagents in 20 mM sodium acetate buffer pH 5.0 alone as 100%. Data showed as the average of three replicates ± SE. Means within a column or across a row that are followed by a different lower case letter are significantly different.

4.10. Determination kinetic parameters

The K_m value was obtained from Lineweaver-Burke plot of xylanase activity at 50 ^{o}C for 30 min in 20 mM sodium acetate buffer pH 5.0 using various concentrations of xylan as substrate. The K_m of xylanase for birchwood xylan was 2.369 mg/ml, and the V_{max} was 2.142 U/mg protein (Figure 4.11). In spite of the narrow high specific activity of this enzyme toward natural xylan, the K_m of this enzyme is similar to that of xylanases from other sources (Camacho and Aguilar, 2003; Araki *et al*., 1998; Gupta *et al*., 2000).

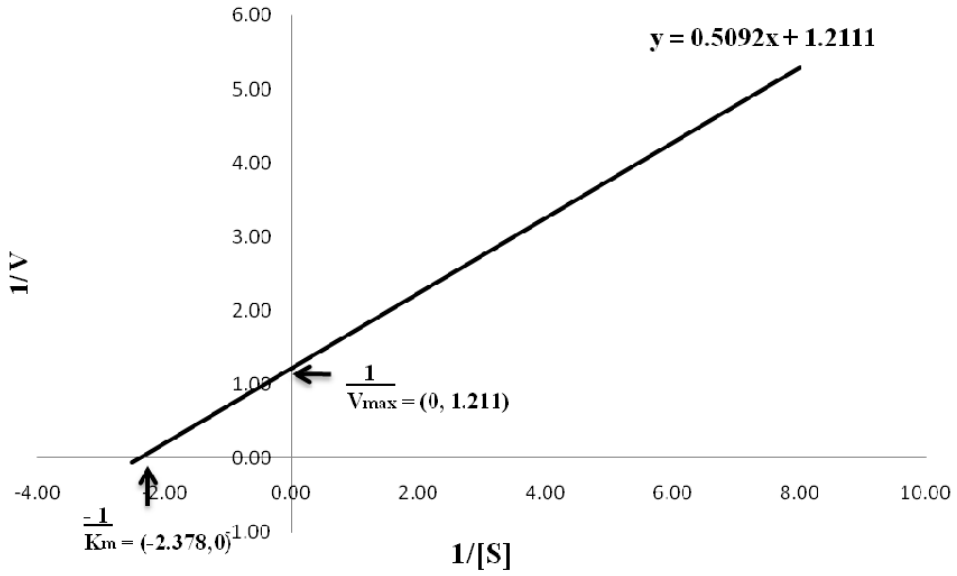


Figure 4.11 Lineweaver-Burk plot of the enriched xylanase fraction from *Alternaria alternata* isolated PTRa9. The xylanase fraction was incubated with different concentrations of birch wood xylan (1.25-20 mg) as substrate. Data are represent as mean of three replicates ± SE.

CHAPTER V

CONCLUSION

In this study, endophytic fungus isolated PTRa9 was determined that was the best isolate for xylanase production and it was identified that is *Alternaria alternata* isolated VC38 (GQ916545.1: 99%). Xylanase production, 2% (w/v) rice bran, 0.1% (w/v) $(NH_4)_2SO_4$ on 4 days were best carbon source, best nitrogen source and best time have highest xylanase activity of 1.95 U/ml. Xylanase purification, 40-60% ammonium sulphate saturation has high specific xylanase activity of 4.70 U/mg protein, DEAE-cellulose chromatography: unbound fraction has high specific xylanase activity of 10.70 U/mg protein, Superdex ^{TM} 75 chromatography: fraction 17 has high specific xylanase activity of 161.1 U/mg protein. The purified xylanase appeared to be homogeneous since the enzyme migrated as a single sharp band of 54.8 kDa. Xylanase characterization, optimum temperature and thermal stability for xylanase activity was around 40^{o}C for 30 min and 40^{o}C for 20 min, respectively, the relative xylanase activity was 124.52% and 131.27%, respectively. The optimum pH was 5.0 at room temperature for 60 min, the relative xylanase activity was 112.82%, and it was maintained over 100% at pH 3.0-11.0 for 60 min. The effect of various metal ions salt, the xylanase was strongly inhibited by almost type of whole concentration of metal ions, however, only 10 mM solution of $CaCl_2 \cdot 2H_2O$ was detected with not inhibition but not activation, the relative xylanase activity was 100.23%. For the kinetic analysis fungus PTRa9 xylanase exhibited K_m of 0.421 mg/ml and V_{max} of 0.826 U/mg protein.

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APPENDICES

APPENDIX A

The media were prepared by sterilization in the autoclave at 121 ^{o}C for 15 minutes.

Potato Dextrose Agar (PDA)

Potato	200 gram
Glucose	20 gram
Agar	20 gram
Distilled water	1 liter

Boil 200 g of peels, dried potato for 1 hr in 1000 ml. of distilled water. Filter, and make up the filtrate to one liter. Add the glucose and agar and dissolve by streaming.

Selective xylan agar

Di-potassium Hydrogen Phosphate (K_2HPO_4)	2 gram
Potassium Di-hydrogen Phosphate (KH_2PO_4)	2 gram
Ammonium Nitrate (NH_4NO_3)	2 gram
Magnesium Sulfate ($MgSO_4$)	0.6 gram
Calcium Chloride ($CaCl_2$)	0.5 gram
Birch wood xylan	10 gram
Agar	20 gram
Distilled water	1 liter

Selective xylan broth

Di-potassium Hydrogen Phosphate (K_2HPO_4)	2 gram
Potassium Di-hydrogen Phosphate (KH_2PO_4)	2 gram
Ammonium Nitrate (NH_4NO_3)	2 gram
Magnesium Sulfate ($MgSO_4$)	0.6 gram
Calcium Chloride ($CaCl_2$)	0.5 gram
Birch wood xylan	10 gram
Distilled water	1 liter

Modified basal medium (Carbon sources affect the production of xylanase)

Dipotassium Hydrogen Phosphate (K_2HPO_4)	2 gram
Potassium Dihydrogen Phosphate (KH_2PO_4)	2 gram
Ammonium Nitrate (NH_4NO_3)	2 gram
Magnesium Sulfate ($MgSO_4$)	0.6 gram
Calcium Chloride ($CaCl_2$)	0.5 gram
Distilled water	1 liter

Table 1A Various carbon sources in modified basal medium

Carbon sources	0.5%	1.0%	2.0%
Chaff Bagasse Rice bran Rice straw Sawdust	5 g/L	10 g/L	20 g/L

Modified basal medium (Nitrogen sources affect the production of xylanase)

Dipotassium Hydrogen Phosphate (K_2HPO_4) 2 gram

Potassium Dihydrogen Phosphate (KH_2PO_4) 2 gram

Magnesium Sulfate ($MgSO_4$) 0.6 gram

Calcium Chloride ($CaCl_2$) 0.5 gram

Best of carbon source and carbon concentration

Distilled water 1 liter

Table 2A Various nitrogen sources in modified basal medium

Nitrogen sources	0.1%	0.2%	0.5%
Ammonium Nitrate, NH_4NO_3 Ammonium Sulfate, $(NH_4)_2SO_4$ di-Ammonium Hydrogen Phosphate, $(NH_4)_2HPO_4$ Ammonium Chloride, NH_4Cl Ammonium Persulfate, $(NH_4)_2S_2O_8$ Peptone Yeast Urea Soybean Corn steep	1 g/L	2 g/L	5 g/L

APPENDIX B

Xylanase activity determination by DNS method

Solutions

1. 20 mM sodium acetate buffer pH 5.0

Sodium acetate ($CH_3COONa•3H_2O$)	2.7218 gram
Distilled water	1 liter

   After thorough mixing, the pH is adjusted to 5.0 with the acetic acid.

2. 1% Xylan in 20 mM sodium acetate buffer pH 5.0

Xylan	1 gram
20 mM sodium acetate buffer pH 5.0	100 millilitre

3. 1% Dinitrosalicylic Acid Reagent Solution

Dinitrosalicylic Acid (DNS)	10 gram
Phenol	2 gram
Sodium sulfite (Na_2SO_3)	0.5 gram
Sodium hydroxide (NaOH)	10 gram
Distilled water	1 liter

4. 40% Potassium sodium tartrate solution

Potassium sodium tartrate ($KNaC_4H_4O_6·4H_2O$)	40 gram
Distilled water	100 millilitre

Assay

1. | | |
   |---|---|
   | Xylanase | 250 µl |
   | 20 mM sodium acetate buffer pH 5.0 | 250 µl |
   | 1% Xylan in 20 mM sodium acetate buffer pH 5.0 | 500 µl |

   Mixed together in tubes using vertex
2. The mixture was incubated in a water bath at 37°C for 15 min and it was stopped the reaction by placing the tubes on ice for 5 min.
3. Centrifuge at 10,000 rpm for 30 min at 4^oC
4. Only 500 µl of the mixture was already centrifuged, reacted with 500 µl of 1% Dinitrosalicylic Acid (DNS) in boiling water for 15 min and it was stopped the reaction by placing the tubes on ice for 5 min.

5. Add 150 µl of 40% Potassium sodium tartrate in tubes. It was mixed together using vertex
6. Only 200 µl of the mixture was pipetted in 96 well microtiter plate then it was measured absorbance at 575 nm

Protein concentration determination by Bradford method

Solutions

1. Bradford Stock solution

95% ethanol	100	millilitre
88% phosphoric acid	200	millilitre
Brillant Blue G	350	milligram

2. Bradford working buffer

Bradford Stock solution	30	millilitre
95% ethanol	15	millilitre
88% phosphoric acid	30	millilitre
Distilled water	425	millilitre

* Store the solution in a tightly stoppered brown glass bottle at room temperature.
Before use, filtered through Whatman No.1 paper into brown glass bottle.

Assay

1. Pipette 100µl protein solution (xylanase) into tube.
2. Add 1 ml bradford working buffer and vertex.
3. Read the absorbance at 595 nm (UV) for each of the samples after 2 minutes but before 1 hour, using the sample without BSA as the blank.

Preparation for denaturing polyacrylamide gel electrophoresis

Stock solutions

1. 2 M Tris-HCl (pH 8.8)

   Tris (hydroxymethyl)-aminomethane 24.2 g

   Adjusted pH to 8.8 with 1 M HCl and adjusted volume to 100 ml with distilled water

2. 1 M Tris-HCl (pH 6.8)

   Tris (hydroxymethyl)-aminomethane 12.1 g

   Adjusted pH to 6.8 with 1 M HCl and adjusted volume to 100 ml with distilled water.

3. 10% SDS (w/v)

   Sodium dodecyl sulfate (SDS) 10 g

4. 50% Glycerol (w/v)

   100% Glycerol 50 ml

   Added 50 ml of distilled water

5. 1% Bromophenol blue (w/v)

   Bromophenol blue 100 mg

   Brought to 10 ml with distilled water and stirred until dissolved.

   Filtration will remove aggregated dye.

Working solution

1. Solution A (30% (w/v) acrylamide, 0.8% (w/v) bis-acrylamide)

   Acrylamide 29.2 g

   N, N,-methylene-bis-acrylamide 0.8 g

   Adjust volume to 100 ml with distilled water

2. Solution B (1.5 M Tris-HCl pH 8.8, 0.4% SDS)

   2 M Tris-HCl (pH 8.8) 75 ml

   10% SDS 4 ml

   Distilled water 21 ml

3. Solution C (0.5 M Tris-HCl pH 6.8, 0.4% SDS)

   1 M Tris-HCl (pH 6.8) 50 ml

   10% SDS 4 ml

Distilled water 46 ml

4. 10% Ammonium persulfate

   Ammonium persulfate 0.5 g

   Distilled water 5 ml

5. Electrophoresis buffer (25 mM Tris, 192 mM glycine, 0.1% SDS)

   Tris (hydroxymethyl)-aminomethane 3 g

   Glycine 14.4 g

   SDS 1 g

   Dissolved in distilled water to 1 litre without pH adjustment (final pH should be 8.3)

6. 5x sample buffer (60 mM Tris-HCl pH 6.8, 25% glycerol, 2% SDS, 0.1% bromophenol blue, 14.4 mM 2-mercaptoethanol)

   1 M Tris-HCl (pH 6.8) 0.6 ml

   Glycerol 5 ml

   10% SDS 2 ml

   1% Bromophenol blue 1 ml

   2-mercaptoethanol 0.5 ml

   Distilled water 0.9 ml

SDS-PAGE

1. 15% Separating gel

   Solution A 10.0 ml

   Solution B 5.0 ml

   Distilled water 5.0 ml

   10% Ammonium persulfate 100 µl

   TEMED 10 µl

2. 5.0% Stacking gel

   Solution A 0.67 ml

   Solution B 1.0 ml

   Distilled water 2.3 ml

   10% Ammonium persulfate 30 µl

   TEMED 5.0 µ

APPENDIX C

Standard curve of xylose by DNS method

Preparation of 10 mg/ml xylose

Xylose 0.1 gram

Distilled water 10 millilitre

Preparation of 1 mg/ml xylose

10 mg/ml xylose 100 µl

Distilled water 900 µl

Table 1C Preparation of standard curve of xylose

1 mg/ml xylose (µl)	Distilled water (µl)	[xylose] µg/ml
0	100	0
10	90	100
20	80	200
30	70	300
40	60	400
50	50	500
60	40	600
70	30	700
80	20	800
90	10	900
100	0	1000

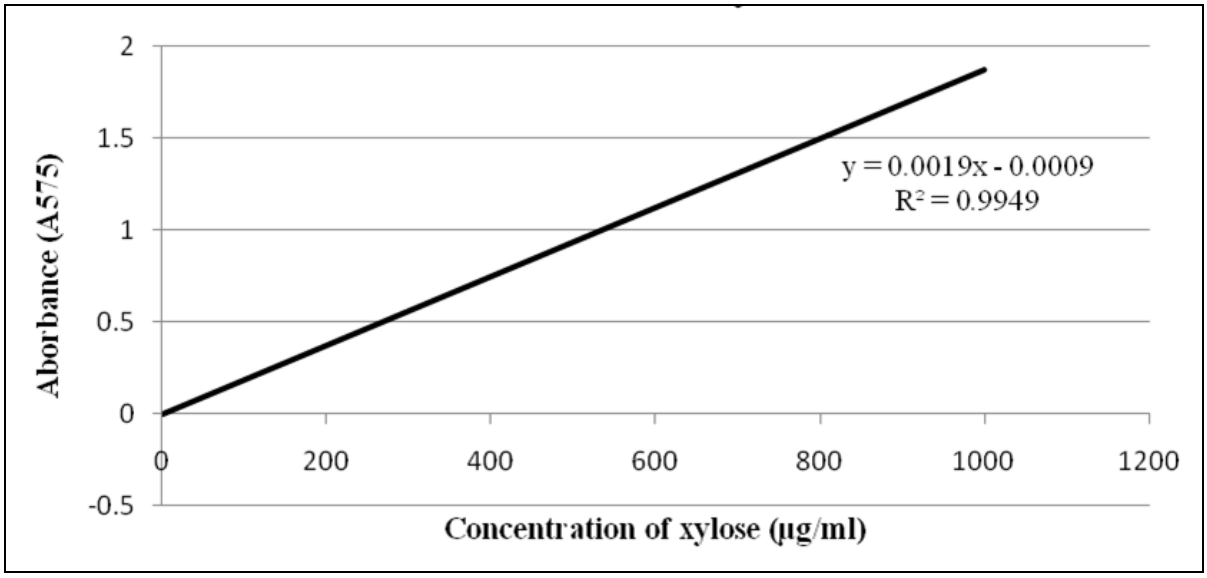

Figure 1C Standard curve of xylose

Standard curve of Bovine Serum Albumin (BSA) by Bradford method

Preparation of 10 mg/ml BSA

BSA 0.1 gram

Distilled water 10 millilitre

Preparation of 1 mg/ml BSA

10 mg/ml BSA 100 µl

Distilled water 900 µl

Table 2C Preparation of standard curve of BSA

1 mg/ml BSA (µl)	Distilled water (µl)	[BSA] mg/ml
0	100	0
2.5	97.5	0.025
5.0	95.0	0.050
7.5	92.5	0.075
10.0	90.0	0.100
12.5	87.5	0.125
15.0	85.0	0.150
17.5	82.5	0.175
20.0	80.0	0.200
22.5	77.5	0.225
25.0	75.0	0.250
27.5	72.5	0.275
30.0	70.0	0.300

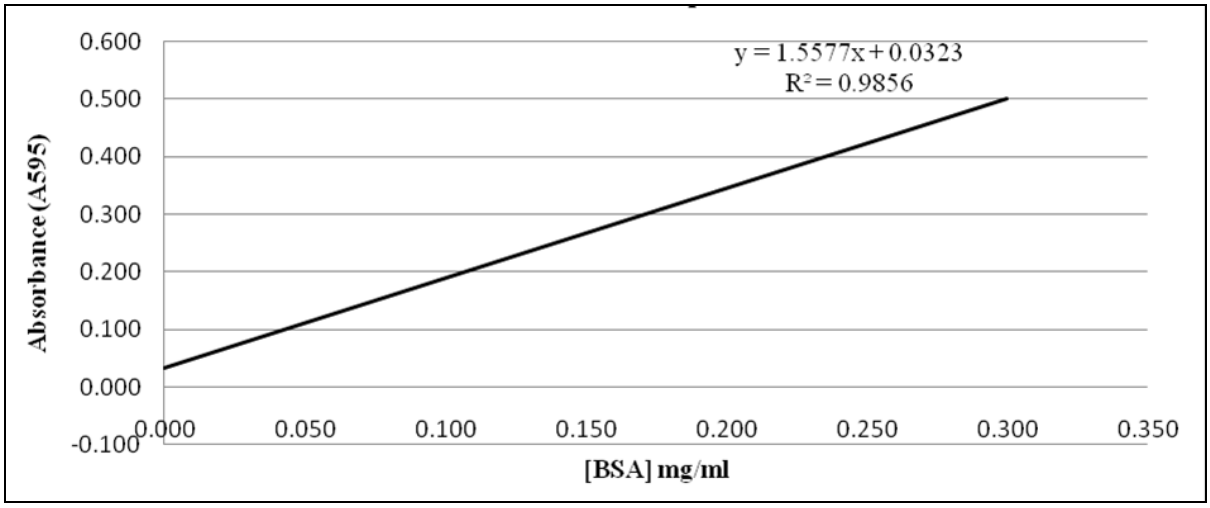

Figure 2C Standard curve of protein

APPENDIX D

Calculation of xylanase activity (U/ml)

Equation of xylose standard curve: y = 0.0019x - 0.0009

y = Absorbance (575 nm)

x = (y + 0.0009)/ 0.0019

= Concentration of xylose (µg/ml)

Concentration of xylose (µg/ml)* total volume (ml) = Δ µg

Δ µg / molecular mass of xylose (g/mol) = □ µmol

□ µmol / total time (min) = ○ µmol / min

Xylanase	A	µl	xylanase activity is	○	µmol / min
Xylanase	1000	µl	xylanase activity is	(○*1000)/A	µmol / min / ml
			=	(○*1000)/A	U / ml

For example

Equation of xylose standard curve: y = 0.0019x - 0.0009

3.933 = 0.0019x - 0.0009

x = 2070.47 µg/ml

(2070.47 µg/ml)* 1.15 ml = 2381.04 µg

(2381.04 µg) / (150 g/mol) = 15.87 µmol

(15.87 µmol) / 15 min = 1.058 µmol/min

Xylanase	250	µl	xylanase activity is	1.058	µmol / min
Xylanase	1000	µl	xylanase activity is	(1.058*1000)/250	µmol / min / ml
			=	4.23	U / ml

Calculation of specific xylanase activity (U/mg protein)

Equation of protein standard curve: $y = 1.5577x + 0.0323$

y = Absorbance (595 nm)

$x = (y - 0.0323)/ 1.5577$

= Concentration of BSA (mg)

Xylanase	A	µl	xylanase activity is	x	mg
Xylanase	1000	µl	xylanase activity is	(x*1000)/A	mg/ml

Xylanase activity (U/ml) / ((x*1000)/A (mg/ml)) = Specific xylanase activity (U/ mg protein)

For example

Equation of protein standard curve: $y = 1.5577x + 0.0323$

$0.174 = 1.5577x + 0.0323$

$x = 0.091$ mg

Xylanase	100	µl	xylanase activity is	0.091	mg
Xylanase	1000	µl	xylanase activity is	(0.091*1000)/100	mg/ml
			=	0.908 mg/ml	
(4.23 U / ml) / (0.908 mg/ml)			=	4.66 U/ mg protein	

BIOGRAPHY

Miss Nichawee Wipusaree was born on July 25, 1985 in Bangkok, Thailand. She graduated with a Bachelor Degree of Science from Department of Microbiology, Faculty of Science, Khon Kaen University in 2007. She had been studies for a Master Degree of Science in Biotechnology, the Faculty of Science, Chulalongkorn University since 2007.

Academic presentation;

1) Wipusaree, N., Sihanonth, P., Plapukiew, J., Sangvanich, P., and Karnchanatat, A. Screening and Production of Xylanase from Endophytic Fungi**,** The 12th Graduate Research Conference Khon Kaen University 2011, 28 January 2011, Khon Kaen, Thailand.
2) Wipusaree, N., Sihanonth, P., Plapukiew, J., Sangvanich, P., and Karnchanatat, A. Screening and Production of Xylanase from Endophytic Fungi, The 22nd Annual Meeting and International Conference of the Thai Society for Biotechnology TSB2010: Biotechnology for Healthy Living, 20-22 October 2010 , Songkla University, Trang, Thailand