Purification and Characterization of Xylanase from *Pichia stipitis* CBS 5773

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ชื่อวิทยานิพนธ์	การทำให้บริสุทธิ์และสมบัติของเอนไซม์ไซลาเนสจากเชื้อ *Pichia stipitis* สายพันธุ์ CBS 5773
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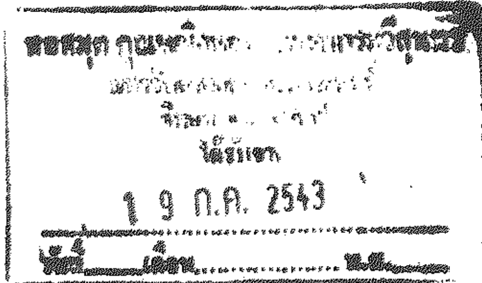

บทคัดย่อ

ยีสต์ Pichia stipitis สายพันธุ์ CBS 5773 สามารถผลิตเอนไซม์ไซลาเนส (EC.3.2.1)ได้ เมื่อเลี้ยงในอาหารที่มีไซแลน 0.5% และเขย่าที่อุณหภูมิ 30 องศาเซลเซียส โดยมีการสร้างเอนไซม์สูงสุดที่ 72 ชั่วโมง เอนไซม์ถูกทำให้บริสุทธิ์โดยวิธี gel filtration ชนิด Sephcryl S-300 เมื่อตรวจสอบความบริสุทธิ์ของเอนไซม์ที่ได้ด้วยวิธี SDS-PAGE เอนไซม์มีน้ำหนักโมเลกุลประมาณ 43.0 กิโลดาลตัน การศึกษาสมบัติของเอนไซม์ดัง-กล่าว พบว่าเอนไซม์ทำงานได้ดีที่สุดที่ pH 5 และอุณหภูมิ 40 องศาเซลเซียส เอนไซม์คงตัวในสารละลายที่มี pH 3 ถึง 6 นาน 30 นาที และมีค่ากิจกรรมของเอนไซม์เหลืออยู่ประมาณ 70% เมื่อบ่มที่อุณหภูมิ 40 องศาเซลเซียสนาน 60 นาที ค่า K_m ของเอนไซม์เท่ากับ 25 มิลลิกรัมต่อมิลลิลิตร และค่า V_{max} เท่ากับ 1.54 ไมโครโมลต่อนาที คอปเปอร์ซัลเฟต และ EDTA ความเข้มข้น 10 มิลลิโมลาร์ รวมทั้ง 1% SDS สามารถยับยั้งการทำงานของเอนไซม์ได้ การติดตามผลการย่อยสารตั้งต้น ด้วยเอนไซม์โดยวิธีโครมาโตกราฟฟีแบบกระดาษ พบว่าเอนไซม์ไซลาเนสดังกล่าวเป็นชนิด exo-xylanase ให้น้ำตาล xylobiose เป็นผลิตภัณฑ์สุดท้าย เมื่อนำเอนไซม์ 500 ไมโครกรัมไปฉีดในกระต่าย สามารถชักนำให้มีการสร้างแอนติบอดีสำหรับเอนไซม์ไซลาเนสได้ และท้ายที่สุดก็สามารถสกัด mRNA ได้ประมาณ 35 ไมโครกรัม ทั้งแอนติบอดี และ mRNA ที่ได้จะนำไปใช้ประโยน์ทาง พันธุวิศวกรรมต่อไปในอนาคต

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Abstract

Xylanase (EC.3.2.1) was elaborated into the culture filtrate of yeast, *Pichia stipitis* CBS 5773, in 0.5% xylan medium. The highest production was detected in 72 hours at 30°C. The enzyme was purified by gel filtration chromatography on Sephacryl S-300. The apparent molecular mass was 43.0 kDa from SDS-PAGE. The purified enzyme showed the maximum activity at pH 5 and optimum temperature at 40°C. The enzyme was stable from pH 3-6 for 30 minutes and stable up to 40°C for 60 minutes with 70% retained activity. The enzyme had a K_m of 25 mg/ml and V_{max} value of 1.54 μmol/min. The enzyme activity was inhibited by 10 mM $CuSO_4$, 10 mM EDTA and 1% SDS. The hydrolysis pattern on xylan by paper chromatography demonstrated that the enzyme was an exo-xylanase which produced xylobiose as a final product. The 500 μg of purified enzyme was further immunized into rabbit for antixylan antibody preparation and thirty five microgram of mRNA was finally isolated. These two products will be used for xylanase gene cloning in the future.

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List of Abbreviations

bp = Base pairs

BSA = Bovine serum albumin

DAB = 3,3'-diaminobenzidine

DNS = 3,5 dinitrosalicylic acid

EDTA = Ethylenediaminetetraacetic acid

kDa = Kilodaltons

K_m = Michaelis-Menten constant

L = Litre

mg = Milligram

ml = Millilitre

mM = Millimolar

MW = Molecular weight

^oC = Degree celsius

O.D. = Optical density

PAGE = Polyacrylamide gel electrophoresis

RBB = Remazol brilliant blue R

rpm = Revolutions per minute

SDS = Sodiumdodecyl sulfate

TEMED = N,N,N',N'-tetramethyl ethylenediamine

TLC = Thin layer chromatography

V_{max} = Maximum velocity

w/v = Weight by volume

YPD = Yeast extract, peptone, dextrose

YPDA = Yeast extract, peptone, dextrose, agar

1. Introduction

After cellulose, hemicellulose is the next most abundant renewable polysaccharide in nature (Biely *et al.*,1985). Appreciable quantities of xylan are present in material released in the pulp processing and food processing industries (Senior *et al.*,1991; Jeffries,1988). It is presently regarded as waste and often deposited in streams and rivers, where it is ecologically harmful. The conversion of xylan to useful products, therefore, presents part of the effort to strengthen the overall economics of the processing of lignocellulose biomass, and also to develop new ways of energy production from renewable resources. The enzymatic hydrolysis of xylan, which is a heteropolymer β-(1,4)-linked D-xylose, α-(1,3)-linked L-arabinose and α-(1,2)-linked D-glucopyranose, is accomplished by the action of endo-(1,4)-β-xylanase (EC 3.2.1.8) and β-xylosidase (EC 3.2.1.37). These two enzymes are widely distributed among fungi and bacteria and properties of the enzymes produced have been studied (reviewed by Coughlan and Hazlewood,1993). Reports on their occurrence in yeast and yeast-like microorganisms are very rare (Stevens and Payne, 1977; Biely *et al.*,1980; Nakanishi *et al.*,1984). Among the yeasts, the information on xylanase is largely derived from *Cryptococcus albidus* (Biely *et al.*,1980). It secretes β-xylanase, a 48 kDa protein which hydrolyzes xylan into oligo-saccharides, mainly to xylobiose and xylotriose. The gene coding for xylanase has been cloned and sequenced (Boucher *et al.*,1988).

Bruinenberg *et al.* (1984) reported the yeast *Pichia stipitis* performed a significant anaerobic alcoholic fermentation on xylose. Although the fermentation characteristics of *Pichia stipitis* on xylose are better than those of other yeasts, the values are far away from those of *Saccharomyces cerevisiae* and *Zymomonas mobilis* on glucose.

Because of many positive properties of *Saccharomyces cerevisiae* with regard to alcoholic fermentation, we decided to explore the feasibility of constructing strains which have the ability to utilize xylose. A main problem lies in the conversion of xylose into xylulose (Hollenberg and Wilhelm,1987). A large number of gene encoding xylanase has been cloned. However, the use of bacterial genes in eukaryotic systems possess several problems (Alexander,1986). It may be more advantageous to clone a eukaryotic xylanase gene instead of a prokaryotic one into *Saccharomyces cerevisiae*. Toward this purpose, we planned to introduce a xylose to xylulose pathway , lacking in *Saccharomyces cerevisiae*.

I would like to refer to purification and characterization of *Pichia stipitis* xylanase, including rabbit antixylanase antibody preparation for further clone this gene into commercial yeast.

Literature Review

1. Lignocellulose

Plant cell walls, compose of three major polymers namely cellulose, hemicellulose, and lignin. As a consequence, these structures are commonly referred to as lignocellulose. The current concept of the structure of lignocellulose is that the cellulose fibers are embedded in a lignin-polysaccharide matrix and xylan may play a significant role in the structure integrity of cell walls by covalent and non-covalent associations. Hydrolysis product of this lignocellulosic component divided into 3 classes.

1.1. Cellulose is the most abundant (30-45% w/w) and although a homopolymer of glucose units linked by a hydrolysable repeating β-(1,4)-linkage (Figure 1), its crystalline structure requires concreted action by several different classes of enzymes to effect hydrolysis (Goodwin and Mercer,1983).

1.2. Lignin is the most recalcitrant and comprises three types of aromatic monomer linked by a range of non-hydrolysable bonds of which β-aryl-ether linkage are the most common (Figure 2). It is generally accepted that lignin degradation is the important rate-limiting step in lignocellulose biodegradation. (McCarthy,1987).

1.3. Hemicellulose is the second most abundant polysaccharide in plant cell walls, accounting for 20-40% of the total carbohydrate fraction. It includes xylan, mannan, galactan and arabinan as the main heteropolymers that the principal monomers present are D-xylose, D-mannose, D-galactose and D-arabinose respectively.

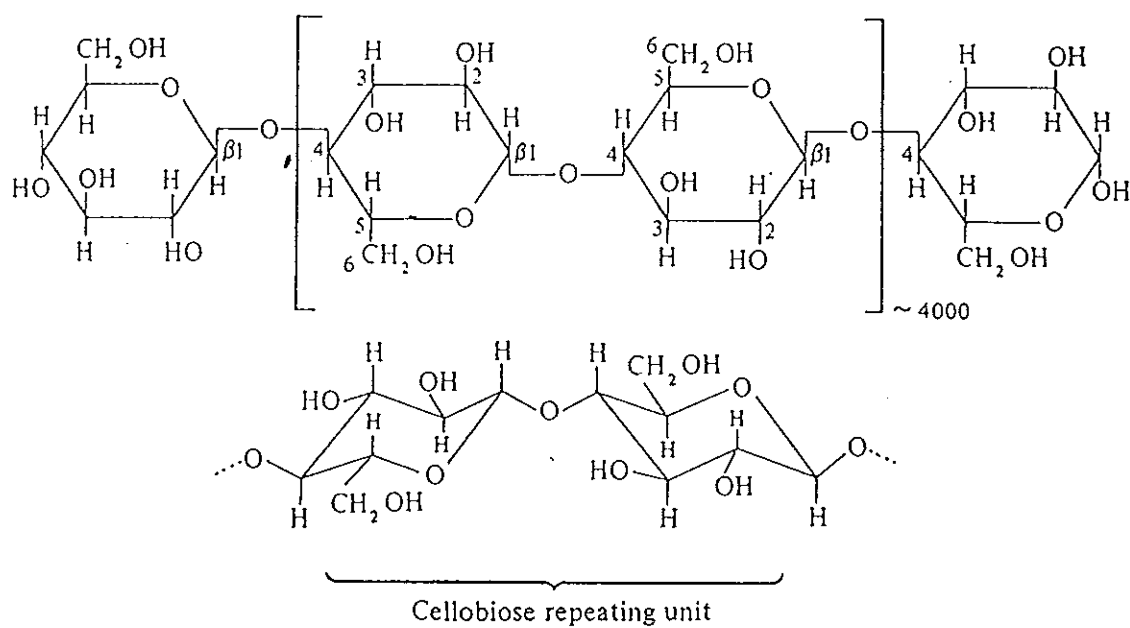

Figure1. Structure and conformation of the cellulose molecule

(Goodwin and Mercer, 1983)

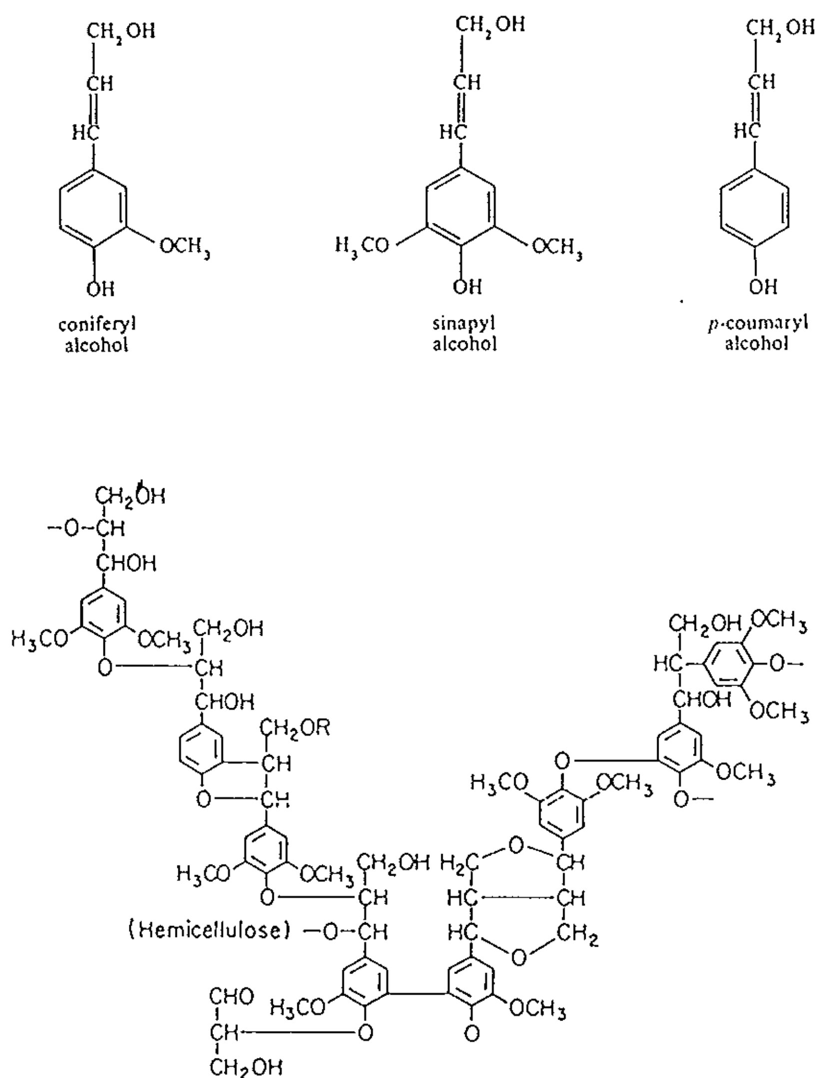

Figure 2. Lignin structure and commonly building unit (Kirk,1983)

2. Structure of Xylan

β-(1,4)-xylan is the predominant hemicellulose. It has a high degree of polymerization and is highly branched. The common substituents found on the β-(1,4)-linked D-xylopyranosyl residues are acetyl, arabinosyl and glucuronosyl residues (Figure 3). The frequency and composition of branches are depended on the source of the xylan. For instant, the young monocot cell wall is a backbone of β-(1,4)-linked D-xylopyranosyl residues carrying side chains of single glucuronic acid units and short branched chains of β-(1,3)- and (1,5)-linked arabinofuranose residues. Hard woods contain acetylated xylan and softwoods contain arabinoxylan, the acetyl and arabinosyl substituents occurring on approximately 70% and 12% of the xylosyl residues, respectively. In addition, xylan is present in some trees and other plants in a partially acetylated form. Homoxylans, consisting only of xylosyl residues, have been isolated from esparto grass and tobacco (Thomson,1993).

3. Source of Xylan

β-(1,4)-D-xylans are mainly found in secondary walls, the major component of mature cell walls on woody tissue. Although they also represent the major hemicellulose in the primary walls of monocots, xylans generally constitute a minor component of the primary walls in dicots. The importance of xylans as a carbon reservoir, is well illustrated in birch wood, of which 35% of the dry weight is xylan. In general, xylan is the major hemicellulose in wood from angiosperms but is less abundant in wood from gymnosperms. It accounts for approximately 15 to 30% and 7 to 12% of the total dry weight, respectively (Wong *et al.*,1988). Potential hemicellulosic sources include sugar crops, such as sugarcane and sorghum, and crops with high starch content, such as corn, sweet potatoes, cassava and other agricultural and forest waste residues. Waste residues from such resources contain up to 40% hemicelluloses as pentose sugars (Bastawde,1992).

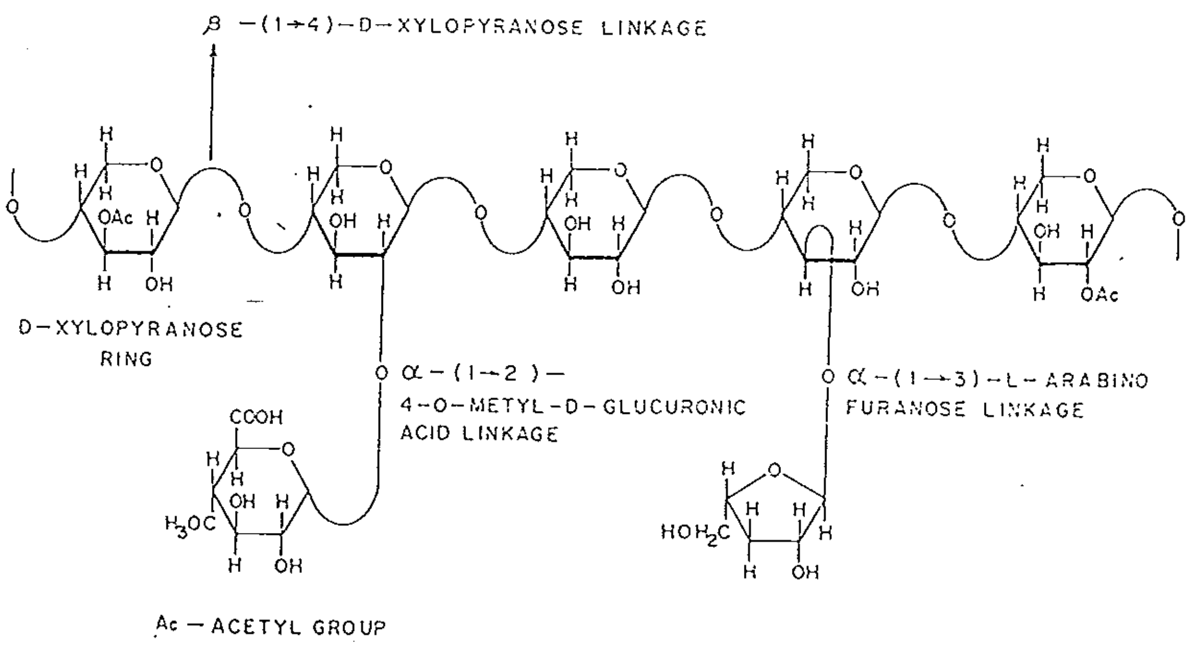

Figure 3. Xylan structure with side chains attached (Bastawde, 1992)

4. Isolation and Extraction of Xylan (Bastawde,1992)

Hemicelluloses are extracted effectively from lignified tissues of grasses and woody plants by alkali treatment. However, partial extraction of hemicellulose from plants is achieved by hot and/or cold water or dilute alkali. Generally 4 to 10% KOH or NaOH is used. However, hemicellulose extraction also used 24% KOH, but the acetyl groups present in the xylans of wood and grasses were hydrolysed during this treatment. Prolonged treatment of rye flour and barley husk with 7% NaOH caused a 20% decrease in the molecular weight of these polymers. The fractions extracted by dilute alkali contained low molecular weight xylan fractions, whereas the concentrated alkali treatment selectively removed the higher molecular weight fractions. Moreover dimethyl sulphoxide (DMSO) was used for hemicellulose extraction from wood homocellulose. Xylan and arabinoxylan is extracted from kraft pulps by removing the glucoside uronic acid residues with $Ba(OH)_2$ Xylan extraction also used acid treatment, a pH of 3.7 to 4.2 was critical for maximum yield before alcohol precipitation of corn cob xylan. Arabinose residues in xylan fractions were removed by 0.2% oxalic acid solution or 0.012% HNO_3 while the xylan fractions of wheat bran and beach wood were isolated with 3% HNO_3.

5. Properties of Xylan (Reviewed by Bastawde, 1992)

5.1. Deacetylated xylans are insoluble in water, but soluble in alkaline and are easily hydrolyzed with acids.

5.2. Acetylated xylans can be extracted by hot water and are more soluble in water.

5.3. Xylan solutions do not reduce Fehling's solution.

5.4. Xylan solutions show high negative optical rotation ranging from $[\alpha]\ D_{20}$ -78.2 to 109.5^{o} C.

5.5. Acetylated xylans are easily degraded by microbial enzymes.

6. Classification of Xylanases

Enzymes which break down hemicelluloses are referred to as hemicellulases : they are defined and classified according to the substrates on which they act. They are collectively grouped as glycan hydrolases (EC.3.2.1). L-Arabinanases degrade only L-arabinans; D-galactanases break down galactans and L-arabino-D-galactans; Mannanases hydrolyse the β-(1,4)-D-mannopyranose linkages of mannans and β-xylanases cleave the β-(1,4)-D-xylopyranosyl linkages of xylans. It appears that xylosidic linkages in lignocellulose are not all equivalent and equally accessible to xylanolytic enzymes. The accessibility of some linkages also changes during the course of hydrolysis. The production of a system of enzymes, each enzyme with specialized functions, is one strategy that a microorganism may use to achieve superior xylan hydrolysis (Bastawde,1992).

Three different types of xylanases are involved in degradation. Those are :

6.1. Endo-β-(1,4)-D-Xylanase. [β-(1,4)-D-Xylano Hydrolase, EC.3.2.1.8]

These enzymes act randomly on xylan to produce large amounts of xylo-oligosaccharides of various chain lengths. They are divided into four types :

6.1.1. Non-arabinose but Xylose Liberating Endoxylanases.

These cannot act on L-arabinosyl initiated branch points at β-(1,4) linkages and produce only xylobiose and xylose as the major end product. These enzymes can break down xylo-oligosaccharides as small as xylobiose.

6.1.2. Non-arabinose and non-xylose Liberating Endoxylanases.

These can not cleave branch points at β-(1,2) and (1,3) linkages and produce mainly xylooligosaccharides larger than xylobiose. These endoxylanases have no action on xylotriose and xylobiose.

6.1.3. Arabinose and Xylose Liberating Endoxylanases.

These can cleave the xylan chain at the branch points and produce mainly xylobiose, xylose and arabinose.

6.1.4. Arabinose but Non-xylose Liberating Endoxylanases.

These can hydrolyze the branch points and produce intermediate size xylooligosaccharides and arabinose.

6.2. Exo-β-(1,4)-D-Xylanase. [β-(1,4)-D-Xylan Xylohydrolases]

These enzymes remove the single xylose units from the nonreducing end of the xylan chain.

6.3. β-Xylosidase or Xylobiase. (EC.3.2.1.37)

These enzymes hydrolyse disaccharides like xylobiose and the higher xylooligosaccharides with decreasing specific affinity.

Endoxylanases (EC.3.2.1.8) are often prevented from cleaving the xylan backbone by the presence of substituents. Therefore, in many cases these must be removed before extensive degradation of the backbone can occur. The enzyme involved include acetylesterases (EC.3.1.1.6), α-L-arabinofuranosidases (EC.3.2.1.55) and α-glucuronidases (EC.3.2.1) as shown in Figure 4.

7. Determination of xylanase activity

Xylanase activity can be determined by measuring the increase in reducing end-groups. Other methods that are not commonly used are RBB-xylan test and turbidity test as the following details.

7.1. Detection of Reducing end of End Product

A common method for the determination of endo β(1,4)-xylanase activity is based on the measurement of reducing sugars released from the corresponding soluble or insoluble polysaccharide using dinitrosalicylic acid reagent. The reaction mixture was heated for 5 minutes in boiling water bath and then cooled under running tap water adjusted to ambient temperature. The colour intensity was measured at 540 nm (Miller,1959). One International xylanase unit (IU) corresponds to 1 μmol. of reducing sugar as xylose produced in 1 minute under the standard assay condition.

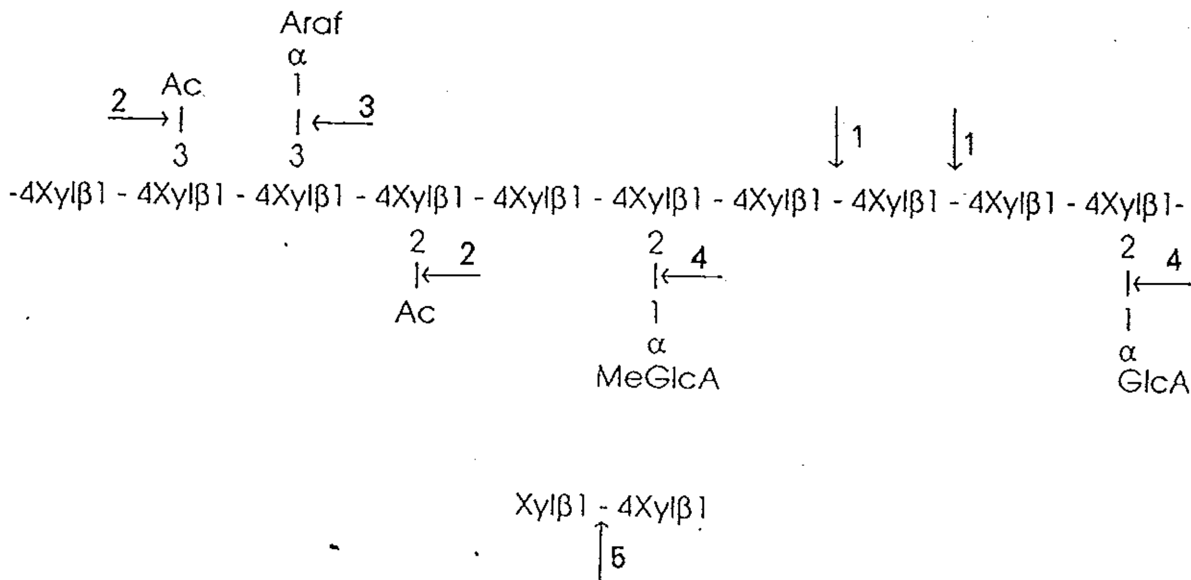

Figure 4. Part of a hypothetical xylan molecule showing sites of hydrolysis by microbial xylanolytic enzyme. 1, endo-*β*-(1,4) D-xylanase; 2, acetyl esterase; 3, α-L-arabinofuranosidase; 4,glucuronidase; 5, *β*-xylosidase (Thomson,1993)

7.2. RBB-xylan Test

A substrate for the enzyme assay is derived from soluble xylan with covalently bounded dyes (RBB: Remazol brilliant blue R). The determination of the enzyme activity on RBB-xylan is done at proper temperature and pH. The reaction was terminated by addition of 2 volumes of 96% ethanol. After 5-30 minutes of standing at a given temperature, the precipitated substrate was removed by centrifugation at 2,000 g for 15 minutes and the absorbance of the supernatant was measured at 595 nm against the respective blank (Biely *et al.*,1985).

7.3. Turbidity Test

Two methods above are difficult to interpret quantitatively if both endo-xylanase and β-xylosidase are present, because they act synergistically. In nature, the first step in the utilization of xylan is solubilization, which is likely to be affected primarily by endoxylanase. Therefore, the accurate measurement of endoxylanase activity is not the amount of reducing sugar produced but the extent of solubilization of a xylan suspension. For this method, the enzyme was incubated with xylan suspension in optimize condition for 30 minutes. After incubation, the reaction was stopped by freeze-thawing and addition of 30% PEG 600. Then the samples were pipetted into cuvettes and left standing at room temperature for at least 15 minutes. The turbidity was measured at 600 nm against blank which consisted of only substrate suspension. One unit of xylan solubilization activity was defined as that which solubilized 1 milligram of the xylan suspension in 1 minute (Wong and Broda,1992)

8. Source of Xylanase

Xylanases are widely distributed. They occur in both prokaryotes and eukaryotes and have been demonstrated in higher eukaryotes, including protozoa, insects, snails and germinating plant seeds. Among the prokaryotes, bacteria and cyanobacteria from marine environments produce xylanases. Extracellular and intra-cellular xylanases from various bacterial protozoa and fungal sources have been studied extensively (Bastawde, 1992).

9. Purification of Xylanase

Summation of xylanases which have been purified to homogeneity and their biochemical properties from various microorganisms are demonstrated in Table 1. Culture broth containing extracellular xylanases can be concentrated either by evaporation under low pressure or by freeze-drying (Bastawde,1992). Other procedures used for concentration are precipitation with organic solvents at lower temperatures (Tsujibo *et al.*,1990) or with 55 to 90% ammonium sulphate saturation (Biswas *et al.*,1990 ; Mujer *et al.*,1991 ; Lumba and Penninckx,1992). The final purification of xylanases is by a combination of anionic (Nguyen-Veit *et al.*,1991 ; Huang *et al.*,1991 ; Faulds and Williamson,1991 ; Simpson *et al.*,1991 ; Nguyen-Veit *et al.*,1991) and cationic (Yamaura *et al.*,1990 ; Lumba and Penninckx,1992 ; Faulds and Williamson,1991) ion exchangers and gel filtration column chromatography (Biswas *et al.*,1990 ; Tsujibo *et al.*,1990 ; Nanmori *et al.*, 1990 ; Nguyen-Veit *et al.*, 1991 ; Bray and Clark,1990 ; Mujer *et al.*,1991), isoelectric focusing (Tsujibo *et al.*, 1990 ; Bachmann and McCarthy,1991 ; Koyama *et al.*,1990). Affinity chromatography (Suzuki and Ashida,1989), and high performance liquid chromatography (HPLC) (Grapinet *et al.*,1988 ; Kluepfel *et al.*,1990 ; Holden and Walton,1992) have also been used recently.

Table 1. Characteristics of xylanases from different microorganisms.

Microorganism	MW (kDa)	Final purification step	optimum		Stability		pI	*Km*	*Vmax*	Reference
			pH	Temp(°C)	pH	Temp(°C)		mg/ml	µmol/min/ml	
Cryptococcus flavus	31.0	Bio-Gel-P-100	4.5	55	3.0-8.0	45	10.0	3.1	UD	Nakanishi *et al.*,1984
Trichoderma koningii G-39	21.5	TSK HW-50F	5.5	60	UD	UD	8.9	0.7	185	Huang *et al.*,1991
Bacillus stearothermophilus	39.5	FPLC Mono G	7.0	60	5.0-1.0	60	5.1	3.8	UD	Nanmori *et al.*,1990
Fibrobacter	53.7	CM-Cellulose	7.6	39.5	5.5-7.0	25-60	7.4	2.6	336	Matte and
succinogens S-85	66.0	CL6B	3.0	50.0	5.5-8.8	15-70	8.0	1.3	118	Forsberg,1992
Thermomonospora fusca	32.0	Isoelectricfucusing	6-8	70	UD	UD	UD	2.6	UD	Bachmann and McCarthy,1991
Aeromonas caviae	22.0	Sephadex G-100	7.0	55	7	50	9.2	UD	UD	Nguyen-Veit *et al.*, 1991

UD = Undetermine

Table1. (continues).

Microorganism	MW (kDa)	Final purification step	optimum pH	optimum Temp(°C)	Stability pH	Stability Temp(°C)	pI	*Km* mg/ml	*Vmax* μmol/min/ml	Reference
Cellvibrio gilvus	40.0	CM-Toyopeal	6.5	55	4.0-9.0	50	5.0	UD	UD	Haga *et al.,* 1991
Streptomyces olivochromogenes	29.0	Mono Q	5.0	30	UD	UD	UD	UD	UD	Faulds and Williamson,1991
Streptomyces sp. *strain EC10*	32.0, 22.0	Sephadex G-100	7.0, 8.0	60	UD	UD	6.8, 8.9	3.3, 4.5	UD	Lumba and Penninckx,1992
Pseudomonas sp. *strainPT-5*	35.0	Butyl-Toyopearl 650 M	7.5	UD	5.5-8.0	UD	UD	UD	UD	Yamaura *et al.,*1990

UD = Undetetrmine

10. Mode of Action of Endoxylanases and End Product Analysis

(Review by Bastawed,1992)

The mode of action of only a few homogenously purified endoxylanases of microbial origin has been studied, either by research on kinetics, determining K_m and V_{max} values, or by end product analysis involved paper chromatography, thin layer chromatography, high permance liquid chromatographgy and subsite mapping techniques. Of the two techniques, end product analysis is suitable for endotype hydrolysing enzyme, while the kinetic method is particularly useful for exotype hydrolases. Study on the mode of action of these enzymes will only be meaningful if pure, homogeneous preparation enzyme and substrate are used. Such studied have been reported for mould, yeast, actinomycete and bacterial endoxylanase

10.1. Endoxylanase of Fungal origin

10.1.1. Rhoszyme HP-150. There were five endoxylanase with different biochemical properties. The first one produced mainly xylobiose and xylose from xylan and xylooligosaccharides, while the other four yielded longer chain length products.

10.1.2. *Aspergillus niger.* Meagher *et al* (1988) used both kinetic and end product analysis techniques to determine the subsite maps of the endoxylanase. Kinetic parameters showed that the endoxylanase had a high affinity as the substrate chain length of xylooligosaccharides increased. On the other hand, the β-(1,4) bond closer to the reducing end was attacked first but as the substrate chain length increased the enzyme attack was shifted towards the inner side of the chain from the reducing end. For example, an acidic endoxylanase from *Aspergilus niger* shown the frequency of 90% at the first β-(1,4)-linkage of xylotriose. Whereas xylotetraose was hydrolysed to xylobiose with 87% bond cleavage frequencies at the second β-1,4 linkage from the reducing end. The endoxylanase cleaved the third β-(1,4)-linkage rather than the second or first xylosidic bond of xylopentose during hydrolysis.

10.1.3. *Ceratocystis paradoxa.* The *C. paradoxa* endoxylanase preferentially cleaved the highly substituted xylan regions with either uronic acids or arabinose rather than the unsubstitiuted one.

10.1.4. *Trametes hirsuta.* The *T. hirsuta* degraded 4-O-methyl-Glc-p-A-xylotriose. The initial hydrolysis products were xylohexaose and xylotetraose but on prolonged hydrolysis, xylobiose and xylose were detected. This indicated that *T. hirsuta* endoxylanase requires five D-xylosyl residue to form the active enzyme substrate complex.

10.1.5. *Sporotrichum dinorphyosporum.* The enzyme produced more complex end products, such as arabinoxylobiose, arabinoxylotriose, glucuronic acid arabino-xylotriose and glucuronic acid arabinoxylotetraose. It was proposed that the enzyme requires trixylooligosacharides to form the productive complex, even though the nonreducing end xylosyl unit was substituted at 3-O region.

10.1.6. *Trichoderma viride.* The *T. viride* endoxylanase produced mainly xylose, xylobiose and xylobiose glucuronic acid arabinose.

Most of fungal strains also produce debranching type endoxylanase, and their bond cleaving specificities are determined as the β-1,4 xylosidic bonds of the xylose units in the main chain of xylans and α-1,3-L-arabinose residues on the sidechain of xylans. Whether this daul action of cleavage of two different linkages in xylan substrate takes place by two different active sites or only one active site is not yet clear and understood. The fungal strain reports having this type of endoxylanase are *Aspergillus niger* strain 14, *Aspergillus niger, Ceratocystis paradoxa, Trichoderma reesei, Talaromyces byssochlamydoides, Oxisporum sp,* and *Trichoderma viride.* Endoxylanase from *A.niger* was studied for its substrate binding site and subsite mapping analysis. It was found that the cleavage frequency of xylotriose to xylose was almost 90% at the first β(1,4)-linkage of xylotriose, 87% at second linkage from reducing end for xylotetraose.

10.2. Bacterial Endoxylanase

The endoxylanase of *Bacillus* and *Streptomyces* fall under this category. There are a few reports published on the mode of action of thes endoxylanase.

10.2.1. *Bacillus circulans WL-12.* Two endoxylanases isolated from *Bacillus circulans* WL-12. Xylanase II did not produce xylose from xylan, but principally xylobiose, xylotriose and xylotetraose. It was shown that this endoxylanase required a minimum of four *β*-(1,4)-D-xylopyranoside linkage residues to form productive complex whereas endoxylanase I could rapidly degrade xylan to xylotetraose, prolonged incubation gave xylose, xylobiose and xylotriose as the main end products in the hydrolysate.

10.2.2. *Bacillus sp.* II-IS. This endoxylanase produced xylose, xylobiose and xylotriose as the end products from rice straw xylan.

10.2.3. *Streptomyces T-7.* Keskar (1990) reported that the major end products of xylan hydrolysis by *Streptomyces T-7* endoxylanase is xylobiose and xylooligosaccharides with traces of xylose residues on short incubation. An interesting observation was that the endoxylanase, similar to the *β*-xylosidase of *Aspergilus niger,* gave only xylose as the end product after 16 hours of xylan hydrolysis.

10.2.4. *Streptomyces* sp. 3137. Three different endoxylanases of this strain was reported by product analysis using TLC. When the xylanases were incubated at 60 to 65°C with xylan and xylooligosaccharides, xylose and xylobiose were the only end products formed.

10.2.5. *Streptomyces* sp. KT-23. Endoxylanase from *Streptomyces* sp. KT-23 produced large amount of xylobiose initially, which was further cleaved to xylose on prolonged incubation.

10.2.6. *Chainia* sp. Endoxylanase was studied for its mode of action on xylan, xylooligosaccharide and ^{3}H-labelled xylohexaose by HPLC. Xylan hydrolysis

mainly produced xylohexose and xylotriose as the end product, but did not produce any xylose even on prolonged incubation.

10.3. Endoxylanase from Yeasts

10.3.1. *Trichosporon cutanum.* Stuttgen and Sahm (1982) studied oat husk arabinoxylan hydrolysis by *Trichosporon cutanum* endoxylanase and found xylose, xylobiose and xylotriose were the main products on prolonged incubation. Arabinose was not detected in the hydrolysates, even after prolonged incubation.

10.3.2. *Cryptococcus albidus.* The bond cleavage frequency studies with ^{3}H-labelled of different xylooligosaccharides from xylotriose to xylopentaose indicate that the substrate binding site for this type of endoxylanase was composed of four subsites. As the chain length of xylooligosaccharide increases, the preference of bond cleavage frequency shifts towards its inner side from the reducing end. Further reported that the bond cleavage of these substrates mainly depends on their concentration. At low substrate concentration, the hydrolysis preceeds at a uni-molecular level but at higher substrate concentration the bond cleavage mechanism change to bimolecular level.

11. Molecular Cloning of the Xylanase Gene

Different research groups have attempted to clone the xylanase gene from bacteria as well as yeast strains into *Escherichia coli* and other hosts. Such studies are essential to produce a more efficient xylanolytic microbial strain which will be useful in biotechnology. The xylanase gene cloning of various microorganisms are summarized in Table 2.

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Table 2. Xylanase gene cloning of various microorganisms.

Microorganism	Gene length (bp)	Vecter	Host	Enzyme MW. (Da)	Reference
Butyrivibrio fibrisolvens 49	2,300	pUC19	*E.coli* JM83	46,664	Mannarelli *et al.*,1990
Cellulomonas sp. CIM 2353	1,420	pUC18	*E.coli* JM 107	45,000	Bhalerao *et al.*, 1990
Caldocellum saccharolyticum	6,067	pNZ1076	*Bacillus* sp. C125	42,000	Luthi *et al.*, 1990
Bacteroides ovatus 0038	3,800	pBT-2	*E.coli* DH5	UD	Weaver *et al.*, 1992
Cryptococcus albidus	UD	pJHS	*Pichia stipitis* PJH53	50,000	Morosoli *et al.*, 1993
Clostridium thermocellum ATCC	1,600	pJX18	*B.subtilis* DB104	45,000	Jung and Pack , 1993
Neocallimastix partriciarum 27	UD	pUC18, pUC19	*E.coli* HB101	58,510	Lee *et al.*, 1993a
Thermoanaerobacterium saccharolyticum B6a-RI	3,471	pHC79	*E.coli* HB101	130,000	Lee *et al.*, 1993b
Bacillus sp. NCIM59	6,500	pLPX6	*B. subtilis* MI111	UD	Shendye *et al.*, 1994

12. Application of Xylanase

12.1. Bioconversion

For bioconversion process that converts lignocellulose to fermentative products, maximal utilization of the various polymeric sugars is desirable. Such complete xylanolytic system, achieve maximum hydrolysis of complex substrate to yield the monomeric residues, would have xylanase, β-xylosidase and other enzyme involved in debranching xylans. The products of such hydrolysis may be convert subsequently into liquid fuels, single-cell protein, solvents and other chemicals by selectively use of specific fermentative microorganism (Wong *et al.*,1988).

12.2. Biopulping

In paper manufacturing, fungi treatment has been suggested because it can reduce energy costs of refining to the paper products, the used of purified xylanases may reduce treatment time of pulps. Xylanases may also yield a range of desirable pulp characteristics by selectively hydrolyzing certain xylan component. Similar considerations also apply to the use of xylanases for preparing other lignocellulosic fibers (Sharma,1987).

12.3. Protein Engineering

The hydrolysis efficiency, hydrolysis specificity, or stability of these enzymes may also be improved by using protein engineering. These goals, would be facilitated by a better understanding of the relationship between the structure and function of xylanases. Stability is a highly desirable characteristic for industrial enzymes because it permits enzyme recycling and simpler handling and storage conditions (Knowles, 1987).

12.4. Waste elimination.

Xylan is generated as a waste product from agriculture, forestry, paper industry and food processing industry (Hollenberg and Wilhelm,1987). Because of problem with the disposal of waste water, bioconversion process is attractive for elimination of this residue.

Aims of thesis

1. To purify xylanase from yeast, *Pichia stipitis*.
2. To study biochemical characteristics of the enzyme.
3. To prepare mRNA and specific antibody against the purified enzyme.

2. Materials and Methods

Materials

1. Chemicals

Name	MW	Company
Acrylamide	71.08	Merck
Ammonium persulfate	228.19	Carlo Erba
Bovine serum albumin	-	Sigma
Bis-acrylamide	154.10	Fluka
Calcium chloride	147.20	J.T.Baker
Chloroform	119.38	May & Baker
Coomassie brilliant blue G-250	826.00	Sigma
Dextrose	180.16	Fluka
Dinitrosalicylic acid	228.19	Fluka
EDTA	372.24	Fluka
Glycine	75.07	Fluka
Lithium chloride	42.40	May & Baker
Magnesium chloride	203.30	Merck
Oat spelts xylan	-	Sigma
Phenol	94.11	Carlo Erba
Sodium hydroxide	40.00	BDH
TEMED	116.20	Sigma
Xylose	150.10	Sigma
Yeast extract	-	Difco
Yeast nitrogen base	-	Difco

2. Instruments

Instrument	Model	Company
Visible Spectrophotometer	Spectronic 21	Milton Roy
UV-Visible Spectrophotometer	Ultrospec-3	Pharmacia
Refrigerated centrifuge	Z 382 K	TLG
Centrifuge	230 A	TLG
Centrifuge	CH-1200 B	Kokusan
pH meter	109	Activon
Analytical balance	Junior 2000C	Precisa
Vortex mixer	G-560 E	Scientific industries Inc.
Hot plate stirrer	NUOVA II	Sybron
Water bath	-	Lab-line instruments Inc.
Larminar air flow	NU 425-400 E	Nuaire
Incubator	IM 550 R	Clayson
Autoclave	HA-300 MII	Hirayama
Hot air oven	OM 1000	Clayson
Fraction collector	2100	BIO-RAD
Microscope	CH-2	Olympus

Methods

1. Yeast Strain

Pichia stipitis strain CBS 5773 was obtained from Professor. Dr. C.P. Hollenberg, Institut fur Mikrobiologie, Heirich-Heine Universitat Dusseldorf, Germany.

The yeast strain was maintained on yeast extract, peptone, dextose, agar (YPDA) plate and grew in liquid xylan media at 30°C.

2. Preparation of Liquid Xylan Medium

Oat spelts xylan was obtained from Sigma Chemical Co. St.Louis. A 0.5% (w/v) solution of oat spelts xylan was autoclaved with 0.67% (w/v) yeast nitrogen base with amino acids.

3. Production of Xylanolytic Enzyme from *Pichia stipitis*

An inoculation culture was prepared by transferring a loopful of cells from a YPDA plate into 5 ml of YPD broth, and shaking at 30°C for 16-24 hours. The cells were harvested by centrifugation at 8,000 rpm for 10 minutes and washed once with sterile distilled water. The pellet was resuspended in 2 ml of sterile distilled water. One millilitre of cell suspension was transferred into 250 ml medium containing 0.67% yeast nitrogen base and 0.5% xylan, and it was shaked at 30°C for 3-5 days. The growth of cells was followed by cell counting under the microscope. The cells were harvested by centrifugation at 8,000 rpm for 10 minutes and the clear supernatant was used for determination of xylanase, xylosidase and cellulase activity.

The harvested cells were washed twice with 50 mM sodium acetate buffer, pH 5.1. They were disintegrated with glass beads by using 11 grams of washed glass beads (0.4 mm diameter) per yeast cells obtained from a 200 ml culture.

Cell breakage was examined under light microscope. When more than 95% of the cells were ruptured, homogenates were separated from ballotini beads after dilution with known volumes of 50 mM sodium acetate buffer (pH 5.1). Cell walls were isolated by centrifugation at 2,500 g for 5 minutes and, the supernatant was used for determination of xylanase and β-xylosidase activity.

4. Growth on Other Carbon Sources in Liquid Media

Inoculation cultures were prepared as in xylan medium, but 0.5% glucose or 0.5% xylose were used instead of 0.5% xylan.

5. Analytical Methods

5.1. Determination of reducing sugar

The reducing sugars liberated as enzyme-hydrolysis products were measured by DNS method (Miller,1959) with xylose as a standard. The color tests were made with 0.4 ml of DNS reagent (Appendix 1.2) added to 0.4 ml aliquots of sample in 16x150 millimetre test tube. The mixture was heated for 5 minutes in a boiling water bath and cooled under running tap water. Four millilitres of distilled water were added and the color intensity was then measured at 520 nm.

5.2. Determination of protein.

Protein concentration was measured by the method of Bradford (1976) with bovine serum albumin as a standard. The color test was done by mixing 0.3 ml of sample with 3 ml of Bradford's reagent (Appendix 1.1) in 13x100 millimetre test tube. The absorbancy at 595 nm was measured after 30 minutes against a reagent blank.

6. Enzyme Assays

6.1. Xylanase

The reaction mixture consists of an appropriated dilution of enzyme, 1mg/ml soluble xylan, and 50 mM sodium acetate buffer, pH 5.1, which make up to the total volume of 0.4 ml. The xylan substrate was prepared from the soluble fraction of a 5 mg/ml suspension of oat spelts xylan in sterile distilled water which was autoclaved and centrifuged at 5,000 g for 20 minutes. A suitable dilution of enzyme in 50 mM acetate buffer, pH 5.1, was incubated with 200 µl of substrate at 40°C for 60 minutes. The reaction was terminated by boiling for 10 minutes and the reducing sugar concentration was determined by the Miller's procedure (Miller,1959), with xylose as a standard. The enzyme activity is expressed as µmol of reducing sugar released per minute per millilitre of the undiluted enzyme.

6.2. β-xylosidase

An activity of *β*-xylosidase was determined in a reaction mixture (total volume of 0.55 ml) consisting of an enzyme suspension with 0.5 ml of 2 mg/ml *p*-nitrophenyl *β*-D-xylopyranoside in 50 mM. acetate buffer, pH 5, incubated at 30°C for 1 hour. The reaction was terminated by addition of 1 ml saturated solution of Na_2B_4O, pH 9.4. The insoluble material was immediately removed by centrifugation at 10,000 rpm for 10 minutes and the supernatant was measured spectrophotometrically at 410 nm. with *p*-nitrophenol as a standard. One unit of *β*-xylosidase is defined as an amount of enzyme which liberates 1 µmol of *p*-nitrophenol per 1 minute from the corresponding substrates.

6.3. Cellulase

Cellulase activity was determined by measuring the reducing sugar liberated from carboxymethylcellulose, CM-23 (Fluka). The substrate solution was 2% carboxymethylcellulose in 50 mM sodium acetate buffer, pH 4.8 . The suspension was autoclaved at 121 pounds for 15 minutes and allowed to cool at room temperature. The activity was determined by incubating a 15 ml reaction mixture containing an

appropriated enzyme suspension and 2 mg/ml of carboxymethylcellulose in 50 mM acetate buffer, pH 5.1, for 180 minutes at 30°C. The liberated reducing group was measured by Miller method (Miller,1959) with an anhydrous D-glucose as a reference standard. One cellulase unit corresponds to 1 μmol of reducing sugar produced in 1 minute.

7. Purification of Xylanase

Step I A culture filtrate (500 ml) from 3 days culture of *Pichia stipitis* (as described in method 3 page 25) was concentrated by lyophilization. The precipitate was dissolved in 7 ml of 50 mM acetate buffer, pH 5.1. The undissolved solid particles were separated by centrifugation and the clear supernatant was further purified in the next step.

Step II The 7 ml portion of this concentrated enzyme was applied to a Sephacyl S-300 column (2x27 cm), which was previousely equilibrated with 50 mM sodium acetate buffer, pH 5.1, and eluted with the same buffer at the flow rate of 0.5 ml/min. Two millilitres per fraction was then collected and measured its absorbancy at 280 nm. The active fractions were pooled and concentrated by lyophilization.

8. SDS polyacrylamide gel electrophoresis

SDS-PAGE was carried out as described by Laemmli (1970).

The slab gel (9x5x0.1cm) with a 12.5% separating gel and 4.5% stacking gel (9x2x0.1 cm) was used.

The protein sample was mixed with a sample buffer containing 1% SDS, 10% glycerol, 5% β-mercaptoethanol and 0.4 mg of bromophenol blue in 0.5 M Tris-HCl buffer, pH 6.8, and boiled for 5 minutes. Electrophoresis was carried out at room temperature using 0.025 M Tris-HCl, 0.192 M glycine, pH 8.3, and 0.1% SDS as an electrophoretic buffer. A constant voltage of 20 milliampere was supplied until

the tracking dye approached the buttom of the gel. Phosphorylase b (MW 94,000), BSA (MW 67,000), ovalbumin (MW 45,000), carbonic anhydrase (MW 29,000), soybean trypsin inhibitor (MW 20,100) and lactalbumin (MW 14,400) were used as reference proteins.

After the electrophoresis was finished, The gel was stained with silver nitrate as the following method.

9. Rapid Silver Staining (Applied from Panbankrade and Udomvorapan,1993)

The gel was immersed in 50 ml of formaldehyde fixing solution, containing 40% methanol and 50 μl of 37% formaldehyde, for 10-30 minutes. After it was washed with two folds of deionized water for 5 minutes, the gel was immersed in 50 ml of sodium thiosulfate (0.2 g/l), containing 3% of sodium carbonate (w/v), 0.0004% of sodium thiosulfate (w/v) and 50 μl of 37% formaldehyde, for 2-3 minutes. It was then washed with two folds of deionized water for 5 minutes and immersed in 20 ml of 0.1% silver nitrate solution for 20 minutes with shaking continuously. The gel was washed with deionized water and immersed in 20 ml thiosulfate developing solution until protein band was produced. The reaction was stopped by addition of 2.3 M. citric acid (1 ml) and shaken slowly for 10 minutes and washed with two folds of deionized water for 10 minutes. The gel was immersed in drying solution for 10 minutes and kept between 2 wet dialysis membranes to dry overnight.

10. Effect of pH on the Enzyme Activity

10.1. Optimum pH

Xylanase activity was measured at different pH values using 0.5 mg/ml xylan solution as a substrate and incubated at 40°C for 60 minutes. The buffers were 50 mM acetate buffer for pH 3-5, 50 mM McIlvaine for pH 6-8 and 50 mM Glycine-NaOH for pH 9-10.

10.2. pH Stability

The initial activity of the enzyme was assayed using 0.5 mg/ml xylan solution as a substrate and incubated at 40°C for 60 minutes. The enzyme solution was preincubated for 30 minutes at various pH (3-10) without the substrate. The pH was then adjusted to 5 with 50 mM sodium acetate buffer, pH 5, and the remaining activity was determined.

11. Effect of Temperature on the Enzyme Activity.

11.1. Optimum Temperature

The enzyme activities were assayed at various temperature (20-60°C) using 0.5 mg/ml xylan solution in 50 mM sodium acetate buffer, pH 5, as a substrate and incubated for 60 minutes.

11.2. Thermostability

The initial activity of the enzyme was assayed using 0.5 mg/ml xylan solution in 50 mM sodium acetate buffer, pH 5, as a substrate and incubated for 60 minutes. The enzyme solution was preincubated for 30-150 minutes at various temperatures (20-60°C) without the substrate, and immediately cooled down on ice. The remaining activity was then assayed.

12. Determination of Kinetic Constant of Enzyme.

The purified enzyme (1.125 mg/ml) was incubated with a varying concentration of soluble xylan (1.75 - 3.25 mg/ml in 50 mM sodium acetate buffer, pH 5.1). The enzyme activity was measured at every 30 minutes of incubation time for 3 hours. The initial velocity was taken as the slope of the linear portion of the graph of reducing sugar formation *versus* time as determined by linear regression. K_m and V_{max} value were calculated by Lineweaver-Burk plot.

13. Effect of Some Ions and Chemicals on Enzyme Activity

The remaining enzyme activity was measured after the reaction mixture was incubated with 10 mM of KCl, NaCl, $CaCl_2$, $MgCl_2$, $ZnSO_4$, $CuSO_4$, EDTA, (dissolved in 50 mM acetate buffer, pH 5.1), 1% SDS and 5% ethanol respectively, at 40°C for 1 hour.

14. Determination of Xylan Degradation Products by Paper Chromatogaphy.

The purified xylanase 0.16 units was incubated with 2 mg of xylan in 0.8 ml of 50 mM acetate buffer, pH 5.1, for different periods of time (0-48 hours). At any given time intervals, 100 µl of each sample was boiled in boiling water bath for 5 minutes. The samples were then spotted on the Whatman paper no.3, with 40 µg xylose and 80 µg xylobiose as reference standards. The paper was placed in a developing tank (for ascending chromatography at room temperature) containing of butanol : acetic acid : H_2O (3:1:1, by volume), for 8-10 hours and allowed to dry at room temperature. The dry paper was immersed in silver nitrate solution (prepared by diluting 0.1 ml of saturated aqueous silver nitrate solution to 20 ml with acetone, and adding water dropwise, with shaking, until the silver nitrate which separates on addition of acetone has redissolved). It was spayed with a 0.5 N NaOH in aqueous ethanol. Brown silver oxide is immediately produced. When reduction is completed, excess silver oxide is removed by immersing the strip in 6 N ammonium hydroxide for a few minutes. The paper was then dried in an oven. Dark brown spots on white background were obtained (Trevelyen *et al.*,1950).

15. Preparation of Antibody Against Xylanase.(Harlow and lane,1988)

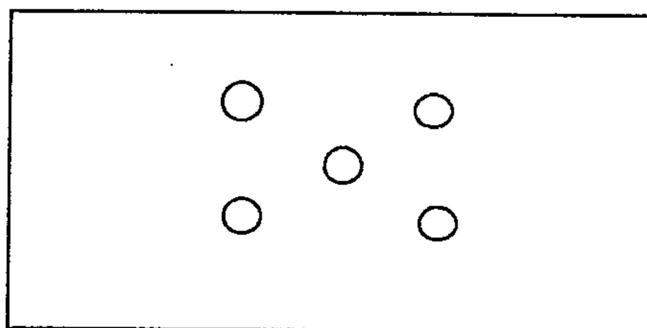
15.1. Immunization of Rabbit

The antigen, approximately 500 µg of purified xylanase, was pipetted into 2 volumes of the adjuvant oil and then transferred into a syringe. The plunger was moved up and down for several times to mix until it was difficult to push. All the air was then removed and a 25 gauge needle was added. The rabbit was placed in a containing chamber and the needdle was inserted into the space which has been created by pulling the rabbit skin at the back of the neck from its body. The end of the needle was moved for a short distance to ensure it was not inserted into muscle. The plunger was then depressed to inject the desired amount of mixed antigen (0.1ml) and then paused for a few second. The injection was moved to the next site and repeated upto 10 sites. The primary injection was given in complete freund's adjuvant while all boosters were done with incomplete freund's adjuvant.

Before each injection, 10 ml of blood was collected from rabbit ear vein to check the production of specific antibody. The first test bleed was taken before the immunization began to prepare a suitable control antibody for further tests. After collection, blood should be allowed to clot for 30-60 minutes at room temperature. The clot was then separated from the side of the collection vessel using a pasteur pipette. The serum was removed from remaining insoluble material by centrifugation at 10,000 rpm for 10 minutes at 4°C and checked for the production of specific antibody. After preparation, the serum was stored at -20°C.

15.2. Determination of Antibody

A 1.4% agarose solution was melted in PBS buffer, pH 7.2 (containing 8.0 g of NaCl, 0.2 g of KCl, 50 mM EDTA, 1.44 g of Na_2PHO_4 and 0.24 g of KH_2PO_4 in 1 L. of distilled water) with a microwave. After it was cooled to 45°C, 3 ml of the agarose solution was pipetted onto the top of a 3x5 clean glass slide and allowed to harden at room temperature. Using a 200 µl capillary pipet. Five small holes were carefully cord as the following pattern.



A 5 µl of rabbit antiserum was added to each of the wells in the outer ring and 5 µl of the antigen (purified xylanase) was added to the middle well. The agar was incubated in a humid atmosphere overnight at room temperature. A positive reaction was scored by the appearance of precipitin line between the well with reactive antibody.

15.3. Purification of Antibody by Sephadex G-75

One millilitre of serum was applied to a 0.5x25 cm. Sephadex G-75 column equilibrated with PBS buffer, pH 7.2, containing 8.0 g of NaCl, 0.2 g of KCl, 1.44 g of Na_2PHO_4 and 0.24 g of KH_2PO_4 in 1 L. distilled water and eluted with the same buffer at the flow rate of 0.6 ml/min. Two millilitres fraction was collected and protein concentration was measured at the absorbancy of 280 nm. IgM can be separated easily from the other antibody at the first peak.

16. Western Blot Analysis (Leonard *et al.*,1994)

16.1. Transfer of protein from SDS-PAGE to Nitrocellulose Membrane

The crude enzyme was performed in 12.5% polyacrylamide gels as described previously (No. 8 page 28). After finish electrophoresis, the gel, nitro-cellulose membrane and paper no.3 were immersed in a dish with transfer buffer containing 25 mM Tris-HCl, 192 mM glycine and 20% methanol, assemble a sandwich of components in the following order : paper no.3, SDS-PAGE gel

(rinsed briefly in transfer buffer), nitrocellulose membrane (prewet in transter buffer for 10 min), paper no.3, carefully remove any air bubbles between layers. The sandwich was placed between support pads provided with the transfer apparatus, inserted the sandwich and support pads into the transfer device so that the membrane was closest to the positive electrode. The proteins were transfered for 2-4 hours in refrigerator using 150 Volts at 175 miliampere (2 gel). After transfer, the membrane was removed from the sandwich and rinsed briefly in distilled water. Dual membranes were prepared (after the transfer finished), one membrane was stained with coomassie brilliant blue and the other was used in the primary antibody reaction.

16.2. Membrane staining with Coomassie Solution to Visualize Protein Transfer

The first transfer membrane (from 16.1) was incubated in coomassie solution, containing 0.1% coomassie blue R-250, 41.6% methanol and 1.68% glacial acetic acid, for 30 seconds at room temperature and then destained in destaining solution (30% methanol and 10% acetic acid) at room temperature with shaking 3-4 times until protein bands could be visualized over background.

16.3. Incubation with Primary Antisera

The second transfer membrane (from 16.1) was incubated in a sealed plastic pouch at room temperature by series of following solutions : TBS buffer (0.025 M Tris, 0.5 M NaCl, pH 7.5) for 10 minutes, blocking agent (TBS buffer and 3% BSA) for 30 minutes, washing buffer (TBS buffer and 0.05% Tween 20) with shaking for 5 minutes, Diluted primary antisera (diluted rabbit antixylanase antibody with TBS buffer 1:300) for 1-2 hours, washing buffer for 5 minutes. The blot was now ready for incubation with a secondary antisera for nonisotopic detection.

16.4. Nonisotopic Detection Using Secondary Antisera Conjugated to Horseradish Peroxidase

The incubated primary antisera membrane (from 16.3) was incubated in diluted secondary antisera (1:1000) with gentle agitation for 1-2 hours, washed twice in washing buffer for 5 minutes each and followed by washing three times in TBS buffer. The washed membrane was placed in suitable plastic tray and 10 ml of probe reagent (50 mg of DAB and 30 μl of 35% hydrogenperoxide in 100 ml TBS buffer) was then added. A suitably brown band was left to develop in the dark, usually involved a 5 to 10 minutes incubation time. The reaction was stopped by washing the membrane 2-3 times in distilled water to remove the hydrogen peroxide. The immunoreactive band would appear brown in color. It was dried at room temperature and the membrane was kept in plastic bag.

17. Yeast RNA Isolation

17.1. Total RNA Isolation (Sherman *et al.*,1986)

One litre of yeast cell in medium at the highest specific activity (3 days) was harvested by centrifuge at 8,000 rpm for 10 minutes. The cells were resuspended in 2.5 ml LETS buffer containing 0.1 M LiCl, 0.01 M EDTA, 0.01 M Tris-HCl (pH 7.4), 0.2% SDS and 0.1% diethyl pyrocarbonate. The cell suspension was added to a 25 ml plastic centrifuge tube containing 10 g of glass beads and 3 ml of phenol equilibrated with LETS buffer. Meniscus should be justed above surface of glass beads for best cell breakage. The centrifuge tube was shaken at top speed alternating 30 seconds, with 30 seconds on ice for a total of 3 minutes. Cell breakages could be checked microscopically under phase contrast which broken cells appeared as non-refractile ghosts. When cells were broken for at least 90%, 5 ml cold LETS buffer was added. It was then vortexed briefly and spinned at 8,000 rpm to break phases. The aqueous phase was transferred to a clean tube and extracted twice with 5 ml phenol/ chloroform/isoamyl alcohol (25:25:1). It was extracted once

with chloroform. Three volumes of absolute ethanol was added and allowed to precipitate for 24 hours at -20°C. The RNA was separated by centrifuged 8,000 rpm for 10 minutes and washed once with 70% ethanol. The precipitate was dried under vacuum and part of these was resuspended to check RNA by agarose gel electrophoresis.

17.2. mRNA Isolation (Method from Clontech Laboratories Inc.)

The total RNA was resuspended to a final concentration of 1 mg/ml in elution buffer (10 mM Tris-HCl, pH 7.4 and 1 mM EDTA) and heated at 70°C for 6 minutes. It was pipetted up and down for several times. The sample was then removed, and allowed to cool at room temperature for 5-10 minutes. The 1/5 volume of sample buffer containing elution buffer and 3 M. NaCl was added into the sample, mixed well, and quickly transferred into the freshly prepared column positioned within a 50 ml conical tube. The sample was mixed with the oligo (dT)-cellulose by pipetting it up and down for several times by a sterile disposable pipette. The column was allowed to stand at room temperature for 10 minutes and centrifuged at 1,500 rpm, 4°C, for 2 minutes. The eluate at the bottom of the 50-ml tube was transferred into another clean tube, 0.3 ml of high salt buffer, containing elution buffer and 0.5% M. NaCl, was applied. The column was centrifuged at 1,500 rpm, 4°C, for 2 minutes and the high salt was discard flow-through. The step was repeated again, and the flow-through between each wash was then discard. The column was washed three times with 0.2 ml aliquots of low salt buffer containing elution buffer and 0.1 M. NaCl, by centrifuging at 1,500 rpm, 4°C, for 3 minutes. The column was lifted out of the centrifuge tube, and discarded any flow-through collected in the centrifuge tube. A sterile 1.5-ml microcentrifuge tube was placed inside a new 50-ml tube to collect subsequent eluates. The lower end of the column was inserted into the microcentrifuge tube and 0.4 ml of elution buffer (prewarm to 65°C) was applied to the column. It was spinned at 1,500 rpm for 2 minutes at room temperature and repeated twice to quantitatively eluate the bound poly A^{+}RNA.

The spin column was removed from the centrifuge tube using sterile forceps and placed the microcentrifuge tube on ice,1/10 volume of 2 M potassium acetate pH 5.0 and 2.5-3 volume of absolute ethanol was added to the polyA$^+$RNA sample. It was stored at -20°C for further use.

17.3 RNA Examination of Agarose Gel (Sambrook *et al.*,1989)

A 0.8 g of powdered agarose was added to 100 ml of TAE buffer (0.040 M. Tris-acetate and 0.001 M. EDTA) in an 250 Erlenmeyer flask. The agarose was heated in a microwave oven for 3 minutes until dissolved and it was allowed to cool to 60°C. The warm agarose solution was then poured into the 3 mm thick mold with no air bubbles. After the gel was completely set (30 minutes), carefully removed the comb and the gel was mounted in the electrophoresis tank. The TAE buffer was added to cover the gel to a depth of about 1 mm. The 5 µl of RNA solution from step 17.1 and 17.2 was mixed with 2 µl of loading buffer containing 20% Ficoll 400, 0.1 M EDTA, 1% SDS, 0.25% Bromphenol blue and 0.25% xylene cyanoland, slowly load the mixture into the slots of the submerged gel using a disposable micropipette. The lid of the gel tank was closed and the electrical leads was attached so that the RNA would migrate toward the anode. A voltage of 100 volts was then applied. The gel was run until the bromophenol blue and xylene cyanoland have migrated to the appropriated distance. The electric current was then turned off and the gel was stained with ethidium bromide solution for 5 minutes. After immersing the gel in distilled water for 5 minutes, the gel was examined by ultraviolet light and photographed.

3. Results

1. Production of Xylanolytic Enzymes from *Pichia stipitis*

A medium containing 0.5% oat spelts xylan was inoculated with an over night culture of *Pichia stipitis* and shaked at $30^{\circ}C$ for 72 hours. The activities of xylanase, cellulase and xylosidase were assayed from cell free cultured fluids and fluids from disintegrated cells. Results in Table 3 demonstrated that *Pichia stipitis* excreted xylanase into the culture medium whereas the activity of xylosidase was detected mainly in the cell. No cellulase activity was found in culture fluid while the activity in cell fraction was not determined.

Table 3 Activity of xylanase, xylosidase and cellulase from *Pichia stipitis* in different fractions of cell culture at 72 hours.

sample	xylanase(U/ml)	xylosidase (U/ml)	cellulase
culture fluid	0.55	0.00	0.00
cell fraction	0.00*	0.12*	ND

ND = not determined

*data obtained from Sivasariyanon,1995

2. Effect of Carbon Sources on Xylanase Production

The production of extracellular xylanase during growth of *Pichia stipitis* on medium using 0.5% oat spelts xylan, 0.5%glucose, and 0.5%xylose as carbon sources were shown in Figure 5. It was found that the synthesis of xylanase from *Pichia stipitis* cells was induced strongly by xlylan, while xylose and glucose had low inducing activity. Xylanase specific activity appeared in the growth medium after a lag of 24 hours and showed the highest activity of 32.35 unit/mg at 72 hours.

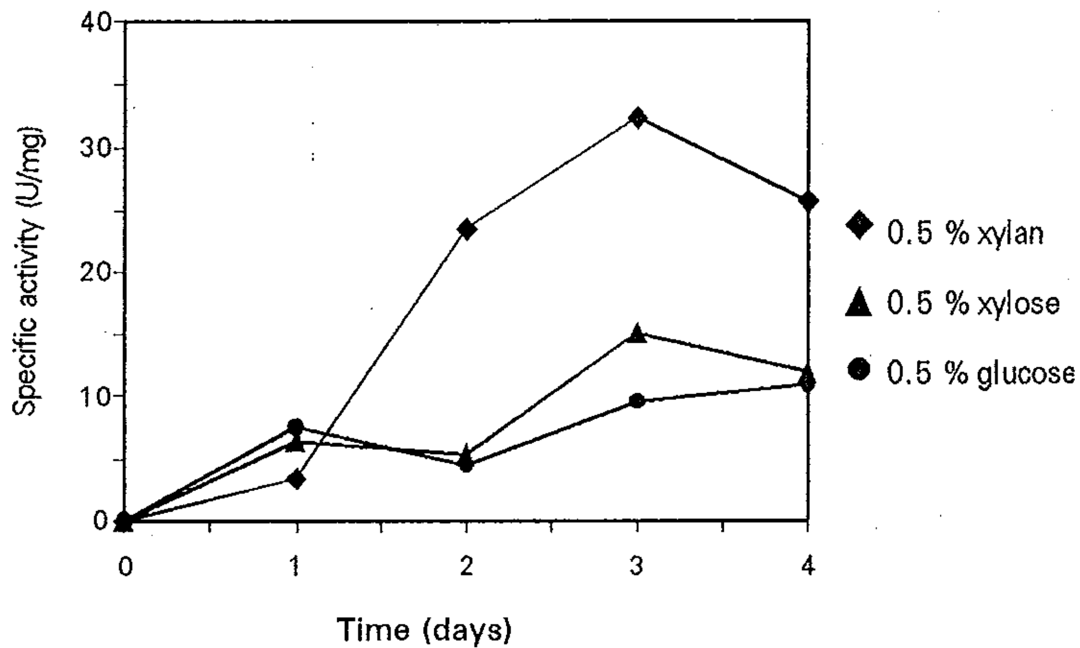


Figure 5. Total xylanase production in culture medium with different carbon sources.

Pichia stipitis was grown in medium containing 0.67% yeast nitrogen base with 0.5% oat spelt xylan, 0.5% glucose and 0.5% xylose as carbon sources and incubated at 30°C.

3. Effect of Carbon Sources on Proteins Production

The crude proteins were prepared from supernatants of yeast cell culture medium containing glucose, xylose and xylan as carbon sources incubated at 30°C for 72 hours. The crude proteins were lyophilized and 30 μg of each samples were loaded on gel containing 12.5% polyacrylamide. Figure 6. showed the electrophoretic patterns of extracellular protein in different carbon source. There was an increased amount of a high molecular weight protein in xylan medium (which was further identified as xylanase).

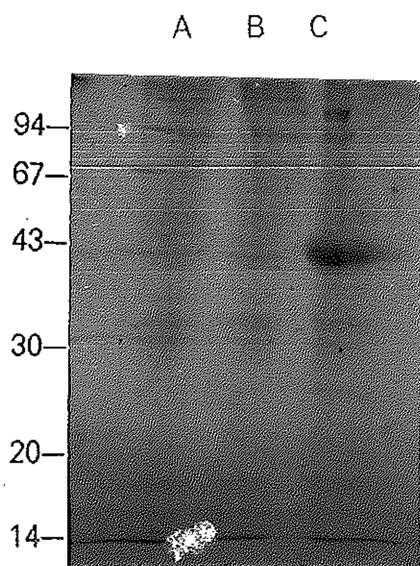


Figure 6. SDS-PAGE analysis of xylanase induction in *Pichia stipitis*

The crude proteins were prepared from supernatants of yeast cell culture medium containing different carbon sources incubated at 30°C for 72 hours : A, 0.5% glucose; B, 0.5% xylose; C, 0.5% xylan and loaded on 12.5% SDS-PAGE. The gel was stained with coomassie brilliant blue.

4. Purification of Xylanase.

Details of enzyme purification are summarized in Table 4. Xylanase was purified 3.2 fold on enzyme specific activity with a recovery yield of 4.46%. Figure 7. showed the final step of xylanase purification on Sephacyl S-300 column chromatography. Proteins were separated into four peaks, the xylanase activity peak showed coincidence with the third protein peak. The fraction no. 25 through 30 had high activity. The purified sample gave a single band on SDS-PAGE (Figure 8).

Table 4. Summary of xylanase purification step.

Purification step	Total protein (mg)	Total activity (units)	Specific activity (units/mg)	Recovery (%)	Purity (fold)
Crude enzyme	7.650	207.01	27.06	100.00	1.00
Lyophilyzed	0.623	11.83	18.99	5.71	0.70
Sephacyl S-300	0.110	9.24	86.52	4.46	3.19

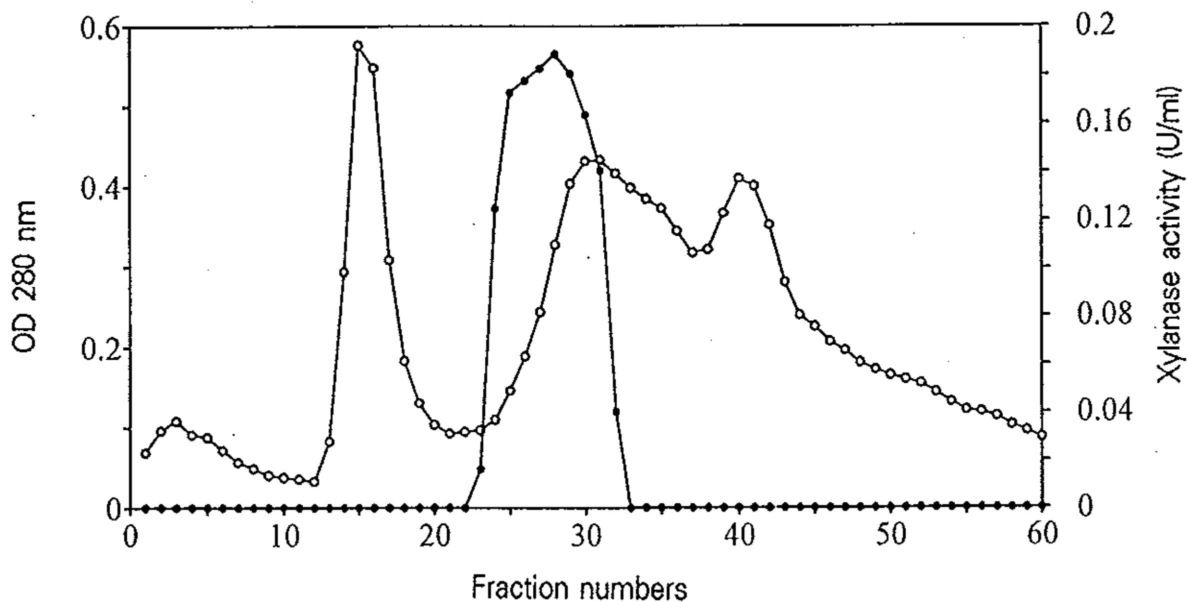


Figure 7. Gel filtration of crude enzyme after lyophilization.

The enzyme after lyophilization was applied to a Sephacyl S-300 column which was previously equilibrated with 50 mM sodium acetate buffer, pH 5.1, and eluted with the same buffer at the flow rate of 0.5 ml/min. Two millilitres per fraction was then collected. Open circle denotes absorbancy at 280 nm., closed circle denotes xylanase activity.

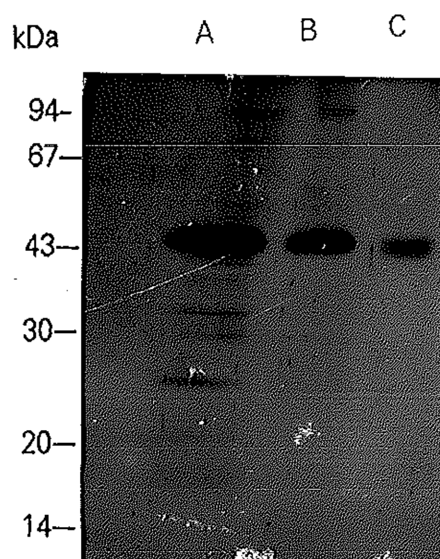


Figure 8. SDS-PAGE of various stages of protein purification.

25 μg of each samples were loaded on gels containing 12.5% polyacrylamide. After electrophoresis, the gel was stained with silver nitrate. Lane A, crude enzyme; Lane B, Crude lyophilized protein; Lane C, a pool from gel filtration (fraction no. 25-30).

5. Molecular Weight Determination

Electrophoretic mobilities of purified xylanase and reference protein on SDS-PAGE were showed *versus* its molecular weight (Table 5). The mobility of the purified xylanase corresponded to a molecular weight of 43.0 kDa.

Table 5 Electrophoretic mobilities of purified xylanase and reference protein on SDS-PAGE

MW (Dalton)	Log MW	R_f
94,000	4.93	0.21
67,000	4.83	0.29
43,000	4.63	0.47
30,000	4.48	0.64
20,100	4.30	0.85
14,400	4.15	0.95
xylanase	4.63	0.47

6. Enzyme Properties

The purified xylanase had an optimum activity at pH 5.0 (Figure 9) and optimum temperature at 40°C (Figure 10). The enzyme was stable from pH 3-6 for 30 minutes at 30°C (Figure 11) and stable up to 40°C for 60 minutes (Figure 12) with 70% retained activity. Effect of various ions and some chemicals on the activity of enzyme were summarized in Table 6. The activity was slightly increased upon the addition of K^{+}, Ca^{++}, Mg^{++}, and Zn^{++} and was markedly inhibited by Cu^{++}, EDTA and 1% SDS.

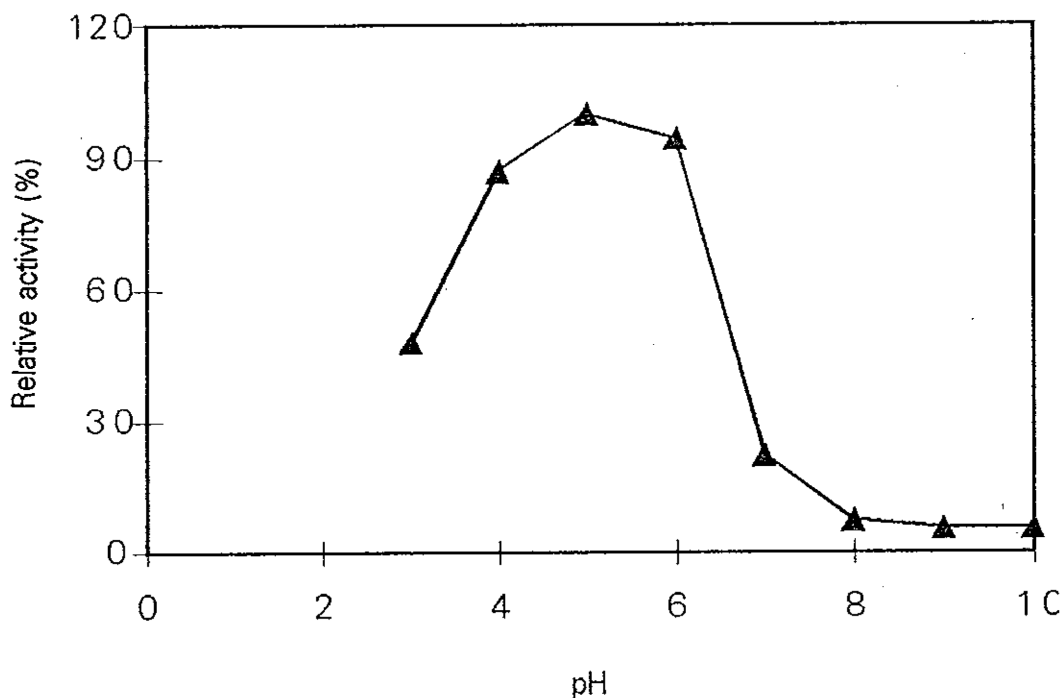


Figure 9. Effect of pH on xylanase activity.

The xylanase activity was measured at different pH values using 50 mM acetate buffer for pH 3-5, 50 mM McIlvaine buffer for pH 6-8 and 50 mM Glycine-NaOH buffer for pH 9-10 and incubated at 40°C for 60 minutes.

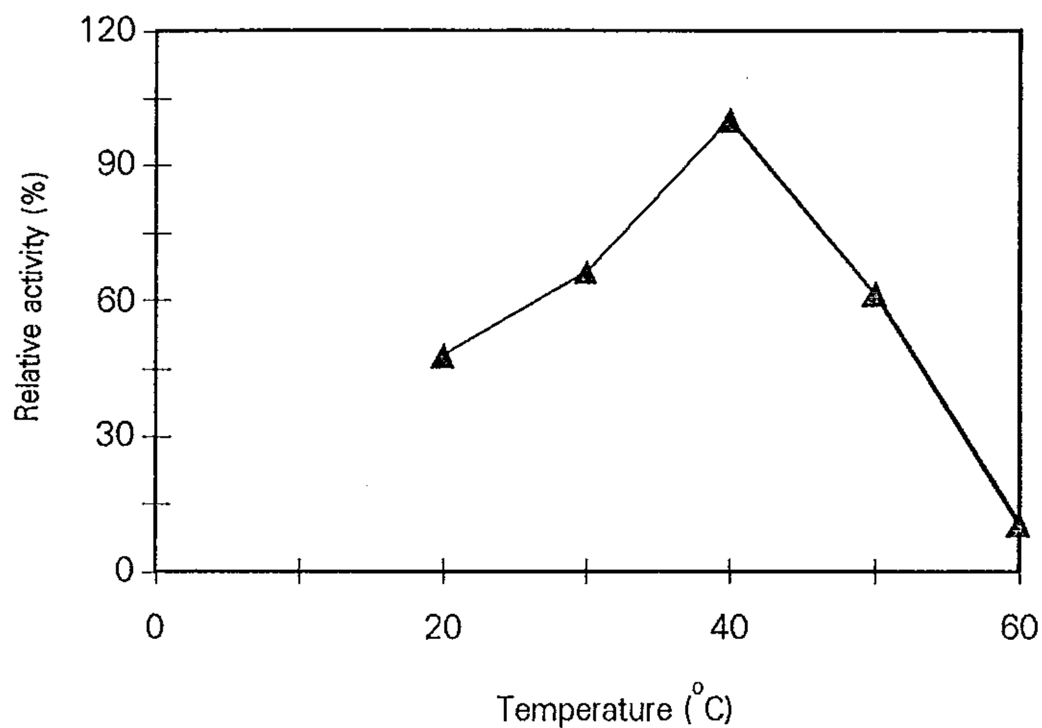


Figure 10. Effect of temperature on xylanase activity.

The enzyme activities were assayed at various temperatures (20-60°C) using 0.5 mg/ml xylan solution in 50 mM sodium acetate buffer, pH 5, as a substrate and incubated for 60 minutes.

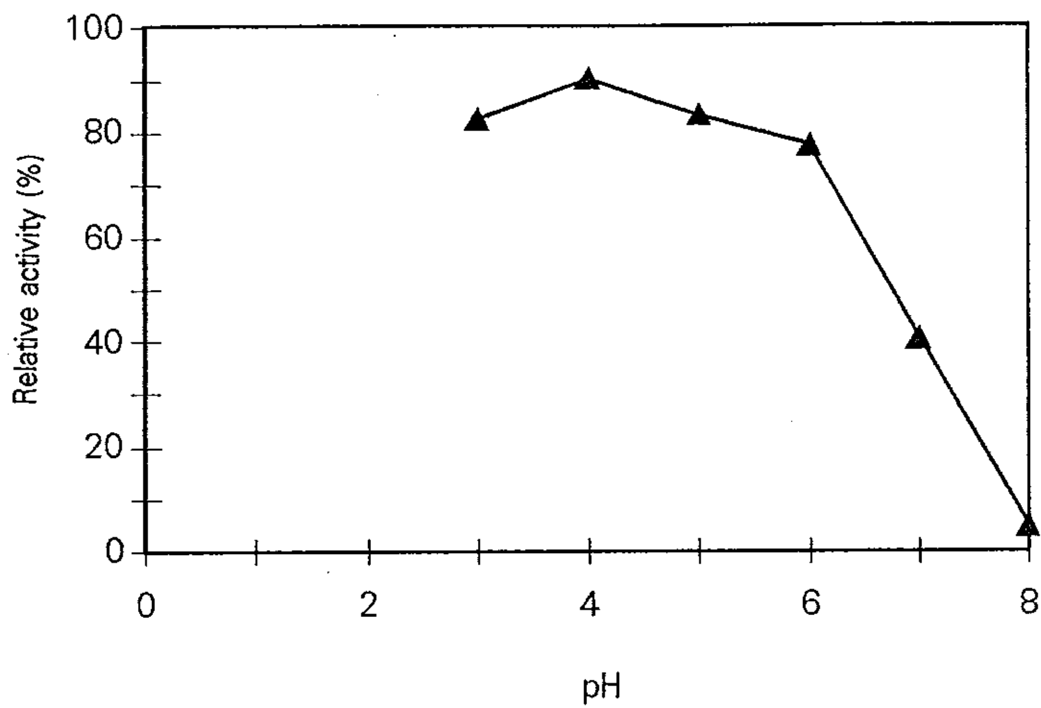


Figure 11. Effect of pH on enzyme stability.

The enzyme solution was preincubated at 30°C for 30 minutes in pH 3,4,5,6,7,8 without the substrate. The pH was then adjusted to 5 with 50 mM sodium acetate buffer, pH 5 and the remaining activity was determined.

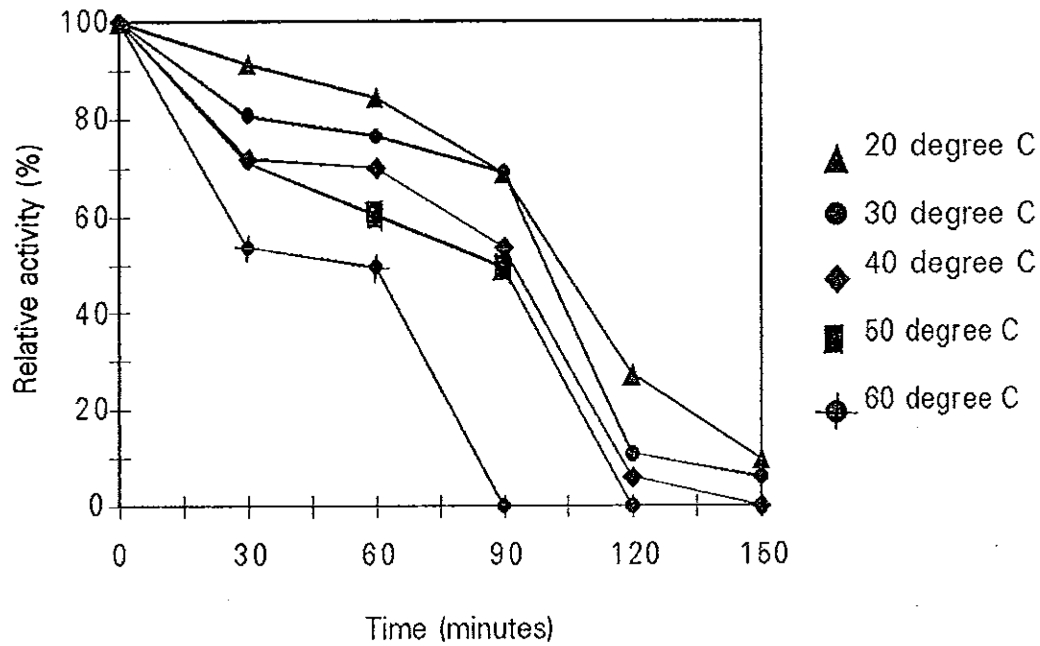


Figure 12. Thermal stability of enzyme.

The enzyme solution was preincubated for 30-150 minutes at various temperatures (20-60 °C) without the substrate, and immediately cooled down on ice. The remaining activity was then assayed.

Table 6 Effect of some ions and chemicals on xylanase activity

Chemicals added	% Activity
none	100
5% Ethanol	91.7
10 mM KCl	110.1
10 mM NaCl	97.2
10 mM $CaCl_2$	119.3
10 mM $MgCl_2$	119.3
10 mM $ZnSO_4$	126.6
10 mM $CuSO_4$	20.2
10 mM EDTA	8.3
1% SDS	13.8

7. Determination of Kinetic Constant of Enzymes

Purified enzyme (1.1125 μg/ml) was incubated with varying concentration of soluble xylan (1.75 mg/ml to 3.25 mg/ml) in acetate buffer, pH 5.1.The Michaelis constant (K_m), and the maximum rate (V_{max}) of enzyme derived from rates of catalysis measured at different substrate concentration. A plot of 1/*V* versus 1/[S] (Lineweaver-burk plot) (Figure13) showed the K_m and V_{max} value of 25 mg/ml and 1.54 μmol/min respectively.

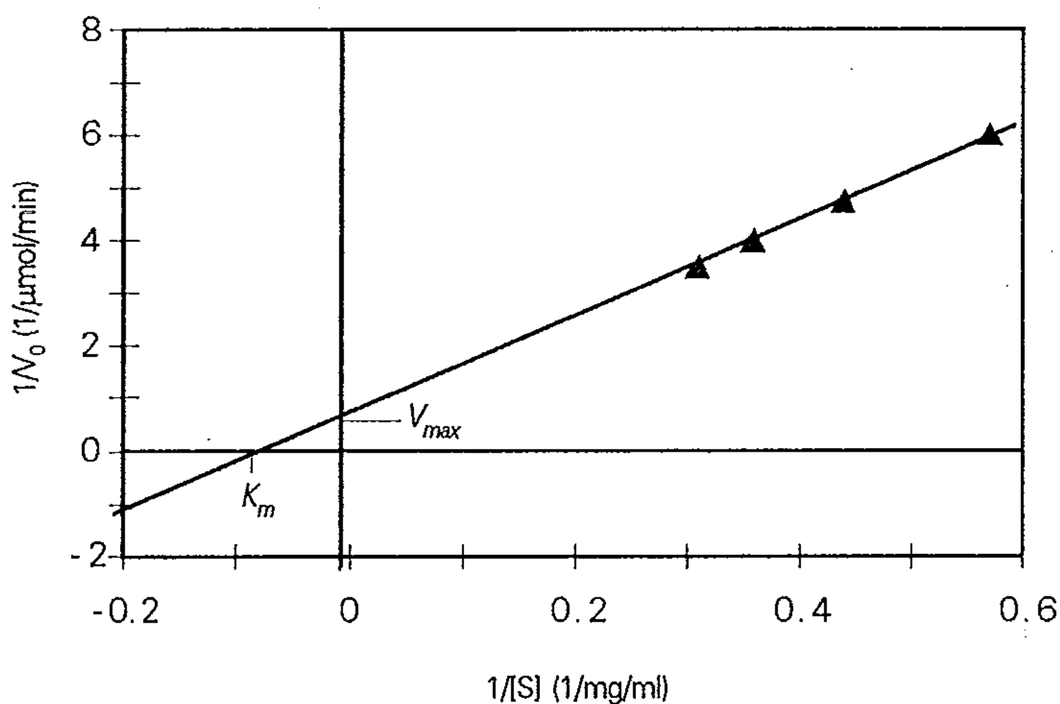


Figure13. Enzyme kinetics of *Pichia stipitis* xylanase in oat spelts xylan.

The purified enzyme (1.125 mg/ml) was incubated with a varying concentration of soluble xylan (1.75 - 3.25 mg/ml in 50 mM sodium acetate buffer, pH 5.1). The enzyme activity was measured at every 30 minutes of incubation time for 3 hours. The initial velocity was taken as the slope of the linear portion of the graph of reducing sugar formation *versus* time as determined by linear regression. K_m and V_{max} value were calculated by Lineweaver-Burk plot.

8. Analysis of the Hydrolysis Product

The purified xylanase was incubated with 2 mg of xylan in 50 mM acetate buffer, pH 5.1, for different periods of time (0-48 hours). The end products were analysed by paper chromatography with a solvent system of n-butanol:acetic acid:water (3:3:1 by volume). At given time intervals, 100 µl aliquots were spotted on the Whatman paper no.3, 40 µg xylose and 80 µg xylobiose were used as standards. Figure 14. shows a paper chromatogram of the hydrolyzate of oat spelt xylan with the xylanase. The end products were oligosaccharide with R_f values corresponding to those of xylobiose (X_2) and higher oligomers. Thus the xylanase of *Pichia stipitis* seemed to be an endoxylanase producing no xylose as major end-product.

9. Specific Antibody against Xylanase

The purified xylanase was immunized into a rabbit by subcutaneous injection for 4 weeks. The serum showed specific antibody against xylanase by double diffusion assay with 1.4% agarose gel onto 3x5 glass slide (data not shown). The serum was further purified by Sephadex G-75 in 0.5x25 cm column. The antibody was tested for its specificity by western blot analysis as shown in Figure 15. The 1:1000 titer of antibody specifically bound to the 43.0 kDa protein, which corresponded to the xylanase.

10. Purification of mRNA

Yeast cells grown in medium containing 0.5% xylan as carbon source at the highest specific activity (72 hours) were used for the purification of mRNA. Total RNA of 874 µg was obtained from 1 L culture and after passing through Olig-dT column, 35 µg of mRNA were remained. The quality of mRNA was observed from agarose gel electrophoresis (Figure 16). The RNA pattern on the agarose gel electrophoresis was different from that observed in *Saccharomyces cerevisiae*.

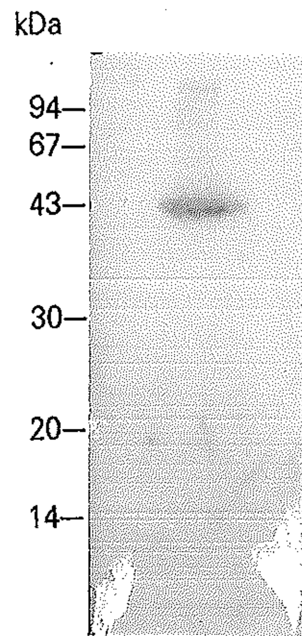


Figure 15. Western blot analysis of crude enzyme.

12.5 μg of crude enzyme prepared from 0.5% xylan medium was loaded on 12.5% SDS-PAGE. Proteins from the gel were transferred to nitrocellulose membrane and hybridized with purified antibody. 1 : 1,000 titer.

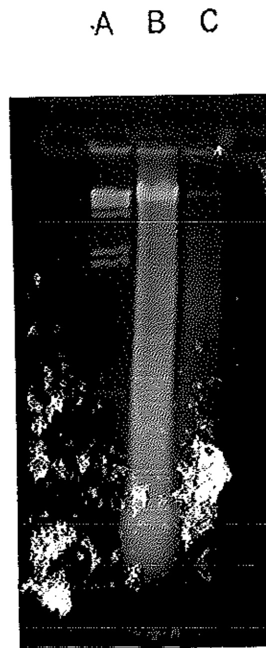


Figure 16. 8 % Agarose gel electrophoretic pattern of *Pichia stipitis* RNA. Lane A, Molecular weight marker lambda hind III; Lane B, Total RNA; Lane C, mRNA.

4. Discussion

1. Production of Xylanolytic Enzyme from *Pichia stipitis*

The xylose-utilizing yeast, *Pichia stipitis,* was grown well on xylan as sole source of carbon (Lee *et al.*,1986). During the growth on oat spelts xylan, the yeast produces at least two enzymes related to xylan degradation. One enzyme, which was secreted into the culture fluid, was identified as an β-xylanase. The other, which was found in cell fraction, was β-xylosidase (Table 3). It was appeared that xylanase production was induced when cells were grown in xylan while glucose and xylose has only slightly effect (Figure 5). This latter finding is in agreement with previous reports on xylanase formation by other xylan-utilizing yeasts, e.g. *Aureobasidium pullulans* (Leather *et al.*,1984) or *Cryptococcus albidus* (Morosoli *et al.*,1989). The xylanase was an inducible enzyme but xylan itself cannot function as a direct inducer as shown by the long lag period before the enzyme appeared in the medium. Xylan in the culture medium was first degraded by the xylanase which was produced constitutively from the yeast cell. The small subunits of which obtained from the degradation entered the yeast cell and induced the production of β-xylanase. The similar mode of xylan utilization was also described in other yeast, e.g. *Cryptococcus albidus* which suggested xylobiose and higher xylose oligosacchrides as the natural inducer of β-xylanase (Biely *et al.*,1980). *Pichia stipitis* was not secreted cellulase into the culture medium after 24 hours of incubation at 30°C. This property was similar to xylanases from *Bacillus pumilus, Bacillus subtilis* (Nguyen-Viet *et al.*,1991) but differred from xylanase of *Clostridium acetobutylicum* (Lee *et al.*,1987).

2. Purification of Xylanase

The enzyme was purified to homogeniety by lyophilization of culture fluid and passed through Sephacyl S-300 column chromato-graphy. The lyophilized was difficult to redissolve due to the presence of a large amount of undegrading xylan and about 5.75 % of pellet was dissolved giving a brown color and sticky solution. A lot of enzyme activity lost at this purification step. There were several attempts to solve this problem, *i.e.* ammonium sulphate precipitation or passing through ion-exchange chromatography, but none of these methods gave satisfied results. Nakanishi *et al.* (1984) used non-metabolic inducer (β-methylxyloside) as an inducer for the production of xylanase by *Cryptococcus flavus*. By lyophilization, they found that it was only slightly viscous and gave high specific activity. However no trial was done by this method since such inducer was not commercially available. After loading the dissolved pellet solution on Sephacyl S-300 column, proteins were separated into 4 peaks and xylanase activity was detected in peak three.Proteins at each step of purification were inspected by SDS-PAGE (Figure 8) Purity of the enzyme from gel filtration was demonstrated by a single protein band on SDS-PAGE with silver staining.

3. Enzyme Properties

The purified xylanase from this study shared many physical properties with xylanase from other microorganisms (Table1). Its molecule mass was 43.0 kDa from SDS-PAGE (Figure 8), which was higher than that from the yeast, *Cryptococcus flavus* (31.0 kDa) or *Trichoderma longibrachiatum* (21.5, 33 kDa). Native form of xylanase was not studied. The enzyme displayed activity with optimal pH at 5 (Figure 9) and optimal temperature at 40°C (Figure 10). This property was similar to xylanase from *Cryptococcus flavus* (pH 4.5 and 55°C) and *Streptomyces olivochromegenes* (pH 5 and 30°C). The enzyme was stable at 40 °C (Figure 12) and at pH range of 3-6 (Figure 10) that were similar to those noted in *Cryptococcus flavus*. (pH 3-8 and

$45^{\circ}C$) but differred from most bacteria (Table 1). The enzyme was activated by four major cations such as K^{+}, Ca^{++}, Mg^{++}, Zn^{++} whereas Cu^{++} inhibited the enzyme activity. The enzyme was also inhibited by EDTA and 1% SDS. Frederick *et al* (1985) reported that two endoxylanases from *Aspergillus niger* were inhibited by Hg^{++} and Cu^{++} while no inhibition was showed by K^{+}, Mn^{++}, Zn^{++} and Ca^{++}. The K_m values of xylanase was 25 mg/ml and V_{max} value was 1.54 µmol/min. It was difficult to compare these kinetic values with other microorganisms because of two reasons. Firstly, oat spelts xylan was not completely dissolved in water or buffer, therefore the real concentration of substrate used in enzyme assay might be lower than the concentration actually used for kinetic calculation. Secondly, since the enzyme assay in this study was based on the detection of reducing sugar, therefore these method do not offer correct results in the present of endoxylanase activity. Purified xylanase seemed to hydrolyze oat spelts xylan producing a single compound with *Rf* value of 0.37 which corresponding to xylobiose on paper chromatography. Transient formation of larger hydrolysis product at the early stage (within one hour) of hydrolysis was not observed. This result indicated that xylanase of *Pichia stipitis* might be an exoxylanase producing xylobiose as the smallest product. A similar mode of action has been reported by xylanase V of *Aeromonas caviae* ME-1 (Kubuta *et al.*, 1994).

4. Antixylanase Antibody Preparation

There are several ways of screening recombinant DNA libraries and one of them is screening with antibody. The purified xylanase protein was used to immunize rabbit and blood was drawn at three weeks after the first immunization. Antibody in the serum was determined by capillary test, microscopic test and double diffusion assay. Three weeks after immunization, the amount of antibody was high enough for further used. Then 5-10 ml blood was drawn from rabbit's vessel and antibody was purified by Sephadex G-75 column (0.5x25 cm). The antibody was tested for their specificity against xylanase protein by western blot analysis.

Although the antibody titer obtained this time (1:500) was not high because the rabbit catched a cold. However, the amount and the specificity of antibody are enough to be used in the future as a probe for screening of the putative gene.

5. Yeast RNA Isolation

There are several methods for gene cloning. Here, we chose to prepare cDNA library, because many yeast genes have intron and exon. In order to obtain a piece of DNA that directly coded for putative protein, cDNA constructed from mRNA is necessary. Moreover, this xylanase protein was induced by xylan. At certain time (about 48-60 hours) in xylan medium, the amount of mRNA for xylanase should higher than other mRNA species. In this work, intact mRNA was purified. Though the RNA pattern obtained from agarose gel electrophoresis was not the same as those observed in *Saccharomyces cervisiae*. However, mRNA was proved to be real RNA by their resistance to Dnase digestion. This mRNA will be useful for construction of cDNA library in the future.

5. Summary

1. Production of Xylanolytic Enzyme from *P.stipitis*

Pichia stipitis CBS 5773 produced extracellular xylanase as the major enzyme in medium containing 0.67% yeast nitrogen base and 0.5% oat spelts xylan (SD-xylan medium). It was found that *Pichia stipitis* excretes xylanase into the culture medium whereas the activity of xylosidase was detected mainly in the cell homogenate. The cultivation was done at 30 ^{o}C with shaking 200 rpm. The highest specific activity was 32.35 Unit/mg at 72 hours.

2. Purification of Xylanase

A xylanase was purified from the culture medium of *Pichia stipitis* by chromatography on Sephcryl S-300. It was purified 3.2 fold with 4.46% recovery yield. The proteins were separated into four peaks. Xylanase activity peak showed coincidence with the third protein peak. The purified sample gave a single band on SDS-PAGE.

3. Properties of Enzyme

The xylanase had an optimum temperature at 40°C. The enzyme retained 70% of the activity on standing at 40°C for 30 minutes. The optimum pH was pH 5 and stable at pH from 3-6 for 30 minutes with 70% retained activity. The apparent molecular mass was 43.0 kDa from SDS-PAGE. The enzyme had a K_m of 25 mg/ml and V_{max} value of 1.54 µmol/min. The activity was slightly increased upon addition of K^{+}, Ca^{++}, Mg^{++} and Zn^{++} and was inhibited by Cu^{++}, EDTA and 1% SDS. The hydrolysis pattern on xylan by paper chromatography demonstrated that the enzyme was an exoxylanase which produces xylobiose as a final product.

4. Antixylanase Antibody Preparation

The protein antigen, purified xylanase, was immunized into a rabbit by subcutaneous injection for 4 weeks. The serum was shown specific antibody against xylanase by double diffusion assay with 1.4% agarose gel onto 3x5 glass slide. The serum was further purified by Sephadex G-75 in 0.5x25 cm. column. It gave positive result on western blot analysis.

5. Yeast RNA Isolation

Yeast cells in culture medium at the highest specific activity (1,000 ml) were harvested and extracted for RNA. The total RNA mass was 874 μg that retained 35 μg mRNA finally.

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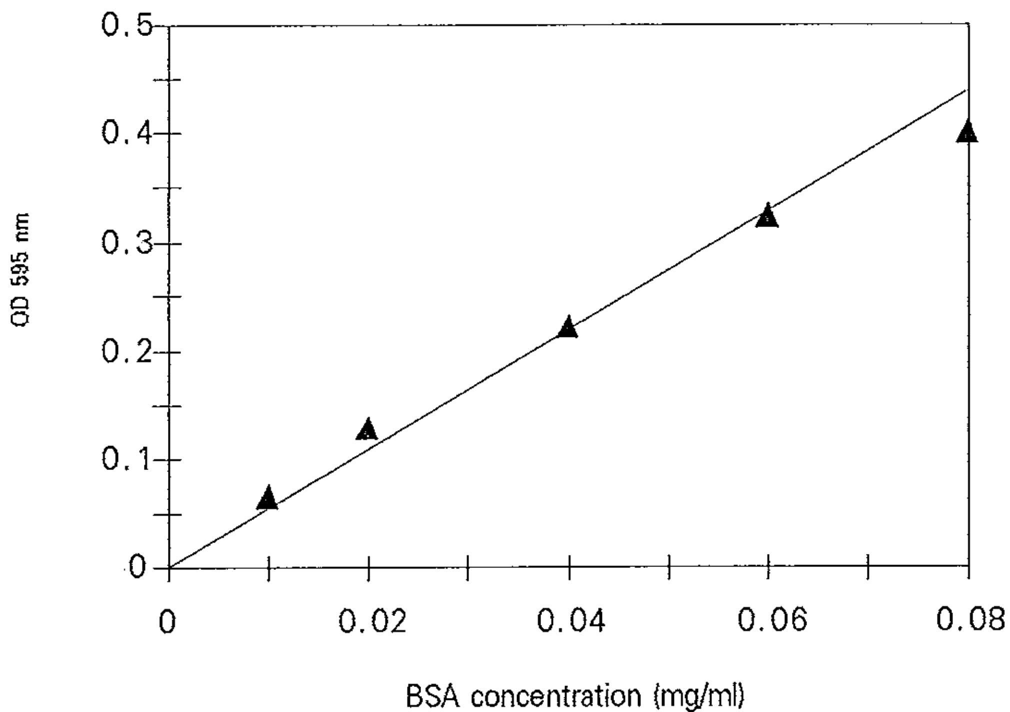
Appendix

1. Standard curve

1.1 Standard curve of protein (by Bradford,1976)

Reagent :

Coomassie Brilliant Blue G	250 mg
95% Ethanol	50 ml
85% (W/V) Phosphoric acid	100 ml
adjust with distilled water to	1000 ml



Protein concentration (mg/ml) = O.D. 595 / slope

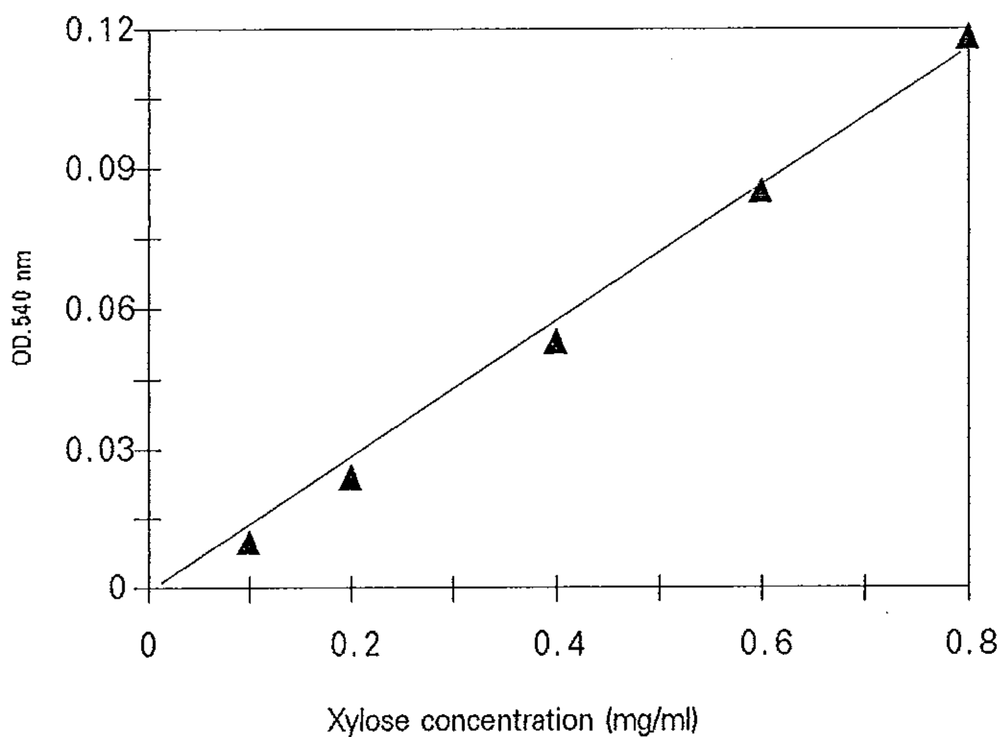
= O.D. 595 / 4.956

Figure 17 Standard curve of BSA at O.D. 595 nm (Bradford test)

1.2 Standard curve of xylose (reducing ends measued by DNS by Miller,1959)

Reagent :

3,5-dinitrosalicylic acid	1.0 g
Distilled water	50 ml
Sodium potassium tartrate tetrahydrate	30 g
2 N NaOH	20 ml
Adjust with distilled water to	100 ml



Xylose concentration (mg/ml)

Enzyme activity (U/ml) $= \text{OD.540} \times 10^3/\text{slope} \times 150.1 \times 60$

$= \text{OD.540} \times 10^3/0.138 \times 150.1 \times 60$

$= \text{OD.540} \times 8.53$

Figure 18 Standard curve of xylose at O.D. 540 nm (DNS test)

2. Medium

2.1 YPD Medium

Yeast extract	1	g
Peptone	2	g
Dextrose	2	g
Adjust with distilled water to	100	ml

2.2 SD-Xylan Medium

Yeast nitrogen base	0.67	g
Xylan	0.50	g
Adjust with distilled water to	100	ml

3. Chemical of Slab Gel Electrophoresis (Panbankrade and Udomvorapan,1993)

12.5 % SDS-PAGE Resolving gel

H_2O	5.80	ml
30% Acrylamide mixture	7.50	ml
1.5 M Tris-HCl (pH 8.8)	4.50	ml
10% SDS	0.20	ml
10% Ammonium persulphate	0.15	ml
TEMED	0.01	ml

4.5 % SDS-PAGE Stacking Gel

H_2O	3.55	ml
30% Acrylamide mixture	0.90	ml
1.0 M Tris-HCl (pH 6.8)	1.50	ml
10% SDS	0.05	ml
10% Ammonium persulphate	0.15	ml
TEMED	0.01	ml

4. Coomassie Briliant Blue R 250 staining (Panbankrade and Udomvorapan,1993)

Coomassie blue R 250 0.1% in water	5 ml
Methanol	5 ml
Glacial acetic acid	2 ml

Staining at least 4 hours at room temperature

5. Destaining (Panbankrade and Udomvorapan,1993)

Methanol	30 %
Acetic acid	10 %
Adjust with distilled water to	100 ml

6. 30% Acrylamide Mixture (Panbankrade and Udomvorapan,1993)

Acrylamide	30 g
Bisacrylamide	0.8 g
Adjust with distilled water to	100 ml

Stored in refrigerator.

7. Electophoresis Buffer (Panbankrade and Udomvorapan,1993)

Tris-HCl	3.00 g
Glycine	14.4 g
10% SDS	10.0 ml
Adjust with distilled water to	1,000 ml

8. Buffer for pH and Metal Ion Control (Dawson *et al.*,1986)

pH	Acid		Salt	
3	0.1 M Citric acid	82 ml	0.1 M Tris-Sodium	18 ml
4	0.2 M Acetic acid	18 ml	0.2 M Sodium acetate	82 ml
5	0.1 M Acetic acid	70 ml	0.1 M Sodium acetate	30 ml
6	0.1 M Citric acid	11.5 ml	0.2 M Sodium phosphate	88.5 ml
7	0.1 M Citric acid	17.8 ml	0.2 M Sodium phosphate	82.2 ml
8	0.1 M Citric acid	2.8 ml	0.2 M Sodium phosphate	97.2 ml
9	0.2 M Glycine	25.0 ml	0.2 M Sodium hydroxide	4.4 ml
10	0.2 M Glycine	25.0 ml	0.2 M Sodium hydroxide	16.0 ml

For buffer concentration 100 mM pH 3,4,5,9,and 10 diluted to 100 ml.

8. Rapid Silver Staining (Panbankrade and Udomvorapan,1993)

8.1. Formaldehyde fixing solution

Methanol	40 %
37 % formaldehyde	0.05 ml
Adjust with distilled water to	100 ml

8.2. Thiosulfate developing solution

Sodium carbonate (w/v)	3 %
Sodium thiosulfate (w/v)	0.0004 %

37% formaldehyde 0.05 ml (Add immediately before use)

8.3. Drying solution

Ethanol	10%
Glycerol	4%

Vitae

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