UNIVERSITI PUTRA MALAYSIA

PURIFICATION AND CHARACTERIZATION OF A THERMOSTABLE XYLANASE CLONED FROM *Bacillus coagulans* ST-6

YOW GEOK YONG

FSAS 1997 16

PURIFICATION AND CHARACTERIZATION OF A THERMOSTABLE XYLANASE CLONED FROM *Bacillus coagulans* ST-6

By

YOW GEOK YONG

Thesis submitted in Fulfilment of the Requirements for the Degree of Master of Science in the Faculty of Science and Environmental Studies, Universiti Pertanian Malaysia.

March 1997

ACKNOWLEDGEMENTS

I would like to express my most sincere thanks and appreciation to my Chairperson, Assoc. Prof. Dr. Abdullah Sipat, Department of Biochemistry and Microbiology, Universiti Pertanian Malaysia, for his guidance, encouragement and constructive suggestions throughout the course of study. I also wish to extend my thanks to Assoc. Prof. Dr. Khatijah Yusoff and Assoc. Prof. Dr. Mohd. Arif Syed for their support and valuable discussion in making this project a success.

I am also very grateful to all the members in the Biotechnology Fundamental Laboratory room 202, friends in Rumen Microbiology Laboratory, UPM, and many others for all their help, cooperation and making my time in UPM an enjoyable and memorable one.

To my beloved parents, brothers and sister, thank you for being understanding and supportive during my entire study in UPM.

TABLE OF CONTENTS

Page

Page

Page

LIST OF TABLES

LIST OF FIGURES

LIST OF PLATES

LIST OF ABBREVIATIONS

The following abbreviations were used in the text:

CFU	colony form unit
CM	carboxymethyl
CMC	carboxymethylcellulose
°C	degrees centrigrade
Da	dalton
DEAE	diethylaminoethyl
DNA	deoxyribonucleic acid
DNS	dinitrosalicylic acid
EDTA	ethylenediamine tetra acetic acid
hr	hour
kb	kilo basepair
kDa	kilodalton
K_m	Michaelis Menten constant
L	litre
mA	milliampere
M	molar
mg	milligram
min	minute

ml	millilitre
mM	millimolar
MW	molecular weight
PAGE	polyacrylamide gel electrophoresis
pI	isoelectric point
RBB	remazol brilliant blue
R_f	relative mobility
rpm	revolutions per minute
SDS	sodium dodecyl sulfate
TCA	trichloro acetic acid
TEMED	N,N,N',N'-tetramethyl-ethylenediamine
TLC	thin layer chromatography
µg	microgram
µl	microlitre
µmol	micromole
V	volt
v/v	volume per volume
V_{max}	maximal velocity of a enzyme reaction
w/v	weight per volume
YT	yeast trypton

Abstact of the thesis submitted to the Senate of Universiti Pertanian Malaysia in fulfilment of the requirements for the degree of Master of Science.

PURIFICATION AND CHARACTERIZATION OF A THERMOSTABLE XYLANASE CLONED FROM *Bacillus coagulans* ST-6

By

YOW GEOK YONG

March 1997

Chairman : Assoc. Prof. Abdullah Sipat, Ph.D

Faculty: Science and Environmental Studies

The recombinant plasmid pBNX contains a xylanase gene cloned from a thermophilic *Bacillus coagulans* ST-6. It was found to produce a high level of intracellular xylanase activity in *Escherichia coli* HB101. This xylanase enzyme was purified to homogeneity *via* a single step chromatography using a Sephadex G-50 column. SDS-PAGE analysis showed a single protein band having a molecular mass of 20 kDa. Zymogram analysis using agarose gel electrophoresis showed a single activity band for both crude and purified enzyme. Isoelectric point of the purified xylanase was pH 9.3.

The optimum temperature and pH for xylanase activity was 55°C and pH 7.2 respectively. The enzyme was found to be stable and retained its activity after 30 min incubation at 60°C. The enzyme showed a broad range of pH stability, in that it retained its activity after a 1 hr incubation at pH 5 to pH 10.

The apparent K_m and V_{max} of the enzyme was 2.18 mg/ml and 147.6 μmol xylose/min/mg protein respectively using oat spelt xylan as the suostrate. The purified enzyme was found to have very low CMCase activity after prolonged incubation of 5 hr to 24 hr (less than 5% that of xylanase activity after 24 hr incubation). No activity towards Avicel and filter paper was observed. No xylose, xylobiose or arabinose was found in the end product analysis using TLC indicating that the xylanase was a nondebranching endoxylanase.

The effect of various metal ions on the measurement of reducing sugar by Somogyi-Nelson and DNS methods was examined. At 1 mM, Ca^{2+}, Mn^{2+} and Pb^{2+} increased colour intensity of the standard glucose measured by DNS method while Mg^{2+}, Zn^{2+}, Hg^{2+} and EDTA decreased the colour formation. For the Somogyi-Nelson assay, only Mn^{2+} and Hg^{2+} decreased colour intensity of standard glucose measured while other compounds showed no effect. A subsequent study on the effect of metal ions on xylanase activity, taking into consideration of these results, showed that only Hg^{2+} was inhibitory while other metal ions (Li^{+}, K^{+}, Na^{+}, Ca^{2+}, Mg^{2+}, Co^{2+}, Mn^{2+}, Cu^{2+}, Zn^{2+}, Pb^{2+} and Fe^{2+}), EDTA, urea and SDS had no effect.

Abstrak tesis yang dikemukakan kepada Senat Universiti Pertanian Malaysia sebagai memenuhi syarat untuk mendapatkan Ijazah Master Sains.

PENULENAN DAN PENCIRIAN ENZIM XILANASE TERMOSTABIL YANG DIKLON DARIPADA *Bacillus coagulans* ST-6

Oleh

YOW GEOK YONG

March 1997

Pengerusi : Prof. Madya Abdullah Sipat, Ph.D

Fakulti : Sains dan Pengajian Alam Sekitar

Escherichia coli HB101 (pBNX), yang mengandungi plasmid rekombinan pBNX xilanolitik yang membawa gen xilanase daripada *Bacillus coagulans* ST-6 termofilik, didapati menghasilkan aktiviti xilanase intrasel yang tinggi . Enzim xilanase in telah ditulenkan sehingga homogen melalui kromatografi menggunakan turus Sephadex G-50. Analisis SDS-PAGE menunjukkan satu jalur protein yang bersaiz 20 kDa. Analisis zimogram menggunakan elektroforesis agarose gel menunjukkan satu jalur aktiviti untuk enzim kasar dan juga enzim tulen. Titik isoelektrik untuk enzim tulen ialah pH 9.3.

Suhu dan pH optima untuk aktiviti xilanase adalah 55°C dan pH 7.2. Enzim ini dapat mengekalkan aktivitinya selepas dieram selama 30 min pada suhu 60°C. Enzim tulen ini juga didapati stabil pada julat antara pH 5 hingga 10.

Anggaran bagi nilai K_m dan V_{max} bagi enzim tulen ialah 2.18 mg/ml dan 147.6 μmol xilose/min/mg protein dengan menggunakan xilan oat spelt sebagai substrat. Enzim tulen didapati mempunyai aktiviti CMCase yang rendah selepas tempoh pengeraman dipanjangkan daripada 5 jam hingga 24 jam (kurang daripada 5% daripada aktiviti xilanase selepas 24 jam). Tiada aktiviti dikesan terhadap Avicel and kertas turas. Tiada xilose, xilobiose dan arabinose dapat dikesan dalam produk terakhir hidrolisis xilan dengan menggunakan TLC, menunjukkan bahawa enzim tulen adalah jenis endo-xilanase.

Kesan pelbagai jenis ion logam ke atas penentuan gula penurun dengan kaedah Somogyi-Nelson dan DNS telah dikaji. Pada kepekatan 1 mM, Ca^{2+}, Mn^{2+} dan Pb^{2+} telah meningkatkan pembentukan warna oleh glukosa piawai dengan menggunakan kaedah DNS, manakala Mg^{2+}, Zn^{2+}, Hg^{2+} dan EDTA telah merendahkan pembentukan warna tersebut. Untuk kaedah Somogyi-Nelson pula, hanya Mn^{2+} dan Hg^{2+} didapati merendahkan intensiti warna dalam pengukuran glukosa piawai manakala kompaun yang lain tidak menunjukkan sebarang kesan. Seterusnya, satu kajian tentang kesan beberapa ion logam ke atas aktiviti xilanase telah dijalankan, dengan mengambil kira keputusan tersebut. Hasil diperolehi menunjukkan hanya Hg^{2+} saja merupakan perencat enzim manakala ion logam lain (Li^+, K^+, Na^+, Ca^{2+}, Mg^{2+}, Co^{2+}, Mn^{2+}, Cu^{2+}, Zn^{2+}, Pb^{2+} and Fe^{2+}), EDTA, urea dan SDS tidak menunjukkan kesan.

CHAPTER I

INTRODUCTION

Dwindling resources of food and fossil fuels have aroused considerable interest in the utilization of renewable plant biomass (Han, 1983). Cellulose and hemicellulose are reported to account for more than 50% of plant biomass on earth. The value of these renewable bioresource is estimated to contain total energy equivalent to 640 billion tonnes of oil (Gilbert and Hazlewood, 1993). Xylan, being the major component of plant hemicellulose found in the cell wall is estimated to constitute more than 30% of the dry weight of plant polysaccharides (Joseleau et al., 1992).

Xylan is largely found as heterogeneous polysaccharides constituting of a β-(1,4) linked D-xylopyranosyl backbone which is substituted with various side chains (Joseleau et al., 1992). Biodegradation of xylan to its monomer often involves microbial xylanase. Bioconversion of xylan found in the lignocellulosic waste to useful products is one of the potential applications of xylanase in the future (Woodward, 1984). Xylanase free of cellulase activity can be used as biobleaching agent in the pulp and paper industry to help to replace the use of hazardous chemical such as chlorine and chlorine dioxide (Wong and Saddler, 1992). Other potential

applications of xylanase enzyme were reported in food industry (Biely, 1985) and feed production (Bedford and Classen, 1992).

Industrial application of xylanase has not been proven to be economically feasible due to the lack of complete understanding of microbial degradation of xylan. Hence, research in improving enzyme production by microorganisms and in increasing the fermentation capabilities of the enzyme under extreme conditions used in industrial process is essential (Wong et al., 1988).

This thesis is a report on the purification and characterization of a thermostable xylanase produced by a recombinant *Escherichia coli* HB101 harbouring a xylanolytic recombinant plasmid pBNX. The xylanase gene in the recombinant *E. coli* (pBNX) has been previously cloned from a thermophilic xylanolytic bacteria, *Bacillus coagulans* strain ST-6 isolated from a local hot spring (Sharifah, 1990; Sipat et al., 1995b, Norwati, 1996).

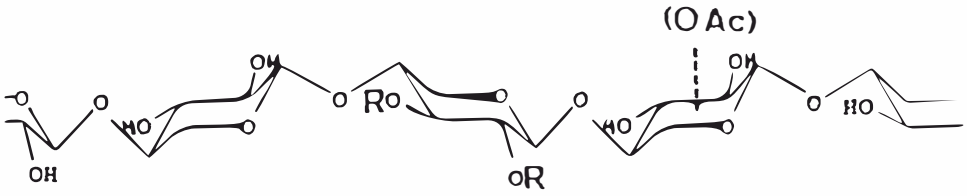
CHAPTER II

LITERATURE REVIEW

Xylan: Chemical Structure

Xylan is found as complex heteropolysaccharides in the plant cell wall (Gilbert and Hazlewood, 1993). The structure of xylan comprises of β-(1,4) linked D-xylopyranosyl polymer, substituted at various points by monosaccharides or short oligosaccharides (Joseluau et al., 1992). Unsubstitued linear xylan (homoxylan) compose exclusively by D-xylose polymer has also been isolated from esparto grass, tobacco stalks and guar seed husk (Wong et al., 1988; Joseleau et al., 1992).

Figure 1 shows the general structure of the xylan from various sources (Joseleau et al., 1992). Xylan accounts for 15 -30% of the dry weight of hardwood (Angiospems) (Timell, 1967; Sjostrom, 1981; Wong et al., 1988) which is usually acetylated at the OH group at C-2 and C-3 of the xylose residues (Fengel and Wegener, 1983). Acetylation occurs at about one per two xylose residues of the hardwood xylan (Bastawde, 1992). The backbone of the acetyled hardwood xylan is additionally substituted with 4-O-methyl-α-D-glucupyranosyluronic acid. The 4-O-methyl-D-glucuronic acid is often linked with glycosidic bond at the C-2 of the D-



R: α-D-GlcpA(1→2)Xyl...
4-OMe-α-D-GlcpA(1→2)Xyl...
α-L-Araf(1→3)Xyl...
α-L-Araf(1→2)Xyl...
β-D-Galp(1→5)α-L-Araf(1→3)Xyl...
β-D-Xylp(1→2)α-L-Araf(1→3)Xyl...
α-L-Araf(1→2, 1→3 and 1→2,3 Araf)$_n$(1→3)Xyl...
Feruloyl
p.coumaroyl
Lignin

Figure 1: General Structure of Xylan (after Joseleau et al., 1992)

xylose residues, though C-3 linkages have also been observed (Whistler and Richards, 1970). Most hardwood xylan has approximately one acidic side chain per ten D-xylose residues (Whistler and Richards, 1970). L-rhamnose and galacturonic acid are also found to linked to the reducing end of hardwood xylan (Sjostrom, 1981; Fengel and Wegener, 1983).

Xylan is less abundant in softwood (Gymnosperms) and accounts about 7-12% of total dry weight of softwood (Wong et al., 1988). Softwood xylan differs from hardwood xylan due to the lack of the acetyl group and the presence of L-arabinofuranose units at an average of 1.3 residues per ten xylose units, linked by α-(1→3) glycosidic bond (Sjostrom, 1981; Fengel and Wegener, 1983). The softwood xylan is also partially substitued with 4-O-methyl-D-glucuronic acid groups at C-2, on an average of two residues per ten xylose units (Sjostrom, 1981).

Xylan from other plants has also been investigated. There are no marked structural differences between the D-xylan from cereals, grasses and wood (Whistler and Richards, 1970). Besides the homoxylan isolated from esparto grass, other grasses like bamboo, barley and Guinea grass are found to be heteroxylan containing arabino-4-O-methyl-glucurono-xylan substitution (Fengel and Wegener, 1983). The cereal D-xylan is also found to have L-arabinose and D-glucuronic acid residues attached to the main chain (Whistler and Richards, 1970). Xylan present in seaweed is different from the terrestrial plants, for example the marine algae are found to contain β-(1,3)-linked D-xylosyl backbone (Joseleau et al., 1992).

Xylan in Plant Cell Wall

Ultrastructural localization studies of xylan in the plant wall showed that it interconnects with other structural cell wall components *via* covalent linkages and also non-covalent secondary forces forces (Wong et al., 1988; Joseleau et al., 1992). Lignin and some phenolic acids appear to covalently link to xylan *via* arabinosyl and glucuronosyl residues (Wong et al., 1988). Covalent linkages between xylan and other xylan chains, galacturonan or pectin subtances have also been suggested. Xylan and cellulose microfibrils are believed to interact *via* hydrogen bonding (Joseleau et al., 1992).

Both these covalent and noncovalent forces have contributed to the strength of lignocellulose having structural functions (Wong et al., 1988; Joseleau et al., 1992). Xylan is also present in the primary wall of growing cells or seed and bulbs and functions as food reserve (Joseleau et al., 1992).

Occurrence and Role of Xylanase

Xylanase plays an important role in plant matter degradation by microorganisms to provide a source of metabolizable energy. β-(1→4)-D-xylanase is reported to be produced by bacteria from marine and terrestrial environment, fungi, rumen bacteria and protozoa, ruminant caecal bacteria, insects, snails, crustaceans,

marine algae, and germinating seeds of terrestrial plants. A β-(1→3)-D-xylanase is also found to be produced by some bacteria and algae from marine environment. Some terrestrial fungi and bacteria are reported to produce both β-(1→3) and β-(1→4)-D-xylanase. However, xylanase has not been found to be produced by vetebrate animal tissue (Dekker and Richards, 1976).

Ruminant depend on xylanase produced by rumen microbial flora and fauna for the degradation of hemicellulose in its diet intake (Woodward, 1984). Xylanase has also been implicated in plant disease caused by bacterial or fungal plant pathogens in which it assists in the infection process by causing disruption of cell wall barrier (Woodward, 1984; Coughlan, 1992).

Classification of Xylanase

Xylanase enzymes can be classified according to their mode of action on the xylan substrate. According to Reilly (1981) and Bastawde (1992), xylanase can be classified as:

1. Endo-β-(1→4)-D-xylanase [β-(1→4)-D-Xylan Xylano Hydrolase, EC 3.2.1.8]

The endo-xylanase attacks the glycosidic bonds within the xylan backbone to produce xylooligosaccharides of various chain lengths. Cleavage is not random but is determined by the length of the substrate, its degree of branching and presence of subtituents. Endo-xylanase can be further divided into 4 types:

a) Non-arabinose Liberating Endoxylanase (Xylobiose and Xylose Producers).

This enzyme cannot cleave L-arabinosyl initiated branch points at β-(1→4) linkages and produces mainly xylobiose and xylose as the end products. These enzymes can break down xylooligosaccharides of size as small as xylotriose but they are more active on larger substrates.

b) Non-arabinose Liberating Endoxylanase (Xylooligosaccharide Producers).

This enzyme cannot cleave branch points at α-(1→2) and α-(1→3) and produces xylooligosaccharides larger than xylobiose as the major end products. It is generally inactive on xylotriose and xylobiose.

c) Arabinose Liberating Endoxylanase (Xylobiose and Xylose Producers).

This enzyme cleaves xylan chain at the branch points and produce mainly xylobiose, xylose and arabinose as the end products.

d) Arabinose Liberating Endoxylanase (Xylooligosaccharides Producers).

This enzyme cleaves branch points and produce mainly xylooligosaccharides of intermediate sizes and arabinose.

The endo-xylanases are the enzymes for general xylan depolymerization and have been extensively studied. A review on xylan and microbial xylanases by Bastawde (1992) showed that endo-xylanases have a broad range of properties and exhibit enzyme multiplicity.

2. Exo-β-(1→4)-D-Xylanase [β-(1→4)-D-Xylan Xylohydrolase]

An exo-xylanase removes single xylose units at a higher rate from the non-reducing end of the xylan chain than short xylooligosaccharides and have no transferase activity. Exo-xylanase is difficult to distinguish from β-xylosidase as both of them are capable of degrading D-xylooligosaccharides. However, only exo-xylanase can attack D-xylan.

Exo-xylanase has remained largely unstudied (Bastawde, 1992). These enzymes deserve more emphasis in industrial use because of their advantageous properties, such as; the absence of transferase activity, low inhibition by xylose and also the ability to attack larger xylooligosaccharides (Reilly, 1981). Observation from the products of hydrolysis has suggested the possible presence of exo-xylanase component in an alkalophilic *Bacillus* sp. (Balakrishnan et al., 1992) and *Aeromonas caviae* ME-1 (Kubata et al., 1994).

3. β-xylosidase [EC 3.2.1.37]

β-xylosidase breaks down short xylooligosaccharides to xylose from the non-reducing end, and have substantial transferase activity which decrease the yield of xylose. These enzyme cannot degrade D-xylan substrate.