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ISOLATION, SCREENING AND MOLECULAR CLONING STUDIES OF XYLANASE PRODUCING BACILLUS PUMILUS STRAINS

AINON BINTI HAMZAH

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ISOLATION, SCREENING AND MOLECULAR CLONING STUDIES OF XYLANASE PRODUCING *BACILLUS PUMILUS* STRAINS

By

AINON BINTI HAMZAH

Dissertation Submitted in Fulfilment of the Requirement for the Degree of Doctor of Philosophy in the Faculty of Food Science and Biotechnology, Universiti Putra Malaysia

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In loving memory of my late parents,

Hamzah bin Haji Mahmud and Nyonya binti Abdul Karim

and to

my family

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ISOLATION, SCREENING AND MOLECULAR CLONING STUDIES OF XYLANASE PRODUCING *BACILLUS PUMILUS* STRAINS

By

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Cellulosic plant materials are an excellent source of hemicellulolytic microorganisms. Five strains of *Bacillus pumilus* PJ19, P2, K52A, K51 and K5B, two strains of *B. subtilis* B2 and PJ18 and one *Cellulomonas* sp. which produced endoxylanase (1,4-ß-D-xylan xylanohydrolase, EC 3.2.1.8) have been isolated locally from plant materials. *B. pumilus* PJ19 produced the highest xylanase activity when grown in shake flask in yeast tryptone broth (YTB) at 200 rpm, 37°C which yielded activity of 265 U/ml. The enzyme was induced in Dubois media by the addition of xylan as carbon source and was repressed by xylose, glucose, fructose, maltose and sucrose. *B. pumilus* strain PJ19 and K5B showed maximum enzyme activity when grown in YTB (pH 7.2), 37°C after 36 hours, P2 (28 hours), while K51 and K52A after 32 hours incubation. The xylanase from *B. pumilus* PJ19 was purified to homogeneity by ammonium sulphate precipitation and gel filtration of CM-Sepharose and Sephacryl S-200. The molecular weight of the purified xylanase was estimated to be 23,000 D by sodium dodecyl sulphate-polyacrylamide gel electrophoresis and

22,515 D by mass spectrometry. The isoelectric point of the enzyme was 9.3. The optimum pH and temperature for hydrolysis of oat spelt xylan were 6.5 and 60°C, respectively. The enzyme was stable at a pH range of 7.5 to 8.5 and at a temperature of up to 45°C but lost 50% of its activity at 58°C after 10 minutes of incubation. The purified enzyme had a K_m of 1.42 mg/ml and V_{max} of 107 μmol/ min/mg for oat spelt xylan and K_m of 2.15 mg/ml and V_{max} of 29.22 μmol/min/mg for birchwood xylan. The major end products of oat spelt xylan hydrolysis were xylobiose, xylotriose and higher oligosaccharides while for birchwood xylan were xylotriose with some xylobiose determined by thin layer chromatography and high performance liquid chromatography. Xylose was not produced as a product of hydrolysis and trans xylosidation was detected. The activity of the enzyme was enhanced in the presence of Mg^{2+}, Ca^{2+} and K^{+} but was inhibited by EDTA, Cu^{2+}, Ag^{+}, Zn^{2+}, Fe^{2+} and Hg^{2+}. The enzyme was competitively inhibited in the presence of xylose with K_i of 1.98 mM. A complete DNA sequence of the xylanase gene was amplified by polymerase chain reaction and cloned into *E. coli* INVαF' using pCRII cloning vector. The complete DNA sequence was also determined. The structural xylanase gene which started from an ATG initiation codon, consists of an open reading frame of 684 bp, which encoded 202 amino acid residues. The molecular weight of the xylanase was estimated from the amino acid composition to be 22,474 D and is in agreement with the results obtained from SDS-PAGE of the purified xylanase. The xylanase was expressed constitutively by the cloned gene in the absence of xylan. The enzyme was located primarily in the cytoplasm probably because of the incompatibility of the Gram-positive signal peptide in *E. coli* to direct the enzyme extracellularly as in the donor strain.

Abstrak disertasi yang dikemukakan kepada senat Universiti Pertanian Malaysia sebagai memenuhi keperluan untuk Ijazah Doktor Falsafah.

PEMENCILAN, PENABIRAN DAN KAJIAN PENGKLONAN MOLEKUL STRAIN-STRAIN *BACILLUS PUMILUS* PENGHASIL XILANASE

Oleh

AINON HAMZAH

April 1997

Pengerusi: Dr Noor Aini Abdul Rashid
Fakulti: Sains Makanan dan Bioteknologi

Bahan tumbuhan berselulosa merupakan sumber terbaik bagi mikroorganisma hemiselulolitik. Lima strain *Bacillus pumilus* PJ19, P2, K52A, K51 dan K5B, dua strain *B. subtilis* B2 dan PJ18, dan satu *Cellulomonas* sp. yang menghasilkan endoxilanase (1,4-β-D-xylan xilanohidrolase, EC 3.2.1.8) telah dipencilkan di Universiti Pertanian Malaysia dari bahan tumbuhan tempatan. *B. pumilus* PJ19 menghasilkan aktiviti xilanase tertinggi apabila dihidupkan dalam kelalang goncangan kaldu yis tripton (YTB) pada 200 rpm, 37°C yang menghasilkan aktiviti pada 265 U/ml. Enzim diaruh dalam media Dubois dengan penambahan xilan sebagai sumber karbon dan direncat oleh xilosa, glukosa, fruktosa, maltosa dan sukrosa. *B. pumilus* strain PJ19 dan K5B, menunjukkan aktiviti enzim yang maksimum apabila dihidupkan dalam YTB (pH 7.2), 37°C selepas pengeraman selama 36 jam, P2 (28 jam), sementara K51 dan K52A selepas pengeraman 32 jam. Xilanase daripada *B. pumilus* PJ19 ditulenkan sehingga homogen dengan pemendakan amonium sulfat dan turasan gel menggunakan CM-Sepharose dan Sephacryl S-200. Berat molekul xilanase tulen dianggarkan 23,000 D dengan

menggunakan elektroforesis gel natrium dodesil sulfat-poliakrilamida, dan 22,515 D dengan kaedah spektrometri jisim. Takat isoelektrik enzim ialah 9.3. Hidrolisis xilan oat spelt adalah optimum pada pH 6.5 dan suhu 60°C. Enzim didapati stabil pada julat pH 7.5-8.5 dan suhu sehingga 45°C, tetapi 50% aktivitinya hilang pada suhu 58°C selepas 10 minit pengeraman. Xilanase tulen mempunyai nilai K_m 1.42 mg/ml dan V_{max} 107 µmol/min/mg untuk xilan oat spelt dan K_m 2.15 mg/ml dan V_{max} 29.22 µmol/min/mg untuk xilan birchwood. Hasil akhir utama hidrolisis xilan oat spelt seperti yang ditentukan dengan kaedah kromatografi lapisan nipis dan kromatografi cecair prestasi tinggi ialah xilobiosa, xilotriosa dan oligosakarida tinggi sementara bagi xilan birchwood pula ialah xilotriosa dengan sedikit xilobiosa. Hidrolisis tidak menghasilkan xilosa kerana berlakunya trans xilosidasi. Aktiviti enzim dapat ditingkatkan dengan kehadiran ion Mg^{2+}, Ca^{2+} dan K^{+} tetapi direncat oleh EDTA, Cu^{2+}, Ag^{+}, Zn^{2+}, Fe^{2+} dan Hg^{2+}. Aktiviti enzim direncat secara kompetitif oleh kehadiran xilosa pada K_i 1.98 mM. Penjujukkan DNA lengkap untuk gen xilanase digandakan secara tindak balas rantai polimerase dan diklonkan ke dalam *E. coli* INVαF' dengan menggunakan vektor pengklonan pCRII. Penjujukan DNA yang lengkap telah juga dilakukan. Gen struktur xilanase yang bermula dari kodon ATG terdiri daripada kerangka pembacaan terbuka 684 bp yang mengekodkan 202 sisa asid amino. Berat molekul xilanase tulen yang dianggarkan melalui komposisi asid amino ialah 22,474 D yang mana bersesuaian dengan nilai yang diperolehi daripada teknik naterium dodesil sulfat dan spektrometri jisim. Gen yang diklonkan menghasilkan xilanase secara konstitutif tanpa kehadiran xilan. Kebanyakan dari xilanase itu dikesan berada di sitoplasma. Ini mungkin disebabkan oleh ketidaksesuaian isyarat peptida gram-positif dalam *E. coli* untuk memberi arahan supaya enzim dibawa keluar sel seperti dalam strain penderma.

CHAPTER I

INTRODUCTION

In recent years, there has been renewed interest in the utilization of plant materials as a source of fuel and chemicals. It is an effort that can partially reduce our total dependence on fossil fuels. An advantage of using plant material is that it is a renewable resource. Total energy content in plant biomass is estimated to be equivalent to 640 billion tonnes of oil (Coughlan, 1985). Furthermore, globally there is an abundance of agricultural residues or agro-wastes such as sugar bagasse, empty fruit bunch from oil palm, coconut husks, paddy straw, wheat straw and forest wastes which accumulate in large quantities every year. From an economic standpoint, it is essential that all of the plant material residues be utilized, or it will cause environmental deterioration and loss of potentially valuable resources.

The main component of plant materials with potential as a source of chemicals is polysaccharides which consists of cellulose (40-60%) with lesser but significant amount of hemicellulose (20-30%) and lignin (15-30%) (Schuerch, 1975). Unlike cellulose, which occurs as homopolymer of glucose, hemicellulose does not have a homogeneous chemical composition. Hemicellulose is a heterogeneous polymer of glucose, mannose, galactose, xylose, arabinose, and uronic acid (Lynch, 1987). Both xylan and cellulose are very difficult to solubilize owing to the ß-1,4-glucosidic linkage.

The degradation of cellulosic and hemicellulosic materials occuring in nature is carried out mainly by microorganisms. They include fungi and bacteria, aerobes and anaerobes, mesophiles and thermophiles, and occupy a variety of habitats. One

of the approaches in handling plant biomass residues is to degrade the complex polysaccharides into simple sugars, alcohols, and other useful products.

Technically, there are three methods which can be used for degrading the lignocellulose components or polysaccharides. Firstly is the physical treatments method which involves primarily ball milling (Mandels et al., 1974), high pressure steaming (Saddler et al., 1982) and irradiation (Han & Ciegler, 1983). These processes have their drawbacks; that is they are not cost-effective and unable to remove noncellulosic substances (Andren & Nystrom, 1976). Secondly, is the chemical treatment method which employ alkali and dilute acids such as sulphuric or hydrochloric acids. Likewise, these treatments also have certain disadvantages. These include formation of hard scale on heated surfaces when $Ca(OH)_2$ is used, the need for expensive corrosion-resistant equipment, contamination of sugars produced by toxic byproducts and often chemicals used are expensive (Marsden & Gray, 1986). Although acid hydrolysis is faster, it can produce toxic compounds which can hinder microbial fermentation. Thirdly, is the employment of biological agents involving enzyme hydrolysis. In fact, this is one of the most important means of converting cellulose or hemicellulose to monosaccharides, which can then be used as fermentation feedstock.

An economic evaluation on the conversion of lignocellulosic materials for ethanol production indicated that the most expensive method is steam explosion coupled with enzyme hydrolysis while the cheapest is acid treatment coupled with enzyme hydrolysis (Parisi & Parisi, 1989). The development of an efficient enzymic hydrolysis offers new prospects for treating hemicellulosic wastes into useful products.

Usage of xylanase

The most widely used enzymes for the hydrolysis of lignocellulose are xylanase and cellulase. Although xylanase enzyme systems for the hydrolysis of xylans have been studied extensively in the past (Dekker & Richards, 1976, Kubicek, 1981), they remain less recognized compared to cellulase systems. This is due to the fact

that cellulose is composed of only D-glucose and can be found in all plants, while the composition of xylan is more complex and it also varies from plant to plant (Biely, 1985; Joseleau et al., 1992). It is also reflected in the literature as there are many reports on enzyme hydrolysis of cellulosic materials to glucose using cellulase from different organisms (Gaden et al., 1976, Gong et al., 1979, Bisaria & Ghose, 1981). However, xylanolytic enzyme systems deserve the same attention as the cellulolytic systems because their biotechnological potential is equally important. This is so as there is a considerable amount of xylan present in agricultural residues, and the conversion of xylan to useful products will strengthen the overall economics. Furthermore, xylan will provide an alternative for energy production from renewable resources.

Enzymes can be derived from various sources, but the use of microbial enzymes for industrial hydrolysis of lignocellulose is advantageous because of the high specificity of the enzyme reactions, the mildness of the reaction conditions, and the absence of substrate loss due to chemical modifications (Wong et al., 1988). That is why this study is focused on the potential use of microbial xylanase. Xylanase is the enzyme for the degradation of xylan, the major component of hemicellulose. According to Gilbert & Hazlewood (1993), xylanase could be exploited in agricultural and industrial processes as listed below:

i. Pre-treatment of forage crops and other cellulosic biomass with cellulase and xylanase to improve the nutritional quality and digestibility of ruminant feeds or to facilitate composting.

ii. Enzymic saccharification of agricultural, industrial and municipal wastes to provide sugar syrups for human or animal consumption or for the production of fine chemicals through industrial fermentations.

iii. Enzymic digestion of industrial wastes as an alternative to landfill deposition.

vi. Addition of cellulase and xylanase to cattle, pig and poultry cereal-based diets to elicit a significant improvement in nutrient utilization through the hydrolysis of barley ß-glucan and arabinoxylans.

v. Xylanase pretreatment of paper pulps to remove certain xylan components and reducing dependence on chlorine in the brightening process.

Xylanases are also applied to facilitate the bleaching of Kraft pulps or to improve fiber properties (Grabski & Jeffries, 1991). Xylanase can also be used in clarification of juices, preparation of dextrans for use as food thickeners, production of fluids and juices from plant materials, in processes of liquefaction of coffee mucilage for the manufacture of liquid coffee, adjustment of wine characteristics and enhancement of pigment astaxanthin (3,3'-dihydroxy-4,4'-diketo-ß-carotene) extraction (Woodward, 1984; Wong et al., 1988; Khasin et al., 1993 and Filho, 1994). The xylanase from *Streptomyces* E86 has been used in the production of xylobiose from commercial hardwood xylan (Kusakabe et al., 1975). Large amounts of hemicellulose which are present in wastes from the pulp and paper industry are in a form that cannot be buried and require expensive cost for disposal. With the advent of the new technology, there is increased interest to use xylan degrading enzymes to reduce these costs. Furthermore, the xylan may be used as raw material to produce xylose that can be used as feedstock or in fermentation of ethanol with or without formation of intermediate xylulose (Gruninger & Fiechter, 1986; Dey et al., 1992 and Filho, 1994).

Objective of the research

With respect to the vast potential in the industrial use of xylanase, this research was done with the ultimate goal of isolating xylanase-producing bacteria from soil. Xylanase is synthesized in large amounts by phytopathogenic soil microorganisms, which are able to degrade plant materials. Bacteria is chosen over fungi due to its fast growth and its ease for scaling up. Physiological and biochemical studies of the bacteria producing xylanase will be carried out to optimize the enzyme production. Through genetic manipulation, superior strains which are able to synthesize enzymes rapidly can be produced. The sequence of the enzyme will show the similarity or difference with other bacterial xylanase.

CHAPTER II

LITERATURE REVIEW

Cellulose, hemicellulose and lignin are the three major components of plant cell wall. Cellulose, the major component of plant cell wall, consists of a linear polymer of anhydroglucose units linked by β–1,4-glucosidic bonds, and do not occur in pure form in any natural resources (Bisaria & Ghose, 1981). In nature, it is always associated with other polysaccharides, such as starch, pectin, lignin and a variety of hemicelluloses. Cellulose has been thoroughly studied by many researchers (Nisizawa, 1973; Bisaria & Ghose, 1981; Coughlan, 1985 and Enari, 1987) and the mechanisms of hydrolysis has been well understood.

As this study is concerned with xylan and the xylanolytic enzyme systems, a comprehensive literature survey on the subjects is presented in this chapter. A brief literature review as background information on specific topics will be presented in each subsequent chapter.

Hemicellulose

Hemicelluloses are heteropolymers composed of various pentoses and hexoses of varying proportions, depending on their botanical origin. Introduced in 1891 by Schulze, they refer to the easily hydrolyzable parts of the cell wall. According to Timell (1964), hemicelluloses are polysaccharides of low molecular weights that occur in plant tissues together with cellulose, and which can be extracted from the original or the delignified material by using alkali or water. Hemicelluloses may

also be defined as polysaccharides present in the cell wall and intercellularly, that can be extracted from higher land-plant lignified tissues by alkali treatment (Wilkie, 1979). Certain carbohydrates in cereal endosperm, namely, non-starch polysaccharides that are described as cereal gums or pentosans are also classified as hemicellulose (Wilkie, 1979). However, they were later redefined to include only plant cell wall polysaccharides that bind noncovalently to cellulose. This definition is based on the chemical properties of these polysaccharides that are relatively easy to measure and are related to their proposed biological formation (Kennedy & White, 1988).

The hemicelluloses are one of the major constituents of lignocellulosic materials, and may comprise 30-40% of the total plant cell carbohydrate. They are important structural components and occur in close association with lignin and cellulose (Zimmermann, 1989; Williams, 1989).

Hemicellulose does not have a homogeneous chemical composition and is a heterogeneous polymer of different types of sugars in the backbone chain and in the side chain or appendages. These may be D-glucose, D-galactose, D-mannose, D-xylose, L-arabinose, D-glucuronic acid, D-galacturonic acid, or sometimes esters of O-acetyl, feruloyl coumaryl linked via L-arabinose residues to the backbone (Woodward, 1984; Puls & Poutanen, 1989). The composition of hemicelluloses in plants can be influenced by various factors such as growth, maturation, nature of soil, climate, length of the day, geographical location, and type of fertilizer used (Wilkie, 1979). However, the main component of hemicellulose is xylan.

Xylans

Xylans form a major component of hemicellulose comprising ß-1,4-linked polymer of D-xylose as a backbone. They are the second biopolymers after cellulose, present in nature in large amounts. ß-1,4-xylans are mainly found in secondary walls of plants, the major component of woody tissue (Timmel, 1967). The amount of xylan varies in different plants, from 35% of the dry weight of birchwood to as little as 7% in some gymnosperms (Whistler & Richards, 1970). Since agricultural

wastes and plant residues are generated on a massive scale each year, xylan represents a considerable potential source of fuel, single-cell protein, solvent and other chemicals by the selective use of specific fermentative microorganisms (Biely, 1985). This also can contribute to the elimination of agricultural wastes. The efficient exploitation of xylans as a source of industrial raw materials requires understanding of the enzyme systems that affect their conversion.

Source of xylan

Xylans are present in many types of terrestrial plants ranging from monocots to dicots and are mainly located in secondary plant cell walls (Timell, 1967). They are the major and minor components of mature cell walls of woody tissue and the primary wall in dicots, respectively (Timell, 1967; McNeil et al., 1984) and are also present as major components of monocot primary cell walls (Dey & Brinson, 1984). Xylan is also present in the primary walls of growing cells (Joseleau & Barnound, 1974), cell walls of the aleurone layer (McNeil et al., 1975) and sometimes in seeds and bulbs (Shaw & Stephen, 1966). 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 (Whistler & Richards, 1970).

In perennial or cereals, xylans are present in wheat straw, wheat leaf, oat straw, corn cobs, wheat bran, maize fiber and rice husk. Xylan is also present in woody plants, such as beechwood, pine, larch, oat, hemlock and spruce (Aspinall, 1959). As such, Malaysian woody plants are an equally good source of xylans.

Structure of xylan

Xylans from land plants are heteropolysaccharides that constitute a large group of related hemicellulose with a great variability in their structures. There is a relationship between the chemical structure of the xylan to their botanical origin and their cytological localization. Therefore, this results in the formation of several different polymers of xylans of related structures.

Most xylans from terrestrial plants have molecular structural which are branched and the degree of branching varies depending on the source (Biely, 1985) and methods of extraction (Wilkie, 1979). The main chain of xylan is a linear backbone structure composed of D-xylose units linked by ß–1,4-xylopyranosyl which may be substituted, depending on the origin of xylan (Dey & Brinson, 1984; Wong et al., 1988; Puls & Poutanen, 1989). Substitutions include: acetylation at C-2 or C-3 of the xylose units, α–1,2-linked glucuronic or 4-O-methylglucuronic acid groups, α–1,3-linked arabinofuranosyl units, and ferulic or coumaric acids esterified to O-5 of arabinose (Coughlan, 1992) as shown in Figure 1.

Very few unbranched linear xylan homopolysaccharides from land plants have been isolated. The best known example is xylan from esparto grass (Aspinall, 1959), which is normally used as a model for chemical and physical studies. It is composed of a straight chain of ß–1,4-linked to D-xylopyranosyl residues. Water soluble, linear xylan, has also been isolated from tobacco stalks which consisting of almost pure ß–1,4-D-xylan without any other sugar components (Eda et al., 1976).

All xylans from terrestrial plants (hardwoods, softwoods and grasses) are linked by ß–1,4-D-xylopyranosyl residues (Timell, 1967). However, those isolated from the marine algae are ß–1,3-linked (Dekker & Richards, 1976), while xylans from sea-weeds, such as *Rhodymenia palmata,* contain unbranched chains of about 80% of ß–1,4-linkages and 20% of ß–1,3-linkages (Kato & Nevins, 1984). In some species of the Chlorophyceae and the Rhodophyceae where cellulose is absent, the xylans appeared to be in the form of only ß–1,3-D-xylopyranose residues which form a highly crystalline fibrillar material (Aspinall, 1959; Joseleau, et al., 1992).

Hardwoods contain acetylated xylan, which made up 10-35% of the total dry weight with a degree of polymerization (DP) of 150 to 200 (Puls & Shuseil, 1993). Seven out of ten xylose units are acetylated on C-2 and/or C-3 position (Puls & Poutanen, 1989; Gilbert & Hazlewood, 1993) and the typical structure is shown in Figure 2.2a. For example, birch xylan contains more than 1 mole of acetic acid per 2 mole of D-xylose. The presence of acetyl groups makes the xylan