SCREENING OF XYLANASE PRODUCER FROM SOIL

WAN RAIMIE BINTI WAN RAMLI

UNIVERSITI MALAYSIA PAHANG

JUDUL **SCREENING OF XYLANASE PRODUCER FROM SOIL**

SESI PENGAJIAN: **2007/2008**

Saya **WAN RAIMIE BINTI WAN RAMLI**
(HURUF BESAR)

mengaku membenarkan tesis (PSM/Sarjana/Doktor Falsafah)* ini disimpan di Perpustakaan Universiti Malaysia Pahang dengan syarat-syarat kegunaan seperti berikut:

1. Tesis adalah hakmilik Universiti Malaysia Pahang.
2. Perpustakaan Universiti Malaysia Pahang dibenarkan membuat salinan untuk tujuan pengajian sahaja.
3. Perpustakaan dibenarkan membuat salinan tesis ini sebagai bahan pertukaran antara institusi pengajian tinggi.
4. **Sila tandakan (✓)

☐	SULIT	(Mengandungi maklumat yang berdarjah keselamatan atau kepentingan Malaysia seperti yang termaktub di dalam AKTA RAHSIA RASMI 1972)
☐	TERHAD	(Mengandungi maklumat TERHAD yang telah ditentukan oleh organisasi/badan di mana penyelidikan dijalankan)
☑	TIDAK TERHAD	

Disahkan oleh

(TANDATANGAN PENULIS)

Alamat Tetap: E-3, Krts IKBN
16300 Bachok
Kelantan

Tarikh: 16 MEI 2008

(TANDATANGAN PENYELIA)

MISS WAN SALWANIS BINTI WAN MD ZAIN

Tarikh: 16 MEI 2008

CATATAN:
* Potong yang tidak berkenaan.
** Jika tesis ini SULIT atau TERHAD, sila lampirkan surat daripada pihak berkuasa/organisasi berkenaan dengan menyatakan sekali sebab dan tempoh tesis ini perlu dikelaskan sebagai SULIT atau TERHAD.
◆ Tesis dimaksudkan sebagai tesis bagi Ijazah Doktor Falsafah dan Sarjana secara penyelidikan, atau disertai bagi pengajian secara kerja kursus dan penyelidikan, atau Laporan Projek Sarjana Muda (PSM).

"I hereby declare that I have read this thesis and in my opinion this thesis is sufficient in terms of scope and quality for the award of the degree of Bachelor of Chemical Engineering (Biotechnology)",

Signature	:…..……...
Supervisor	: Wan Salwanis binti Wan Md Zain
Date	: 16 May 2008

SCREENING OF XYLANASE PRODUCER FROM SOIL

WAN RAIMIE BINTI WAN RAMLI

A thesis submitted in fulfillment of the requirements for the award of the degree of Bachelor of Chemical Engineering (Biotechnology)

Faculty of Chemical & Natural Resources Engineering
Universiti Malaysia Pahang

MAY 2008

DECLARATION

"I declare that this thesis entitled 'Screening of Xylanase Producer from Soil' is the result of my own research except as cited in references. The thesis has not been accepted for any degree and is not concurrently submitted in candidature of any other degree",

Signature :…….
Name of candidate : Wan Raimie binti Wan Ramli
Date : 16 May 2008

DEDICATION

Special dedicated to my family, my friends, my fellow colleague,
and to all faculty members

For all your care, support, and believe in me.

Sincerely;
Wan Raimie binti Wan Ramli

ACKNOWLEDGEMENT

I would like to forward my very appreciation to my supervisor, Miss Wan Salwanis binti Wan Md Zain for the guidance and support. I also would like to express my gratitude to my academic advisor, Mr Mior Ahmad Khushairi bin Mohd Zahari for his support and believe in me during my studies.

I am very thankful to Universiti Malaysia Pahang and FKKSA for providing good facilities in campus and laboratory specifically. A very big thank you dedicated to all the staff of Faculty of Chemical and Natural Resources Engineering. Also not forgeting my family and colleagues.

ABSTRACT

The purpose of this study is to screen and to characterize the bacterial producing xylanase. The scopes include screening of xylanase producer and characterization of microorganisms and analysis of enzyme. This research was about an alternative to produce xylanase instead of using agricultural and forestry wastes, paper industry wastes, and various fruit wastes. The screening procedure was started from collecting soil sample until growth of bacteria on selective agar plate to screen the best xylanase producer from sample. After that, xylanase producer was characterized based on morphology using four types of morphological test. Besides that, the enzyme needs to be analyzed in different pH and temperature in term of protein content and xylanase activity. The optimum xylanase activity obtained was 72.667 U/ml at pH 6 and 65˚C.

ABSTRAK

Tujuan utama kajian ini adalah untuk memencilkan dan mencirikan bakteria yang menghasilkan enzim xylanase. Skop kajian termasuklah pemencilan bakteria yang menghasilkan xylanase dan pencirian bakteria dan enzim. Kajian ini adalah tentang alternatif lain untuk menghasilkan xylanase selain menggunakan sisa pertanian dan perhutanan, sisa daripada perindustrian kertas, dan sisa buah-buahan. Prosedur pemencilan bermula daripada pengumpulan sampel tanah sehingga pembiakan bakteria pada agar untuk pemencilan bakteria yang terbaik yang menghasilkan xylanase daripada sampel tanah tersebut. Selepas itu, bakteria yang menghasilkan xylanase dicirikan mengikut morfologi menggunakan empat jenis ujian morfologi. Selian itu, enzim xylanase juga telah dianalisa dalam pelbagai perbezaan pH dan suhu berdasarkan kandungan protein dan aktiviti xylanase. Keputusan kajian mendapati aktiviti xylanase optimum ialah 72.667 U/ml dalam pH 6 pada suhu 65˚C.

TABLE OF CONTENT

LIST OF TABLES

LIST OF FIGURES

LIST OF SYMBOLS/ABBREVIATIONS

ABS	-	absorbance
BSA	-	bovine serum albumin
cm	-	centimeter
g	-	gram
g/l	-	gram per liter
hr	-	hour
kD	-	kilodalton
mg	-	milligram
ml	-	milliliter
mm	-	millimeter
nm	-	nanometer
OD	-	optical density
rpm	-	rate per minute
sp.	-	species
sec	-	second
U/ml	-	Unit per milliliter
v/v	-	volume per volume
w/v	-	weight per volume
w/w	-	weight per weight
µg	-	microgram
µg g^{-1} hr^{-1}	-	microgram per gram per hour
µg/min/ml	-	microgram per minute per milliliter
˚C	-	degree Celsius
˚F	-	degree Fahrenheit
%	-	percent

LIST OF APPENDICES

CHAPTER 1

INTRODUCTION

1.1 Introduction

1.1.1 Screening Xylanase Producer From Soil

Xylan is a polysaccharide found in plant cell walls. It is found in almost all parts of the plant. In plant biology, xylan is known as plant cell wall polysaccharide containing a backbone of β-1,4-linked xylose residues. Side chains of 4-O-methylglucuronic acid (glucuronoxylan) and arabinose (arabinoxylan) are present in varying amounts, together with acetyl groups. Xylan can be successfully applied to almost any clean, dry, oil-free surface. In fact, the only materials to which it will not adhere are those, which have inherent release characteristics such as polyolefins and fluoropolymers.

Endoxylanase (1,4-β-D-xylan xylohydrolase; EC 3.2.1.8) are the main enzyme involved in xylan hydrolysis. Endoxylanase degrade plant cell wall polysaccharides by cleaving internal glucosidic bonds of xylan (Coughlan and Hazlewood, 1993). Xylanase deconstructs plant structural material by breaking down hemicellulose, a major component of the plant cell wall. Plant cell walls are necessary to prevent dehydration and maintain physical integrity. They also act as a physical barrier to prevent attack of plant pathogens. In nature, some plant consumers or pathogens use xylanase to digest or attack plants. Many microorganisms produce xylanase, but mammals do not. Some herbivorous insects and crustaceans also produce xylanase.

The xylanase enzyme (endo-1,4-β-xylanase, EC 3.2.1.8) from *Trichoderma* sp. has a pI of 9.0 and is produced by fermentation. Xylanase consists of 190 amino acids and has a molecular weight of 21 kD. Xylanases belong to the glucanase enzyme family and are characterized by their ability to break down various xylans to produce short-chain xylo-oligosaccharides. Xylanase readily crystallizes in ammonium sulfate and sodium/potassium phosphate across pH 3.5 to 9.0. Xylanase can also be crystallized with other salts, polymers, and organic solvents. Xylanase solubility increases with increasing temperature in moderate concentrations of ammonium sulfate. Xylanase solubility in phosphate pH 9 decreases in the temperature range of 0 to 10˚C but remains constant in the range of 10 through 37˚C. Xylanase has been extracted from many different fungi and bacteria. It is commonly used in animal feeds, paper production, and food production [2].

There are a few organisms that have been reported as xylanase producer such as *Bacillus circulans* (Heck *et al.,* 2005), *Enterobacter* sp. MTCC 5112 (Khandeparker and Bhosle, 2004), *Staphylococcus* sp. SG-13 (Gupta *et al.,* 2001), *Trichoderma harzianum* 1073 D3 (Seyis and Aksoz, 2005), and *Streptomyces* sp. (strain Ib 24D) (Rawashdeh *et al.,* 2005). *Bacillus circulans* and *Enterobacter* sp. MTCC 5112 are isolate from aquatic ambient while *Staphylococcus* sp. SG-13 is isolated from agricultural residues. *Trichoderma harzianum* 1073 D3 is isolate from natural wastes (orange pomace, orange peel, melon peel, hazelnut shell) but *Streptomyces* sp. (strain Ib 24D) is isolate from soil samples.

1.2 Problem Statement

In the last decade, production of xylanase enzyme has attracted the attention of many researchers, as these enzymes are essential for the degradation of plant biomass. Xylanases have potential applications in the pulp and paper, food, feed, and beverages industries. For commercial applications, xylanases should ideally be produce quickly and in large quantities from simple and inexpensive substrates. Natural xylan sources such as agricultural and forestry wastes, paper industry wastes, and various fruit wastes are potential raw material for xylanase production. As

xylanases have a wide range of application, economical production of these enzymes is of great importance (Seyis and Aksoz, 2005).

Fungi produced most of the enzymes. Due to the fact that fungi are hazardous, other methods in producing xylanase was found in bacteria. The bacteria can replace the fungi that give slower effect of degrading waste. Besides, bacteria are more compatible and robust compare to fungi. In this study, it is focus on bacteria in soil as a new source for producing xylanase. The bacteria will be cultivated to extract the enzymes needed and then being produced in a large quantity for industrial purpose in order to minimize raw material costs.

1.3 Objective

The objective of this study is to screen and to characterize the bacterial producing xylanase.

1.4 Scopes

The scopes of the research consist of three parts:

i) Screening of xylanase producer.

ii) Characterization of microorganism.

iii) Analysis of enzyme.

CHAPTER 2

LITERATURE REVIEW

2.1 Hemicellulose

Hemicellulose is a group of complex carbohydrates that, with other carbohydrates (*e.g.,* pectins), surround the cellulose fibres of plant cells. While cellulose is crystalline, strong, and resistant to hydrolysis, hemicellulose has a random, amorphous, structure with little strength. Hemicellulose consists of shorter chains compare to cellulose – around 200 sugars unit as opposed to 7000-15000 glucose molecules per polymer seen in cellulose. In addition, hemicellulose is a branched polymer. The most common hemicelluloses contain xylans (many molecules of the five-carbon sugar xylose linked together), a uronic acid (*i.e.,* sugar acid), and arabinose (another five-carbon sugar). Hemicelluloses have no chemical relationship to cellulose [8]. Hemicelluloses have the property of being soluble in dilute alkali. They are usually classified according to the sugar residues present xylan, mannans, arabinans, and galactans. Most hemicelluloses do not occur as homopolysaccharides but as in the side chain or appendages. These may be D-xylose, L-arabinose, D-mannose acid, O-acetyl groups or feruloyl and coumaryl ester linked via L-arabinose residues to the backbones (Coughlan, 1989).

2.1.1 Xylan

Xylan is a yellow, water-soluble, gummy polysaccharide found in plant cell walls and yielding xylose upon hydrolysis. Xylans can be hydrolyzed by β-xylanase. Xylan is the most abundant hemicelluloses, ranking second only to cellulose and constitutes up to 35% of the total dry weight plants. Xylans consist of a homopolymeric backbone of 1-linked α-D-xylopyranose units depending on its origin the backbone may be sustituted (Coughlan, 1989). The xylans are the major hemicelluloses of many plant materials where they often contribute to the rigidity of plant cell walls. Wood xylans are either O-accetyl-4-O-methylglucuronoxylans (in hardwoods) or arabino-4-O-methylglucuronoxylans (in softwoods). The degree of polymerization hardwoods xylan (150-200) is higher than that of softwoods (70-130) (Gray and Michael, 1991).

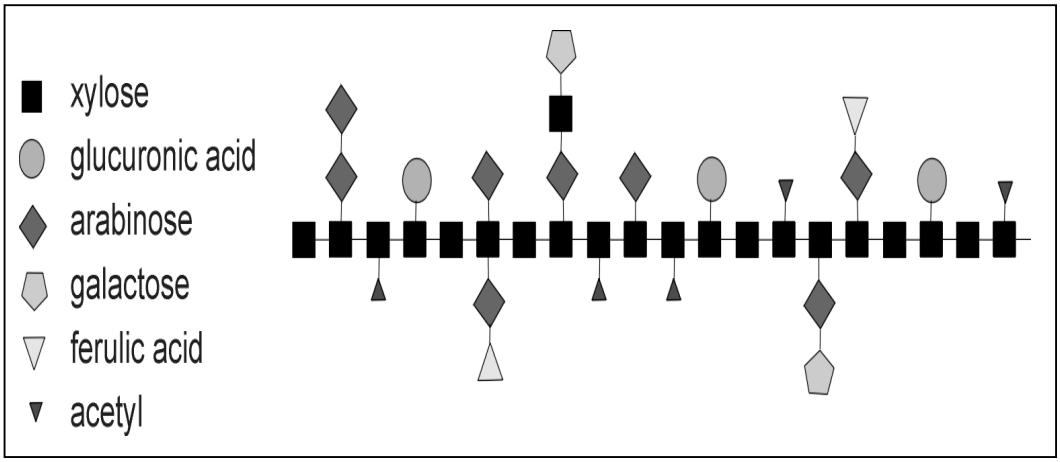

Figure 2.1 : Structure of the xylan [11]

2.1.2 Application of xylan

Xylan can be successfully applied to almost any clean, dry, oil-free surface. In fact, the only materials to which it will not adhere are those, which have inherent release characteristics such as polyolefins and fluoropolymers [12].

2.1.2.1 Metals

Almost every structural metal can be coated successfully with xylan, including steel (carbon and stainless), aluminum (wrought and cast), copper (and alloys), and titanium. High nickel- and chrome-bearing alloys, and some platings of nickel, can also be coated if abrasive blasting is used and the coatings are applied within an hour or two of preparation.

2.1.2.2 Plastics

Many plastic materials can be coated successfully with xylan, including nylon, PEEK, PEK, PPS, ABS, polycarbonate, epoxy, polyester and phenolic. The exceptions are the polyolefins and fluoropolymers - both of which have natural release characteristics. Also, vinyl products containing a high content of plasticizer can cause adhesion problems. Parts made of these materials must be cured at temperatures well below the softening temperature of the substrate to avoid distortion and polymer degradation.

2.1.2.3 Elastomers

Some xylan coatings may be applied successfully to elastomeric parts not expected to elongate more than about 30% in service. Elongation greater than this may cause the coating to crack. If a discontinuous coating is not objectionable, elongation far greater than 30% is permissible.

Elastomeric parts successfully coated with xylan include bushings, mounts, automotive door and window seals, and vibration dampers. Suitable substrates include natural rubber, EPDM, SBR, butadiene and its derivatives.

2.1.2.4 Glass and ceramics

Fluoropolymer coatings will adhere to clean ceramic or glass surfaces, but curing the coating without cracking the substrate can be difficult. (If possible, use glass or ceramic intended for high temperatures). In most cases, a low-temperature cure (below 150°C/300°F), followed by a slow cool-down period, will not crack the substrate. For glass parts, coating adhesion may be improved by fluorine.

2.1.2.5 Fabrics and composites

Xylan coatings are being increasingly used on woven and non-woven industrial textiles made from such modern materials as carbon fibre for low friction and release at elevated temperatures. One of the most successful applications of xylan involves a fabric bearing which is woven from a nylon/glass blend, then coated and cured.

These composite bearings are used under the compressor blades of large fanjet engines. The natural porosity of fabrics forms sponge-like "wells" into which the coating can penetrate. In service, this extra supply can continue to provide PTFE to a wear surface long after the coating has worn away from a smooth substrate. Xylan adheres well to other composites too, provided release agents have not been applied to the material.

2.1.2.6 Paper and wood

Xylan adheres well to uncoated or unvarnished paper products as well as wood. As unlikely as it may seem, the coatings perform every bit as well as they do on metal and other substrates. Cure temperature should not exceed 180°C/350°F.

2.2 Enzymes in Soils

Enzymes are proteins that act as catalysts by accelerating rates of reaction without undergoing permanent change. Enzymes are specific activators because they combine with their substrates in stereo specific fashion that decreases the stability of certain susceptible bonds, which reduces the energy of activation of reactions.

In soils, enzymes can exist intracellularly (inside the cytoplasmic membrane), which is of course important in cellular life processes. In addition, enzymes can exist outside the cytoplasmic membrane, in the periplasmic space or cell surface, and as extracellular enzymes in soil solution or stabilized in the soil matrix. It is generally assumed that soil enzymes are largely of microbial origin, but it is also possible that animals and plants may contribute enzymes to soils. The activity of approximately 100 enzymes has been identified in soils. The soil enzymes most often studied are oxidoreductases, transferases, and hydrolases. Some hydrolases and transferases have been extensively studied because of their role in decomposition of various organic compounds and thus are important in nutrient cycling and formation of soil organic matter. These include enzymes involved in: the C cycle, i.e., amylase, cellulase, xylanase, glucosidase, and invertase; the N cycle, i.e., protease, amidase, urease, and the aminase; the P cycle, i.e., phosphatase; and the S cycle, i.e., arylsulfatase.

A relatively small amount of any enzyme can be directly extracted from soil; therefore enzymes are mainly studied by measuring activity. The activity of enzymes varies temporally (seasonal), which often corresponds to microbial community responses to the environment, vertically (decreasing from the surface), at microscales, according to microbial community distribution, and at landscape level, where soil type is a major controlling factor (particularly textural distribution and organic matter).

Table 2.1 : The response of enzyme activities to the type of vegetation and soil (Daniel *et al.*, 2005)

Soil enzyme activity	Range of activities	Vegetation/soil type
Xylanase activity	13-24 (mg glucose g^{-1} per 24hr)	Spruce forest/n.k.
	0.28-8.0 (mg glucose g^{-1} per 24hr)	Beech forest/n.k.
	1.8-3.0 (mg glucose g^{-1} per 24hr)	Grassland/orthic luvisol
	0.24-1.83 (mg glucose g^{-1} per 24hr)	Agricultural land/haplic luvisol,entisol
β-Glucosidase	20-55 (μg ρ-nitrophenol g^{-1} hr^{-1})	Grassland/pachic arguistoll
	36-160 (μg ρ-nitrophenol g^{-1} hr^{-1})	Forest/Haplohumult
	130-310 (μg ρ-nitrophenol g^{-1} hr^{-1})	Crop rotation/hapludalf
	71-86 (μg ρ-nitrophenol g^{-1} hr^{-1})	Crop rotation/pachic ultic argixerolls
	41-253 (μg ρ-nitrophenol g^{-1} hr^{-1})	Crop, manured soil, pasture/typic haploxeroll
Protease activity	150-520 (μg tyrosine g^{-1} per 2 hr)	Agricultural land/haplic chernozem
	224-514 (μg tyrosine g^{-1} per 2 hr)	Pasture/typic dystrochrept
	120-430 (μg tyrosine g^{-1} per 2 hr)	Wheat seeds/loamy sand
	198-288 (μg tyrosine g^{-1} per 2 hr)	Crop rotation/haplic luvisol
Arginine deaminase activity	2.5-5.0 (μg N g^{-1} hr^{-1})	Grassland/pachic arguistoll
	1.7-2.0 (μg N g^{-1} hr^{-1})	Crop rotation/phaeozem, lithosol, cambisol
	4.0-11.0 (μg N g^{-1} hr^{-1})	Forest/sandy soils
	0.1-1.3 (μg N g^{-1} hr^{-1})	Crop rotation/fluventic ustochrept
Arylsulfatase activity	30-50 (μg ρ-nitrophenol g^{-1} hr^{-1})	Grassland/pachic arguistoll
	115-340 (μg ρ-nitrophenol g^{-1} hr^{-1})	Agricultural land/hapludoll
	6.9-213 (μg ρ-nitrophenol g^{-1} hr^{-1})	Pasture/typic dystrochrept
	21-49 (μg ρ-nitrophenol g^{-1} hr^{-1})	Forest/podzol
	12-58 (μg ρ-nitrophenol g^{-1} hr^{-1})	Crop, manured soil, pasture/typic haploxeroll
Alkaline phosphatase	40-80 (μg ρ-nitrophenol g^{-1} hr^{-1})	Grassland/pachic arguistoll

	40-790 (μg ρ-nitrophenol g^{-1} hr^{-1})	Agricultural land/aeric vertic epiaqualfs
	100-500 (μg ρ-nitrophenol g^{-1} hr^{-1})	Crop rotation/hapludalf
	181-225 (μg ρ-nitrophenol g^{-1} hr^{-1})	Crop rotation/ustochrept
Dehydrogenase	114-155 (μg TPF g^{-1} 24 hr)	Crop rotation/haplumbrepts, hapludalfs
	0.6-0.9 (μg TPF g^{-1} 24 hr)	Crop rotation/fluvisol
	68-97 (μg TPF g^{-1} 24 hr)	Crop rotation/ustochrept
	148-207 (μg TPF g^{-1} 24 hr)	Crop rotation/fluventic xerochrept

n.k., soil type not known

2.2.1 Xylanase

Xylanase is the name given to a class of enzymes which degrade the linear polysaccharide β-1,4-xylan into xylose, thus breaking down hemicellulose, which is a major component of the cell wall of plants. As such, it plays a major role in the digestive system of herbivorous microorganisms (mammals, conversely, do not produce xylanase). Additionally, xylanases are present in fungi for the degradation of plant matter into usable nutrients. Commercial applications for xylanase include the chlorine-free bleaching of wood pulp in the papermaking process, and the increased digestibility of silage (in this aspect, it is also used for fermentative composting). Additionally, it is the key ingredient in the dough conditioners s500 and us500 manufactured by Puratos. These enzymes are used to improve the dough's workability and absorption of water. In the future, xylanase may be used for the production of biofuel from unusable plant material (William, 1997).

Xylanase is used to break down plants as well as the sugar xylose. It has been found in many different fungi and bacteria. The enzyme is from *Trichoderma* sp. and consists of 190 amino acids. Xylanase belongs to the glucanase enzyme family, which is characterized by their ability to break down various xylans to produce short-chain xylo-oligosaccharides. Enzymes are important for a proper

nutrition and digestion. Without the proper levels of enzymes from foods or supplements, humans are susceptible to excessive gas and bloating, diarrhea, constipation, heartburn, low energy, acne, arthritis, allergies, insomnia, high cholesterol and many other discomforts [15].

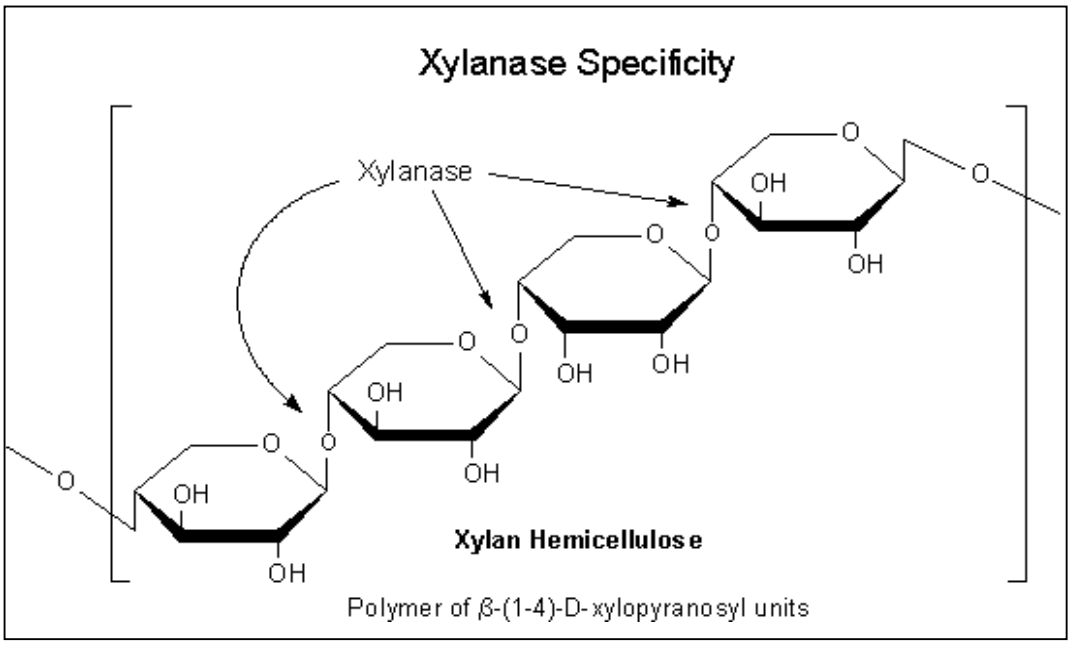

Figure 2.2 : Random image of xylanase [16]

2.2.2 Application of xylanase

2.2.2.1 Biobleaching paper pulp

The use of xylanase leads to a reduction in organo-chlorine pollutants such as dioxin from the paper making process. In addition, chlorine-free bleaching (such as peroxide or ozone bleaching) can achieve brighter results with the addition of xylanase. Because xylanase does not harm cellulose, the strength of the paper product is not adversely affected.

2.2.2.2 Improving animal feed

Adding xylanase stimulates growth rates by improving digestibility, which also improves the quality of the animal litter. Xylanase thins out the gut contents and allows increased nutrient absorption and increased diffusion of pancreatic enzymes in the digesta. It also changes hemicellulose to sugars so that nutrients formerly trapped within the cell walls are released.

2.2.2.3 Improving silage (or enhanced fermentative composting)

Treatment of forages with xylanase (along with cellulase) results in better quality silage and improves the subsequent rate of plant cell wall digestion by ruminants. There is a considerable amount of sugar sequestered in the xylan of plant biomass. In addition to converting hemicellulose to nutritive sugar that the cow or other ruminant can digest, xylanase also produces compounds that may be a nutritive source for the ruminal microflora.

2.2.3 Xylan hydrolysis reaction

Xylanase (endo-1,4-beta-xylanase; E.C. 3.2.1.8) is an enzyme that catalyses xylan hydrolysis reaction of xylan. Xylanase effectively convert xylan into xylooligosaccharides (mainly xylobiose, which is a major product of xylan degradation). Xylobiose is the smallest molecule to induce the production of xylanolytic enzymes (Mensfield *et al.,* 2003). Xylanase attacks the main chain, most easily and therefore most rapidly at non-substituted regions, generating non-substituted and branched or esterified oligosaccharides. Acid pretreatment of biomass is necessary in many cellulosic ethanol processes. This step hydrolyzes the hemicellulose to form fermentable sugars and exposes cellulose for subsequent enzymatic hydrolysis steps.

A key to the economic success of cellulosic ethanol production is the efficient hydrolysis of xylans into the monomer, xylose (Nimlos *et al.,* 2007). During xylan hydrolysis, xylanase acts synergistically with accessory enzyme. Xylanase release substituted xylooligosaccharides, which are more readily diffusible and more favorable substrates for accessory enzyme. On the other hand, the removal of side chains substituents by accessory enzyme creates new sites on the main chain for productive binding with xylanase (Mensfield *et al.,* 2003). Xylanase activity is highly dependent on the presence of debranching enzymes. The highest affinity was found for the triouronic acid (4-O-Me-Glc)$X_{2,}$ the nature intermediate product of xylanase hydrolysis of many fungal and bacterial producing xylanase (Nimlos *et al.,* 2007).

2.3 Xylanase producer

Actinomycete is a group of heterotrophic filamentous eubacteria tending to form extremely find-ramified mycelia (1.0 to 1.5 μm). They are gram positive bacterial found in large number of habitats including in natural environmental such as soil, composts, and decaying plants. They multiply by mere breaking of the mycelium or by hyphea. The most common genera in soil (*Streptomyces* and

Nocardia) are particularly able to degrade organic substances, which cannot easily be decomposed, thereby producing vitamins, and antibiotics (Lozet and Mathieu, 1991). They play important role in the degradation of lignocellulosic materials. Since hemicellulose represents an important substrate in these habitats, actinomycetes can be considered as a potential source of hemicellulolytic enzyme. Strains with hemicellulolytic activities have been isolated from different soil compost, animal manure, hay or decaying woods. Hemicellulose degrading enzyme is produced by mesophilic, thermotolerant or thermophilic actinomycetes. Being aerobic, their presence in a soil signifies good structure and good aeration. They can be symbiotic with some higher plants such as Alnus and Myrica (Lozet and Mathieu, 1991). Actinomycetes belong to the microorganisms producing most of the antibiotics applied in medicine and many other compounds of practical importance (Kurytowicz *et al.*, 1971). Table 2.2 shows example of some actinomycetes produce xylanase (Coughlan, 1989). Table 2.3 shows properties of actinomycete xylanase and Table 2.4 shows example of microorganisms' xylanase producer.

Table 2.2 : Example of some actinomycetes produce xylanase (Coughlan, 1989)

Producing strain	Reference
Actinomadura sp.	Zimmermann *et al.,* (1988)
Cellulomonas sp.	Peiris *et al.,* (1982)
Cellulomonas flavigena	Rajoka and Malik, (1984)
Cellulomonas uda	Rapp and Wagner, (1986)
Chainia sp.	Srinivasan *et al.,* (1984)
Microbispora rosea	Kusakabe *et al.,* (1969)
Microbispora bispora	Ball and McCarthy, (1988)
Micromonospora chalcea	Sorensen, (1957)
Micromonospora sp.	Ball and McCarthy, (1988)
Pseudonocardia thermophila	Zimmermann *et al.,* (1988)
Saccharomonospora viridis	McCarthy *et al.,* (1985)
Streptomyces albus	Sorensen, (1957)
Streptomyces xylophagus	Iizuka and Kawaminami, (1965)
Streptomyces spp.	Kusakabe *et al.,* (1969)
Streptomyces viridochromogenes	Kusakabe *et al.,* (1969)
Streptomyces mistakaensis	Kusakabe *et al.,* (1969)
Streptomyces albogriseolus	Kusakabe *et al.,* (1969)
Streptomyces coelicolor	Kusakabe *et al.,* (1969)
Streptomyces purpurascens	Kusakabe *et al.,* (1969)
Streptomyces scabies	Kusakabe *et al.,* (1969)
Streptomyces netropsis	Kusakabe *et al.,* (1969)
Streptomyces shindenensis	Kusakabe *et al.,* (1969)
Streptomyces olivaceus	Iizuka and Kawaminami, (1965)
Streptomyces olivochromogenes	Kusakabe *et al.,* (1969)
Streptomyces afghaniensis	Loginova *et al.,* (1981)
Streptomyces flavogriseus	Ishaque and Kluepfel, (1981)
Streptomyces exfoliatus	Sreenanth and Joseph, (1982)
Streptomyces osteogriseus	Park and Toma, (1982)
Streptomyces albosporus	Sreenanth and Joseph, (1983)
Streptomyces cyaneus	Sreenanth and Joseph, (1983)
Streptomyces caiusiae	Sreenanth and Joseph, (1983)
Streptomyces sp.	Marui *et al.,* (1985)
Streptomyces viridosporous	Deobald and Crawford, (1987)
Streptomyces spp.	Yasui *et al.,* (1988)
Streptomyces spp.	Ball and McCarthy, (1988)
Streptomyces lividans	Kluepfel *et al.,* (1986)
Streptomyces cyaneus	Zimmermann *et al.,* (1988)
Streptomyces rubiginosus	Taiana *et al.,* (1989)
Streptosporangium roseum	Kusakabe *et al.,* (1969)
Thermomonospora fusca	McCarthy *et al.,* (1985)
Thermomonospora curvata	McCarthy *et al.,* (1985)
Thermomonospora chromogena	McCarthy *et al.,* (1985)

Table 2.3 : Properties of actinomycetes producing xylanase

Producing strain	pH opt.	Temp. opt.	pI	MW (kD)	Reference
Chainia sp.	5.0	65	8.0	-	Arnold and Julia, (1999)
Saccharomonospora viridis	5.0-8.0	60	-	-	Biely *et al.,* (1985)
Streptomyces xylophagus	6.2	55-60	-	-	Bocchini *et al.,* (2005)
Streptomyces flavogriseus (CD45-2)	6.5	50	-	-	Dekker (1985)
Streptomyces exfoliates	5.5	50	-	-	Gray and Micheal, (1991)
	7.0	55	-	-	Gray and Micheal, (1991)
	5.5	55	-	-	Gray and Micheal, (1991)
Streptomyces sp. (KT23)	5.5	55	6.9	43	Rifaat *et al.*, (2005)
Streptomyces sp. (3137)	5.5-6.5	60-65	7.1	50	Benson (1994)
	5.0-6.0	60-65	10.1	25	Benson (1994)
	5.0-6.0	60-65	10.3	25	Benson (1994)
Streptomyces lividans (1326)	5.5-6.5	55-65	5.2	-	Tsujibo *et al.,* (2004)
Streptomyces sp. (E86)	5.5-6.2	55-60	7.3	40	[8]
Thermomonospora sp.	5.5-7.7	65-80	-	-	Benson (1994)
Thermomonospora curvata	5.0-8.0	60	-	-	Biely *et al.,* (1985)
Thermomonospora fusca	5.0-8.0	70	-	-	Biely *et al.,* (1985)
Thermomonospora chromogena	5.0-8.0	75	-	-	Biely *et al.,* (1985)

Table 2.4 : Example of microorganisms' xylanase producer.

Microorganisms	Source	Xylanase molecular weight	Optimum condition	Optimum activity	References
Bacillus circulans BL53	Aquatic ambient	-	80˚C at pH 4-7	0.95 U/mg protein	Heck *et al.*, (2005)
Arthrobacter sp. MTCC 5214	Aquatic ambient	~ 20 Kd	100˚C at pH 9	240 U/ml in 2 days	Khandeparkar and Bhosle (2006)
Bacillus circulans D1	Aquatic ambient	-	37˚C	0.437 U/mg protein in 5 days	Bocchini *et al.*, (2005)
Enterobacter sp. MTCC 5112	Aquatic ambient	~ 43 Kd	50˚C at pH 9	49 U/ml	Khandeparkar and Bhosle, (2004)
Streptomyces sp. (strain Ib 24D)	Soil	25-50 Kd	60˚C at pH 6.5	1447 U/ml in 3 days	Rawashdeh *et al.*, (2005)
Paecilomyces thermophila J18	Soil	25.8 kD	75˚C at pH 7	998 U/ml in 5 days	Li *et al.* (2005)
Streptomyces sp. AMT-3 strain	Soil	-	55-65˚C at pH 6	70.0 U/ml in 10 days	Nascimento *et al.*, (2002)

2.3.1 Bacterial

Streptomyces bacteria are gram positive, soil inhabiting, and filamentous, with a high G+C content in their DNA (Tsujibo *et al.*, 2004). *Streptomycetes* grew most in pore spaces which were humid and air-filled, and growth was reduced in water-logged pores. Spores of *Streptomycetes* survived for long periods in dry soil (Williams *et al.*, 1972). There are three different journals which show bacterial *B. circulans* (Heck *et al.*, 2005), *Enterobacter* sp. MTCC5112 (Khandeparkar and Bhosle, 2004), and *Streptomyces* sp. (strain Ib 24D) (Rawashdeh *et al.*, 2005) are xylanase producer. Table 2.5 shows the survey of xylanase producer.

Table 2.5 : The survey of xylanase producer (bacterial)

Microorganisms	Condition	Media	Basic liquid medium	References
Bacillus circulans BL53	18 hr, 37˚C and 125 rpm	Basal salt solution (BSS) supplemented with xylan	In (g/l) $MgSO_4$ 0.2, KH_2PO_4 1.0, K_2HPO_4 1.0, NH_4NO_3 1.0, $CaCl_2$ 0.02, and $FeCl_2$ 0.05	Heck *et al.*, (2005)
Enterobacter sp. MTCC 5112	At 50˚C and pH 9.0 for 48 hr	Basal salt solution (BSS) supplemented with xylan (0.5%)	In (w/v) NaCl, 30.00 g; KCl, 0.75 g; $MgSO_4$, 7.00 g; NH_4Cl, 1.00 g; KH_2HPO_4 (10%) 7.00 ml; KH_2PO_4 (10%) 3.00 ml	Khandeparkar and Bhosle, (2004)
Streptomyces sp. (strain Ib 24D)	At 28˚C for 4 days	Oat spelt xylan agar medium	0.1 ml trace salt solution: 1 ml/l: $FeSO_4$. $7H_2O$, 0.1 g; $MnCl_2$. $4H_2O$, 0.1 g; $ZnSO_2.7H_2O$, 0.1 g; distilled water, 100 ml	Rawashdeh *et al.*, (2005)

2.4 Previous research on xylanase producer

2.4.1 Fungi as Xylanase Producer

Haltrich *et al*., (1996) gave an overview of fungal xylanases and showed that the enzyme can be produced by a number of microorganisms including bacteria, yeasts and filamentous fungi such as *Trichoderma, Bacillus, Cryptococcus, Aspergillus, Penicillium, Aureobasidium, Fusarium, Chaetomium, Phanerochaete, Rhizomucor, Humicola, Talaromyces* and many more. These fungi produced xylanase enzymes extracellularly with a wide range of activities from 4-400 IU/ml using various substrates both in submerged and solid state fermentation (SSF) processes. Isolate USM A1 I which was identified to be *Aspergillus niger* was selected as a potential producer of xylanase via a solid state fermentation system (SSF) using palm kernel cake (PKC) as substrate (Pang and Ibrahim, 2004).

2.4.2 Isolating xylanase producer from inside part of plants

Manabu *et al.,* (2001) claimed that the inside part of plants is a novel and good source for isolating xylanase producers in comparison with soil. The host plant collected for the research are *Aconitum yesoense, Gnaphalium japonicum, Hedysarum vicioides, Humulus lupulus, Ilex crenata, Kerria japonica, Miscanthus sienensis, Pueraria lobata, Rosa rugosa, Rubus idaeus, Syringa vulgaris, Trillium apetalon, Trillium tschonoskii,* and *Ulmmus davidiana.* Healthy branches showing no disease symptoms were collected. The activity of xylanase produced by the isolates was as high as 0.191 U/ml when they were cultivated at 27˚C for 5 days. It is noteworthy that xylanase producers were found in endophytes in large numbers although the isolation was not carried out with a selective medium for xylanase producers.

Table 2.6 : Numbers of endophytic isolates from plants in Hokkaido and the xylanase-producing microorganisms among them (Manabu *et al.,* 2001)

Host plant	**Endophytic fungus**		**Endophytic bacterium**	
	Total	**Xylanase producer**	**Total**	**Xylanase producer**
Aconitum yesoense	7	7	10	9
Gnaphalium japonicum	5	5	5	5
Hedysarum vicioides	4	4	7	5
Humulus lupulus	3	3	2	1
Ilex Crenata	6	4	4	1
Kerria japonica	9	8	2	1
Miscanthus sienensis	18	12	8	2
Pueraria lobata	2	2	3	1
Rosa rugosa	78	74	20	17

CHAPTER 3

MATERIALS AND METHODOLOGY

3.1 Chemicals

All chemicals are obtained from Fluka Company (Aldrich Chemical). All chemicals are in analytical grade.

3.2 Culture Condition for Xylanase Production

3.2.1 Source of Xylanase Producer

Three soil samples were collected from around Universiti Malaysia Pahang. After approximately removed 3 cm of soil from the surface, samples were taken to a depth of 30 cm. Each soil sample was crushed, mixed thoroughly, and sieved through a 2 mm sieve to get rid of large debris, and the sieved soil used for isolation of xylanase producer. A 1-g aliquot of each soil sample was dried at 35°C for 24 hours in an oven (Rawashdeh *et al.*, 2005).

3.2.2 Preparation of Cultivation Medium

3.2.2.1 Preparation of Nutrient Agar Plate

Agar medium is considered as solid media as gelatin or silica gel. It serves as food for culturing bacteria. This agar supports all types of bacteria. It usually comprise of water, carbon, and minerals. A total of 0.2% (w/v) nutrient agar was used. The agar powder was dissolved into 1-liter solution when the solution was heated. The Schott bottle was covered with aluminum foil and prior to sterilization. Sterilization is carried out at 121˚C for 15 minutes. After sterilization completed, the bottle was removed and allowed to cool enough so that the agar will begin to solidify. After that, the agar was poured into Petri dishes until the agar just covered the bottom of the Petri dish (approximately 20 ml). The Petri dish cover was placed on the Petri dish immediately and the agar was allowed to solidify (fully gel). After the plate of agar has solidified, the plate was turned upside down and sealed using parafilm tape. The plate was stored in 4˚C freezer for further usage.

3.2.2.2 Preparation of Selective Agar Plate

The selective agar plate containing KNO_3 2 g/l, K_2HPO_4 1 g/l, $MgSO_4$ 0.5 g/l, $CaCO_3$ 3 g/l, $FeSO_4$ 0.01 g/l, xylan 0.80 g/l, and agar powder 20 g/l were prepared (Rifaat *et al.*, 2005). The medium was sterilized at 121˚C for 15 minutes. The same method as nutrient agar plate preparation is used to prepare selective agar plate.

3.2.2.3 Preparation of Seed Culture Medium

The seed culture medium composed of KNO_3 2 g/l, K_2HPO_4 1 g/l, $MgSO_4$ 0.5 g/l, $CaCO_3$ 3 g/l, $FeSO_4$ 0.01 g/l, and xylan 0.80 g/l.

3.2.2.4 Preparation of Bacterial Inoculum

A loopful of microbe from the selective agar plate medium was transferred into the 250 ml shake flask containing 30 ml of seed culture medium. The culture was incubated at 33˚C for 36 hours with rotary shaking 200 rpm. The cells were centrifuged at 5000 rpm for 5 minutes. The cell pallets were washed once with 0.85% (w/v) saline solution. Finally, the cells were suspended again in saline solution. The mixture was homogenized and the optical density (OD) was read at 600nm using spectrophotometer until it reaches 0.5.

3.2.2.5 Storage of Microbial

Actinomycetes may preserved as agar plugs under 10% glycerol for short term to intermediate term preservation (Khandeparkar *et al.,* 2004). 10% (w/w) solution of glycerol was prepared and 2 ml was dispensed into 4 ml capacity Wheaton borosilicate glass vials fitted with rubber-lined caps. The vials were autoclaved twice for 45 minutes each. Transfer tubes were autoclaved for 25 minutes. Several plugs were cut and deposited into each of the vials, which were then frozen and stored at -70˚C (Demain and Devies, 1999).

3.2.3 Preparation of Crude Enzyme

Mixture of 10 ml inoculum with 90 ml seed culture medium in 250 ml shake flask was incubated 48 hours at 200 rpm in temperature 33˚C. The mixture then was centrifuged at 5000 rpm at 4˚C for 5 minutes. The supernatant was taken as crude xylanase.

3.2.4 Preparation for Screening

3.2.4.1 Preparation of Primary Screening Plate

Sub samples of 1 g were suspended in 100 ml sterile distilled water then incubated in an orbital shaker incubator at 28˚C with shaking at 140 rpm for 30 minutes. Mixtures were allowed to settle then serial dilutions up to 10^{-6} were prepared. From each dilution, 0.1 ml was taken and spread evenly over the surface of nutrient agar with sterile L-shaped glass rod then incubated at 27˚C for 3 days (Saadoun *et al.,* 2003).

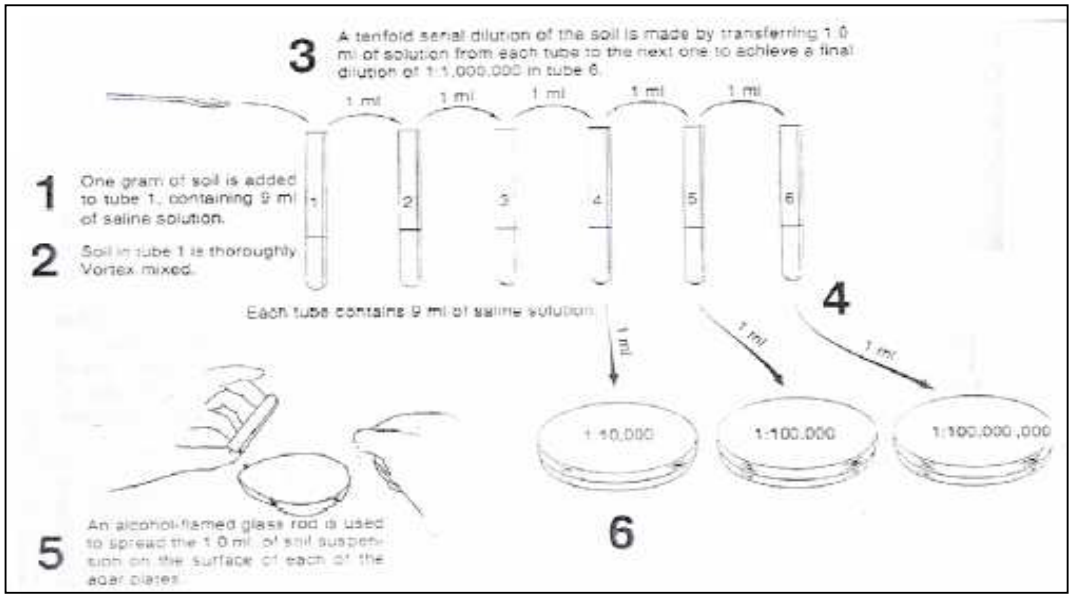

Figure 3.1 : Example sample dilution and primary screening (Benson, 1994)

3.2.4.2 Second and Third Screening

From the first screened plate, each colonies being selected and each was streaked for isolation onto selective agar plate. The plates were then being incubated for 4-6 days at 33˚C. The colony was then transferred for 10 times to ensure that the culture was pure enough to morphology and physiological test.

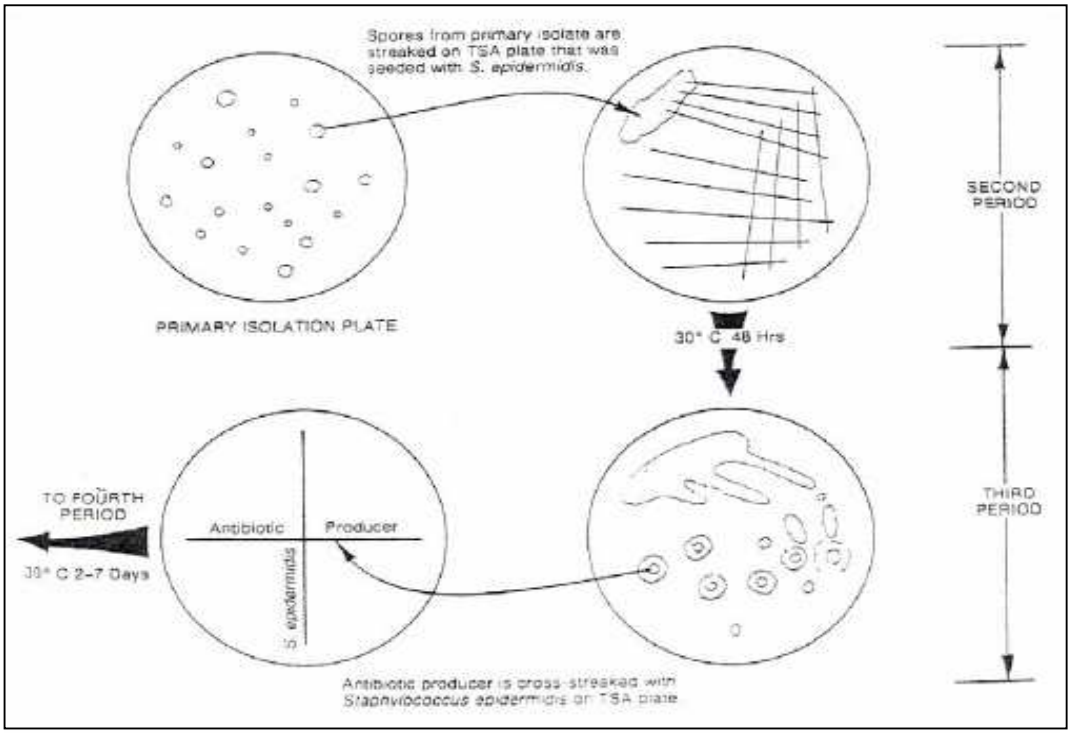

Figure 3.2 : Example second and third screening (Benson, 1994)

3.3 Characterization of Bacteria

3.3.1 Morphological Test

3.3.1.1 Simple stain

Microscope slide was cleaned and dried thoroughly. The surface in which the smear is to be spread was flamed. The inoculating loop was flamed. A loop full of tap water was transferred to the flamed slide surface. The loop was reflamed to make sure the entire length of the wire that will enter the tube has been heated to redness. After the inoculating loop was cooled, a pinhead size sample of the bacterial growth was picked up without digging into the agar. The bacteria on the loop was dispersed in the drop of water on the slide and spreaded the drop over an area the size of a dime. It should be a thin, even smear. The inoculating loop was reflamed to redness including the entire length that entered the tube. The smear was allowed to dry thoroughly. The smear was heat-fixed cautiously by passing the underside of the slide through the burner flame two or three times. The temperature of the slide was tested after each pass against the back of the hand. It has been heated sufficiently when it feels hot but can still be held against the skin for several seconds.

Then, flooding it with methylene blue to stain the smear and allowed it to remain covered with the stain for one minute. During the staining, the slide held in the fingers. At the end of the designated time, the excess stain was rinsed off thoroughly gently with distilled water. The back of the slide was wiped and the stained surface was blotted with bibulous paper. The stained smear was placed on the microscope stage smear side up and focuses the smear using the 10X objective. An area of the smear in which the cells are well spread in a monolayer was chosen. The area to be studied was centered, applied oil directly to the smear, and focused the smear under oil with the 100X objective. The cells observed were captured.

3.3.1.2 Gram staining

Bacterial smear was prepared on a clean slide. The slide was heated for few seconds until it becomes hot to the touch so that bacteria are firmly mounted to the slide. The primary stain crystal violet was added and incubated 1 minute. This step colored all cells violet. Gram's iodine was added, for 1 min. All cells remain violet. The Decolorizer was washed with ethanol and acetone. The secondary stain, safranin, was added and incubated 1 min, then washed with distilled water for a maximum of 5 seconds. If the bacteria is Gram-positive then the cell will retain the primary stain, will not stain, and will appear black-violet. If the bacteria is Gram-negative then the cell will lose the primary stain, take secondary stain, and will appear red-pink. Gram stain is 2 g of 90% crystal violet dissolved in 20 ml of 95% ethyl alcohol while Gram's iodine is 1 g of iodine, 2 g of potassium iodide, dissolved in 300 ml of distilled water. Decolorizer is 50% ethyl alcohol and 50% acetone.

3.3.1.3 Acid-fast stain

1/2 a drop of water was placed on a clean slide using the dropper bottle of distilled water by touching the dropper to the slide. A small amount of the bacterium was aseptically removed from the agar surface and mixed it with the water. The loop was flamed and let it cool. The mixture was spreaded over the entire slide using the loop to form a thin film. This thin suspension was allowed to completely air dry. The slide (film-side up) was passed through the flame of the bunsen burner 3 or 4 times to heat-fix. Then, the smear was covered with a piece of blotting paper and flooded with carbol fuchsin. The smear was steamed for 5 minutes by passing the slide through the flame of a gas burner. The slide was allowed to cool and washed with water. The acid-alcohol decolorizing was added slowly dropwise until the dye no longer runs off from the smear. Rinse with water and then counterstain with methylene blue for 1 minute. Wash with water, blot dry, and observe using oil immersion microscopy. Acid-fast bacteria will appear red; non-acid-fast will appear blue.

3.3.1.4 Spore staining

Smears of organisms to be tested for endospores were prepared. The smears were heat-fixed. The smears were covered with a piece of absorbent paper cut to fit the slide and placed the slide on a wire gauze on a ring stand. The paper was saturated with malachite green and holding the Bunsen burner in the hand heated the slide until steam can be seen rising from the surface. The heats removed and reheat the slide as needed to keep the slide steaming for about three minutes. As the paper begins to dry, a drop or two of malachite green was added to keep it moist, but don't add so much at one time that the temperature is appreciably reduced. Do not overheat because the process is steaming and not baking. The paper was removed with tweezers and rinsed the slide thoroughly with tap water. The slide was drained and counterstained 45 seconds with 0.5% safranin. Wash, blot, and examine. The vegetative cells will appear red and the spores will appear green.

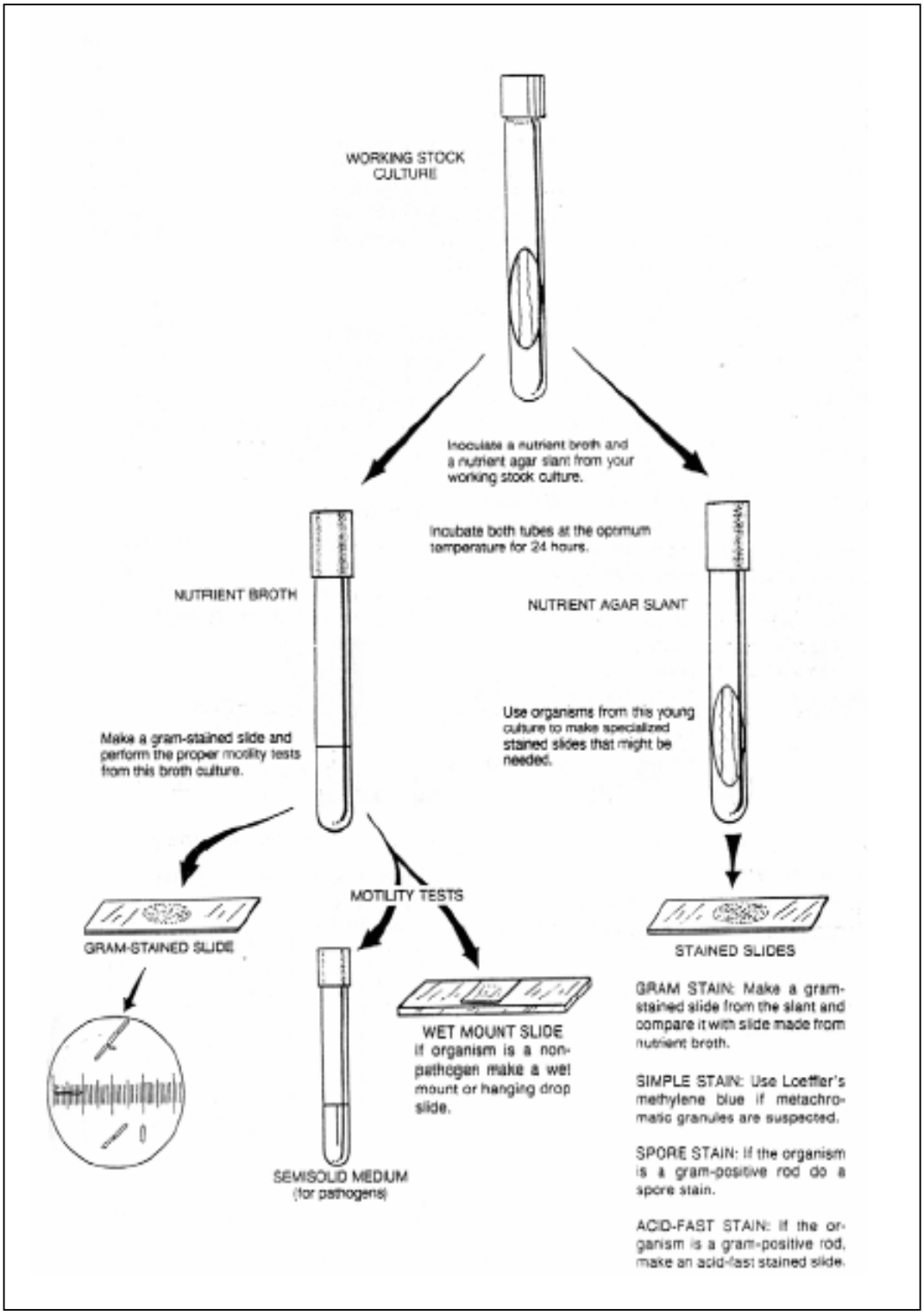

Figure 3.3 : Example of Morphology Study (Benson, 1994)

3.4 Analysis on Enzyme Activity

3.4.1 Effect of pH

To determine the optimum medium pH for maximum enzyme activity, selected medium of different pH (pH 5.8, pH 6, pH 7, pH 8, and pH 9) was inoculated with the crude enzyme at 65˚C for about 15 minutes. The range of buffers were used at 50 mM in preparing 0.5% (w/v) xylan solution for detection of xylanase activity was phosphate buffer (pH 5.8, 6 and 7), Tris buffer (pH 8 and 9). The reaction conditions composed of 1.8 ml substrate and 0.2 ml crude xylanase. The effects of medium pH on xylanase activity were recorded. The pH value giving the highest enzyme activity was used in further enzyme assays (Rawashdeh *et al.*, 2005).

3.4.2 Effect of Temperature

Usually, the optimal temperature for enzyme activity was determined by performing the standard assay procedure at a range of temperature from 4˚C to 100˚C by using the same method as mentioned above (Rawashdeh *et al.*, 2005). The determination of the optimum temperature for xylanase activity was done at temperature ranges from 40-70˚. The effects of temperature on xylanase activity were recorded.

3.5 Analysis Procedure and Enzyme Assay

3.5.1 Modified Lowry Method

Solution A (carbonate buffer) was prepared by dissolved 0.4 g sodium tartrate and 20 g sodium carbonate 1 N Na_2 CO_3 in 100 ml alkaline and dilute with water to 200 ml. For solution B (alkaline copper tartrate solution), 2 g sodium potassium tartrate and 1 g $CuSO_4.5H_2O$ was dissolved in 90 ml water and added 10 ml 1 N NaOH. Solution C (Folin–Ciocalteau reagent) is 1 part Folin-Ciocalteau reagent and 15-part of water. Solution A and B stable at room temperature while solution C was prepared daily.

0.2 ml of crude xylanase was pipetted into labeled test tube. 0.2 ml of the diluents was used as blank. At 15 seconds intervals, 1.0 ml of the solution A and B was added to the tubes, mixed, and allowed to set at room temperature for 10 minutes. At the end of the incubation period, 0.1 ml of solution C was immediately mixed with the reaction samples, and leave at room temperature for another 30 minutes. The absorbency of sample was measured at 650 nm. A standard curve was prepared by plotting the average blank corrected at 650 nm reading for each BSA standard versus its concentration in μg/ml. The protein concentration in the crude xylanase was determined using the standard curve.

3.5.2 3,5-Dinitrosalicylic acid (DNS) Method

The mixture was incubated in a water bath at certain temperature for 15 minutes. The released reducing sugar was measured using the 3,5-dinitrosalicylic acid (DNS) method in which the reaction was stopped by adding 3 ml of DNS reagent. A reddish brown colour developed after placing the reaction tubes in a boiling water bath for 5 minutes. After cooling the reaction tubes to room temperature, the O.D. was measured at 575 nm with xylose as the standard, where one unit (U) of xylanase activity is defined as the amount of enzyme that releases 1 μg xylose/min/ml under the above mentioned conditions (Rawashdeh *et al.*, 2005).

CHAPTER 4

RESULTS AND DISCUSSIONS

4.1 Screening Results

4.1.1 Primary screening

The primary screening plates were prepared from samples that were diluted up to 10^{-6}. The bacteria were grown on nutrient agar plate for 3 days in incubator at 33˚C before transferred to selective xylan agar plate.

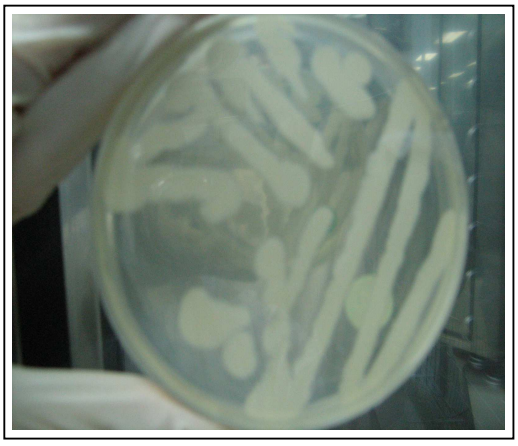

Figure 4.1 : Result of primary screening

4.1.2 Third Screening

Selective xylan agar plates were used in third and the next screening process to screen colonies from the nutrient agar. Positive results showed some remarked activities of xylanase enzyme.

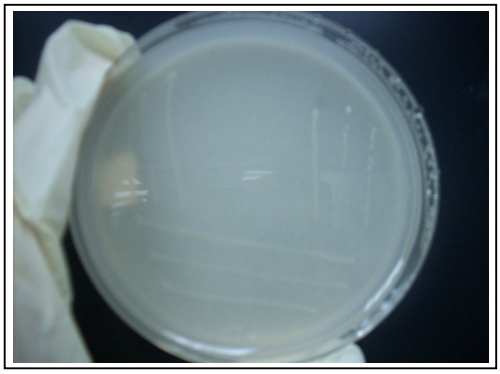

Figure 4.2 : Positive result

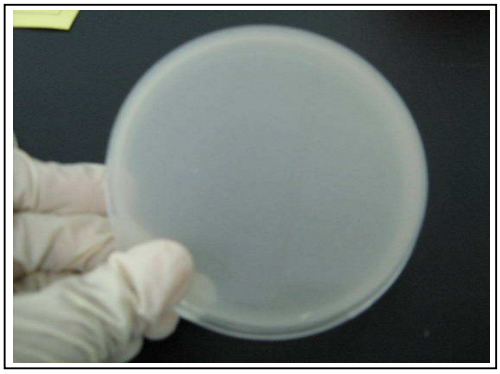

Figure 4.3 : Negative result

4.1.3 Tenth Screening

Figure 4.4 showed the tenth screening plate. There are two single colonies that can be observed from the figure. The single colony bacterial will be generate for further experiments.

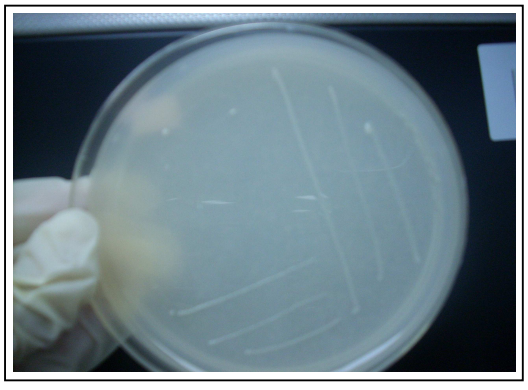

Figure 4.4 : Tenth screening result

4.2 Morphological Test Results

4.2.1 Simple staining

The shape of bacterial that can be observed under microscope (magnification 100x) is in coccus and in chain arrangement. So, the result showed that the bacterial was near to *Streptococci* type bacterial.

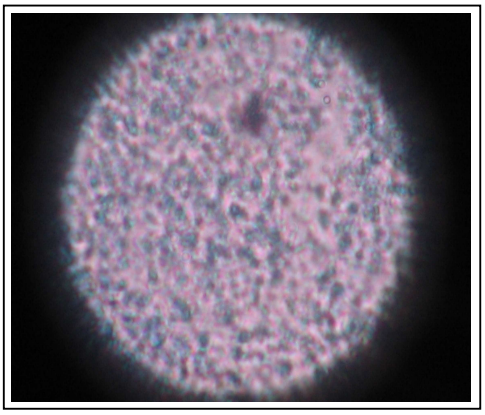

Figure 4.5 : Simple stain result

4.2.2 Gram staining

Gram positive and gram negative organisms are distinguished each other by differences in their cell walls. The result showed the cells appeared pink to red color. So, the bacterial was gram negative cell (observed under microscope 100x magnification). In gram negative organisms, there were peptidoglycans present in the cell walls but they only comprise 10-20% of the cell wall. Gram negative cells also have an outer layer which gram positive organisms do not have. Exposing gram negative cells to the decolorizer dissolves the lipids in the cell walls, which allows the crystal violet-iodine complex to leach out of the cells and allow the cell to be stained with safranin and finally giving reddish-pink color to the cell.

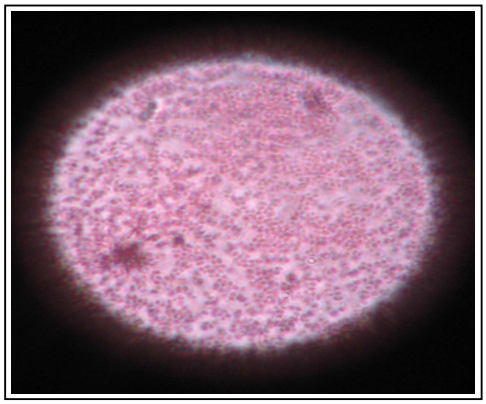

Figure 4.6 : Gram Stain result

4.2.3 Acid-fast stain

Acid-fast stain used to identify acid-fast microorganisms such as members of the genus *Mycobacterium.* Acid-fast organisms are highly resistant to disinfectants and dry conditions. Figure 4.7 showed the bacterial stained blue which remarked as non acid-fast bacterial (observed under microscope magnification 100x). So, the bacterial was not a member of genus *Mycobacterium.*

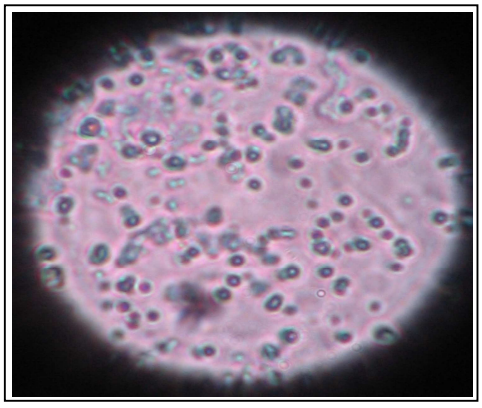

Figure 4.7 : Acid-fast Stain result

4.2.4 Spore staining

Spore staining was used to visualize bacterial endospores. By forming spore, bacteria can survive in hostile conditions. The result in Figure 4.8 (observed under microscope 100x magnification) illustrated that the cells were stained red. So, the type of bacteria was vegetative cell, which cannot produce endospores. As claimed by Benson, (1994), *Bacillus* and *Clostridium* type of bacteria were endospore bacteria. So, this bacterial was not a member of genus *Bacillus* or *Clostridium.*

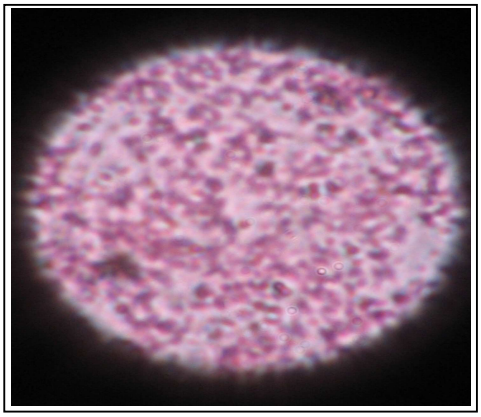

Figure 4.8 : Spore staining result

4.3 Enzyme Analysis

Xylanase activity was studied in two physical parameters including pH and temperature effect. Modified Lowry Method was used to determine the protein content in the crude enzyme and DNS test was used to assay xylanase activity. For the effect of pH, different pH xylan substrate from pH 5.8 to pH 9 was prepared and the reaction mixture composed of 1.8 ml substrate and 0.2 ml crude xylanase was incubated at temperature 65˚C. For the effect of temperature, optimal temperature for xylanase activity was determined at range of temperature 40 to 70˚C.

4.3.1 Modified Lowry Method

The crude xylanase was analyzed using Lowry method to determine the protein content.

Table 4.1 : Protein concentration in crude xylanase

OD	Protein concentration (µg/ml)
0.104	148.571

4.3.2 3,5-Dinitrosalicylic acid (DNS) Method

4.3.2.1 Effect of pH

To determine the effect of pH on xylanase activity, the substrate was diluted in different pH of buffer solution and incubated in water bath at 65˚C for 15 minutes. The release reducing sugar was measured using DNS method.

The results as illustrated in Figure 4.9 and 4.10 showed that the enzyme had high activity from pH 5.8 to pH 7, with a maximum activity at pH 6. This result

agrees with finding of Rawashdeh *et al.*, (2005) who reported that xylanase from *Streptomyces* sp. (strain lb 24D) had high activity at pH 5-8. The activity decreased from pH 6 to pH 9. This showed that the optimum condition is in slightly acidic medium. Since the source of the sample is from soil, which the pH condition is at pH 6-7, so the findings is acceptable. The maximum xylanase activity is about 72.667 U/ml at pH 6. Table 4.2 showed the result of effect of pH to enzyme activity.

Table 4.2 : Result for effect of pH

PH	OD	Xylose concentration (µg/ml)	Activity (U)
5.8	0.170	170	56.667
6.0	0.218	218	72.667
7.0	0.196	196	65.333
8.0	0.104	104	34.667
9.0	0.034	34	11.333

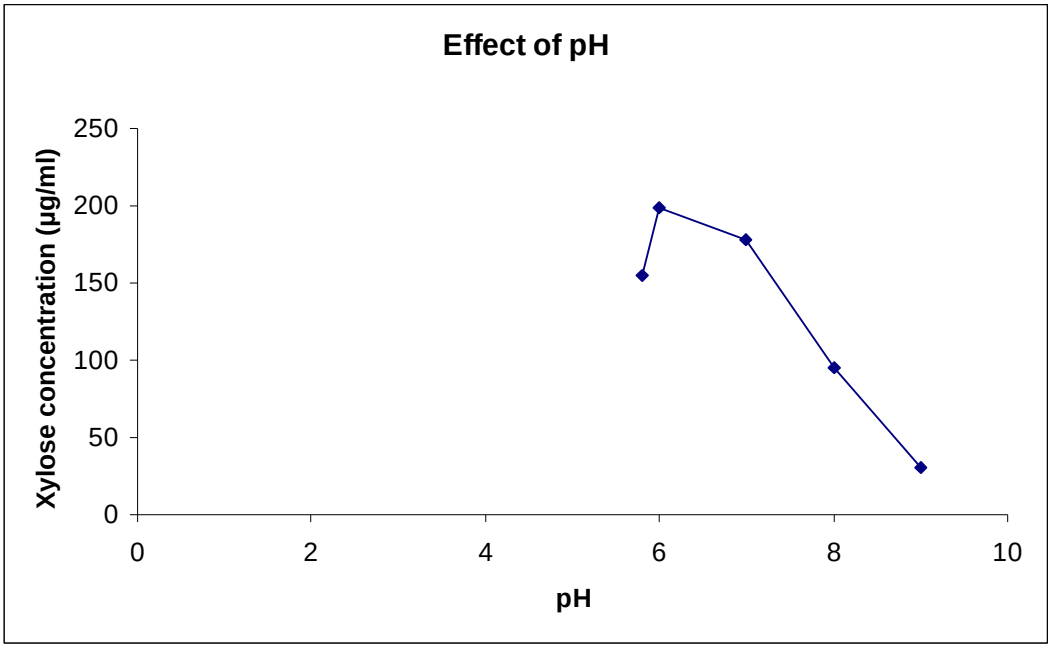

Figure 4.9 : Effect of pH at 65˚C

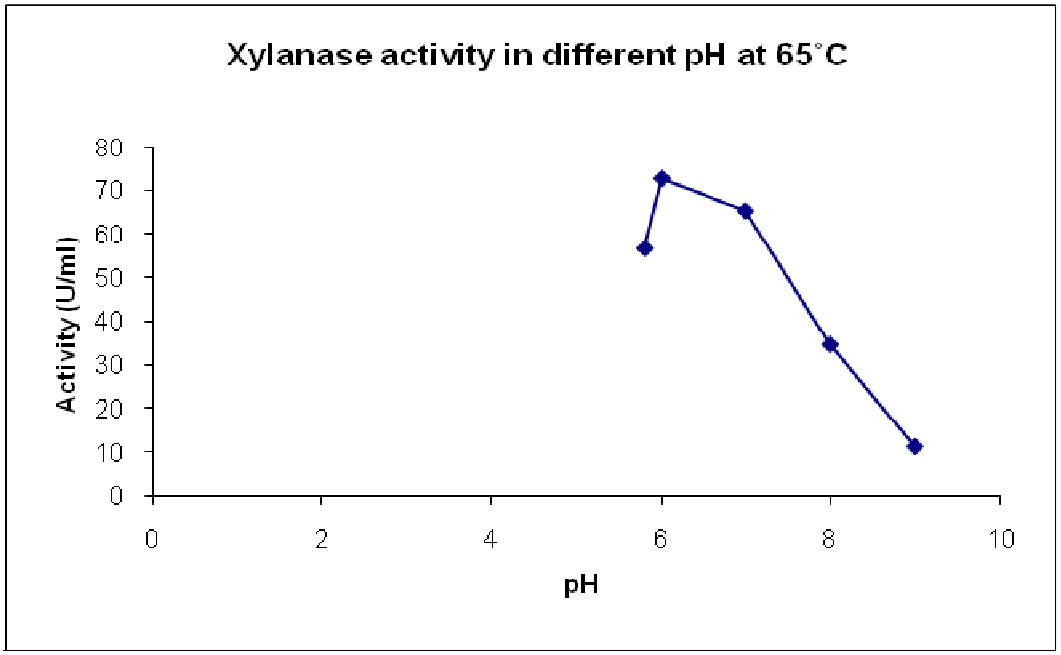

Figure 4.10 : Effect of different pH on xylanase activity at 65˚C

4.3.2.2 Effect of temperature

As observed in the Figure 4.11 and 4.12, the xylanase activity increased slowly from 40^{o}C to 60^{o}C, and rocketed at 65^{o}C. But, a sharp decrement in xylanase activity was observed at temperature 65^{o}C to 70^{o}C. The maximum activity is peaked at 65^{o}C and this agreed with Rawashdeh *et al.*, (2005), who reported that the optimum temperature for xylanase activity (U/ml) in *Streptomyces* sp. (strain lb 24D) was 65^{o}C. The enzyme is thermophile since the optimum condition is at range 60-80^{o}C which considered as quite high temperature but not extreme temperature. The xylanase activity is 72.667 U/ml at 65^{o}C. As done by Rawashdeh *et al.*, (2005), tomato pomace medium was used and the result showed that the maximum activity is high (1447 U/ml) after 3 days compared to Nascimento *et al.*, (2002) that reported the activity is only 116 U/ml in larchwood medium after 3 days. Table 4.3 showed the result for effect of temperature to enzyme activity.

Table 4.3 : Result for effect of temperature

Temperature (˚C)	OD (pH 6)	Xylose concentration (µg/ml)	Activity (U)
40	0.084	84	28.000
50	0.104	104	34.667
60	0.144	144	48.000
65	0.218	218	72.667
70	0.076	76	25.333

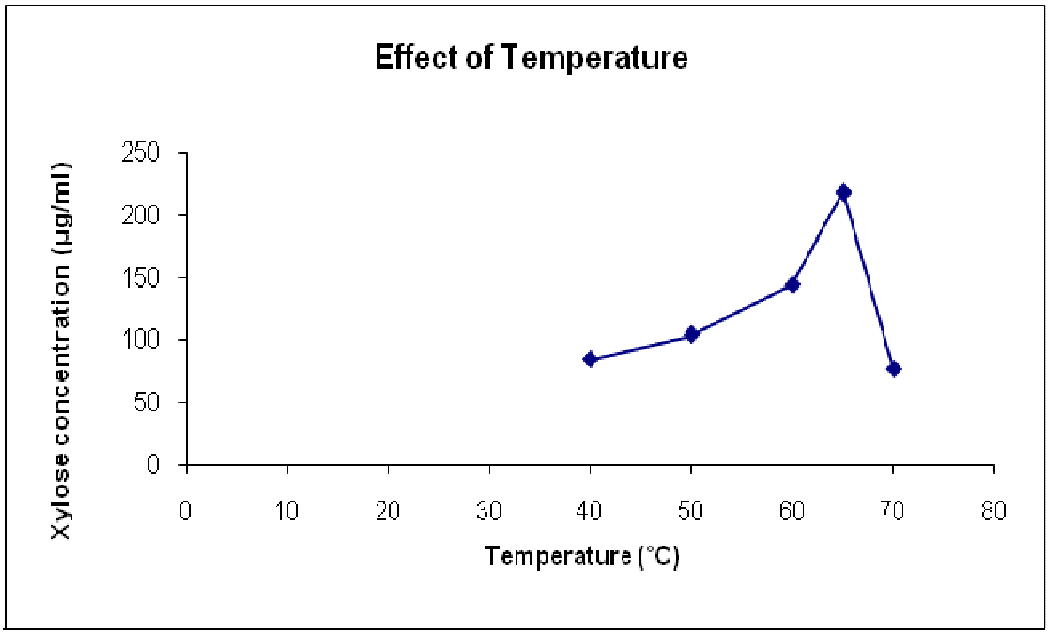

Figure 4.11 : Effect of temperature at pH 6

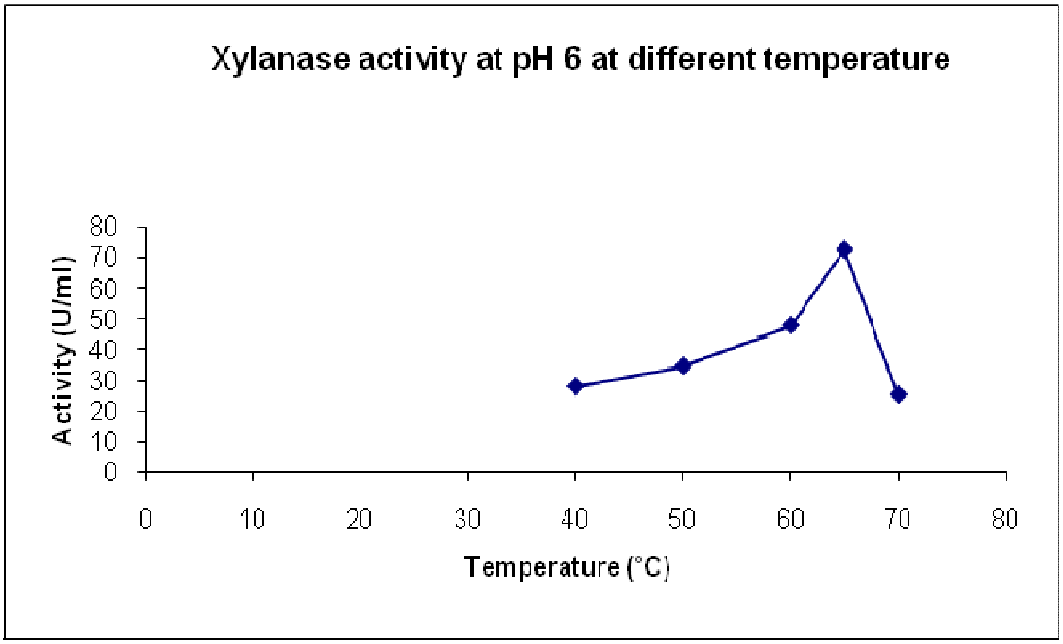

Figure 4.12 : Effect of temperature on xylanase activity at pH 6

CHAPTER 5

CONCLUSION AND RECOMMENDATIONS

5.1 Conclusion

From the sample soil, the screen procedure had done and gives the positive result in selective xylan agar plate. The selective colony bacteria had basic structure in coccus. For gram staining, the result showed that the bacterial is gram negative type. The test also gave negative result in acid-fast and stained red color for spore staining which showed that the bacterial is vegetative cell. From the enzyme analysis, the protein content in the crude enzyme is 148.571μg/ml after 48 hours incubation. The results for enzyme characterization showed that the optimum pH and temperature for maximum xylanase activity were obtained at pH 6 and 65˚C respectively with activity 72.667 U/ml.

5.2 Recommendations

The production of xylanase from bacteria in soil has unique characters and has a wide potential to be exploited in both microbiology and biotechnology fields. The ability of this microbe to survive should be explored extensively. Several recommendations for further studies are as follow:

i) Instead of using xylan selective agar to isolate xylanase producer, other method can be used that is by using a mixture of insoluble chromogenic

substances. It provides a specific, reliable, and rapid simultaneous detection of corresponding polysaccharide-degrading microorganisms (Ten *et al.*, 2004).

ii) For specific characterization of microorganisms, DNA identification of microorganisms can be done using polymerase chain reaction (PCR).

iii) Further study on the growth medium and physical condition. Low cost of medium but with high production is important in order to increase the quality of production.

REFERENCES

1. Coughlan M. P. and Hazlewood G. (1993). *B-1,4-D-Xylan-degrading enzyme system: Biochemistry, Molecular biology, and Applications.* Biotechnology Applied Biochemistry (17): 259-289.

2. http://www.greatvistachemicals.com/biochemicals/xylanase.html (May 31, 2007).

3. MaesJu´ Lio Xandro Heck, Simone Hickmann Floˆres, Plinho Francisco Hertz, marco Antoˆnio Za´chia Ayub (2005). *Statistical Optimization of Thermotolerant Xylanase Activity from Amazon Isolated Bacillus circulans on Solid-state Cultivation.* Bioresourse Technology.

4. Rakhee Khandeparkar and Narayan B. Bhosle (2004). *Purification and Characterization of Thermoalkalophilic Xylanase Isolated from the Enterobacter sp. MTCC 5112.* Research in Microbiology. Volume 3(3).

5. Gupta Saurabh, Kuhad R.C., Bushan Bharat, and Hoondal G. S. (2001). *Improved Xylanase Production From a Haloalkalophilic Staphylococcus sp. SG 13 Using Inexpensive Agricultural Residues.* World Journal of Microbiology and Biotechnology. Volume 17 (1): 5-8(4).

6. Seyis I. and Aksoz N. (2005). *Xylanase Production from Trichoderma harzianum.* Food Technology Biotechnology. 43 (1): 37–40.

7. Rifaat Rawashdeh, Ismail Saadoun, and Amjad Mahasneh (2005). *Effect of Cultural Conditions on Xylanase Production by Streptomyces sp. (strain Ib 24D) and Its Potential to Utilize Tomato Pomace.* African Journal of Biotechnology. Volume 4 (3): 251-255.

8. http://www.britannica.com/eb/topic-260780/article-9039958 (August 8, 2007).

9. Coughlan M. P. (1989). *Enzyme System for Lignocellulose Degradation.* Elseiver Applied Science: 151-181.

10. Gray F. Leatham and Micheal E. Himmel (1991). *Enzymes in Biomass Conversion A.C.S* : 505-533.

11. Brillouet and Joseleau, 1987; Bajpai, 1997; Schooneveld- Bergmans et al., 1998.

12. http://www.whitfordww.com/design/xylan-substrates.html (August 8, 2007).

13. Daniel Hillel (editor-in-chief), Dick R. P., and Kendeler E. (2005). *Encyclopedia of Soils in the Environment Volume 1,* Elsevier Academic Press: 448-455.

14. William V. Dashek (1997). *Methods in Plant Biochemistry & Molecular Biology.* CRC Press: p.313.

15. http://www.webvitamins.com/Nutrient.aspx?id=2156 (August 8, 2007).

16. http://www.sigmaaldrich.com/img/assets/19500/hemicullulase_xylanase.gif (August 14, 2007).

17. Shawn D. Mensfield and John N. Saddler (2003). *Application of Enzymes to Lignocellulosics.* ACS Symposium Series 855: 360-375.

18. Mark R. Nimlos, Heidi M. Pilath, David K. Johnson, Michael E. Himmel, Xianghong Qian, and Haitao Dong. (2007). *Kinetics And Modeling Of Xylan Hydrolysis And Xylose Dehydration And Reversion During Acid Pretreatment.* (1) National Renewable Energy Laboratory, 1617 Cole Boulevard, Golden, CO 80401, (2) Mechanical Engineering, Colorado State University, Mechanical Engineering, 1374 Campus Delivery, Fort Collins, CO 80523.

19. Jean Lozet and Clement Mathieu (1991). *Dictionary of Soil Science.* Second Edition. Rotterdam: A.A. Balkema.

20. Kurytowicz W., Gause G. F., Alina Paszkiewicz, Tatiana P. Preobrazhenskaya, Irena Wojewódzka, Tatiana S. Maximora, Wanda Woźnicka, W. Brzosko, K. Malinowski, and S. T. Williams (1971). *Atlas of Spores of Selected Genera and Species of Stertomycetacea.* Baltimore, Maryland: University Park Press.

21. Biely P., Mislovicova D., and Toman R. (1985). Analytical Biochemistry: *Soluble Chromogenic Substrates for Assay of Endo-1,4-β-xylanase and Endo-1,4-β-glucanase*: 142-146.

22. Bocchini D. A., Oliveira O. M. M. F., and Gomes R. Da Silv (2005). Process Biochemistry 40. *Use of sugarcane bagasse and grass hydrolysates as carbon sources for xylanase production by Bacillus circulans D1 in submerged fermentation*: 3653-3659.

23. Dekker R. F. H. (1985). Biodegradation of Hemicellulose. In: T. Higuchi. *Biosynthesis and Biodegradation of Wood Components.*

24. Rifaat H. M., Nagieb Z. A., and Ahmed Y. M. (2005). *Production of Xylanase by Streptomyces Species and Their bleaching Effect on Rice Straw Pulp.* Applied Biology and Environmental Research. 4(1): 151-160.

25. Harold J. Benson, Professor Emeritus (1994). Microbiological Application. Sixth Edition.WCB.56-58.

26. Hiroshi Tsujibo, Mitsuo Kosaka, Sadao Ikenishi, Takaji Sato, Katsushiro Miyamoto, and Yoshiniko Inamori (2004). *Molecular Characterization of a High-Affinity Xylobiose Transporter of Streptomyces thermoviolaceus OPC-520 and Its Transcriptional Regulation.* Journal of Bacteriology. Volume 186 (4):1029-1037.

27. Khandeparkar R. D. S. and Narayan B. Bhosle (2006). *Screening, Purification and Characterization of Xylanase Produced by Arthrobacter sp.*

MTCC 5214 when grown in Soli-state Fermentation. Enzyme and Microbial Technology.

28. Lite Li, Hongmei TianYongqiang Cheng, Zhengqiang Jiang, Shaoqing Yang (2005). *Purification and Characterization of A Thermostable Cellulase-free Xylanase from The Newly Isolated Paecilomyces themophila,* Enzyme and Microbial Technology.

29. Nascimento R. P., Coelho R. R. R., Marques S., Alves S., G` irio F. M., Bonc E. P. S., and Amaral-Collaco M. T. (2002). *Production and Partial Characterization of Xylanase from Streptomyces sp. strain AMT-3 Isolated from Brazillian Cerrado Soil,* Enzyme and Microbial Technology 31: 549-555.

30. Dietmar Haltrich, Bernd Nidetzky, Klaus D. Kulbe, Walter Steiner, and Silvia ZupanImageiImage (1996). *Production of Fungal Xylanases.* Bioresource Technology Volume 58 (2): 137-161.

31. Pang Pei Keng and Ibrahim Che Omar (2004). *Xylanase Production by a Local Fungal Isolate, Aspergillus niger USM AI 1 via Solid State Fermentation Using Palm Kernel Cake (PKC) as Substrate Songklanakarin.* Journal Science Technology 27 (2): 325-336.

32. Manabu Suto, Miho Takebayashi, Katsuichi Saito, Michiko Tanaka, Atsushi Yokota, and Fusao Tomita (2001). *Endophytes as Producers of Xylanase.* Journal of Bioscience and Bioengineering. Volume 93 (1): 88-90.

33. Arnold L. Demain and Julia E. Devies (1999). *Manual of Industrial Microbiology.* Second Edition. ASM Press Washington D.C.

34. Ismail Saadoun and Raad Gharaibeh (2003). *The Streptomyces flora of Badia region of Jordan and Its Potential as A source of Antibiotics Active against Antibiotic-resistant Bacteria.* Journal of Arid Environments (53): 365-371.

35. Leonid N. Ten, Wan-Taek Ima, Myung-Kyum Kima, Myung Suk Kanga, Sung-Taik lee (2004). *Development of a Plate Technique for Screening of*

Polysaccharide Degrading Microorganisms by Using a Mixture of Insoluble Chromogenic Substrates. Journal of Microbiological Methods (56): 375-382.

36. Miller G. L. (1959). *Use of Dinitrosalicylic Reagent for Determination of Reducing Sugar*. Analytical Chemistry (31): 426-428.

37. Lim Ting Chin (2006). *Screening of Xylanase Producer from Soil.* Universiti Malaysia Pahang.

38. http://faculty.ccbcmd.edu/courses/bio141/lecguide/unit1/prostruct/acidfast.html (August 24, 2007).

39. http://homepages.wmich.edu/~rossbach/bios312/LabProcedures/Endosproe_stain_results.html (August 24, 2007).

40. http://homepages.wmich.edu/~rossbach/bios312/LabProcedures/Gram%20Stain%20results.html (August 24, 2007).

41. http://homepages.wmich.edu/~rossbach/bios312/LabProcedures/Simple%20stain%20results.html (August 24, 2007).

42. http://webhost.bridgew.edu/fgorga/courses/biochem_I/Exper%202%20.%20Lowry.pdf (August 26, 2007).

APPENDICES

A 1 Preparation of 50mM Phosphate Buffer at 25˚C

0.5 L of 1 M K_2HPO_4 at 174.18 g mol^{-1} = 87.09 g

0.5 L of 1 M KH_2PO_4 at 136.09 g mol^{-1} = 68.045 g

Table A.1 : Preparation of 50mM Phosphate buffer

Ph	Volume of 1 M K_2HPO_4 (ml)	Volume of 1 M KH_2PO_4 (ml)
5.8	8.5	91.5
6.0	13.2	86.8
7.0	61.5	38.5

Dilute the combined 1 M stock solutions to 2 L with distilled water.

A 2 Preparation of 50mM Tris Buffer at 25˚C

0.1 L of 1 M Tris at 121.14 g mol^{-1} = 12.114 g

Table A.2 : Preparation of 50mM Tris buffer

pH	Volume of 1 M Tris Base (ml)	Volume of 1 M HCl (ml)
8.0	37	2
9.0	19.6	3.2

10 ml of the combined 1 M stock solutions was diluted to 0.2 L with distilled water.

A 3 Preparation of DNS Reagent

In order to prepare dinitrosalicyclic acid (DNS) reagent, 100 ml of 5% (w/v) of 3, 5- Dinitrosalicyclic acid is mixed in 2 M Sodium Hydroxide (NaOH) with 250 ml of 60% (w/v) sodium tartrate and made the total volume up to 500 ml with distilled water.

A 4 Xylose Standard Curve

Standard curve for DNS method has been made for test of glucose concentration in solution. The concentration and absorbance were recorded in order to plot the curve. Table A.3 showed the data collected to calibrate the curve. While standard curve for xylose concentration was illustrated in Figure A.1.

Table A.3 : Xylose concentration and OD

Xylose concentration (µg/ml)	OD (abs)
500	0.582
400	0.373
300	0.268
200	0.229
100	0.091
50	0.037
0	0

Figure A.1 : Xylose standard curve

A 5 BSA Standard Curve

Standard curve for Modified Lowry method has been made for test of protein concentration in solution. The concentration and absorbance were recorded in order to plot the curve. Table A.4 showed the data collected to calibrate the curve. While standard curve for BSA concentration was illustrated in Figure A.2.

Table A.4 : BSA concentration and OD

BSA concentration (µg/ml)	OD (abs)
500	0.371
400	0.251
300	0.211
200	0.190
100	0.094
50	0.034
0	0

Figure A.2 : BSA standard curve