SCREENING FOR MANNANASE ACTIVITIES OF BACTERIA FROM SOIL AND WATER SAMPLES OBTAINED FROM MAITRI, ANTARCTICA AND AROUND SABAH.

TAM HENG KEAT

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ABSTRACT

Palm kernel cake is an important ingredient for the formulation of animal feed but it has a high percentage of hemicellulose (mannan) contents that hardly consumed by nonruminant animals. In addition, mannan is also an anti-nutritive agent that prevents the nutrients in PKC to be fully absorbed by poultry when used as feed. Mannan can be removed by mannanases to improve nutrition. Mannanases are produced by plants, fungi and bacteria. The objectives of this project are to screen and identify mannanase-producing bacteria obtained from Maitri, Antarctica and around Sabah. Bacteria were isolated and grew on various agars. Isolated bacteria were subjected screening for mannanases using 0.15% guar gum labeled Remazol Brilliant Blue (RBB) or 0.15% locust bean gum labeled RBB with yeast extract, polypeptone, ammonium nitrate, potassium phosphate monobasic and magnesium chloride. Haloes formation is visible if bacteria have a mannanase activity. Mannanase positive bacteria were differentiated using RAPD-PCR with 9 pirmers (A10, A11, A13, A17, E1, E3, E4, E5, and E6). Only primers E3 and E5 produced sufficient data to differentiate these bacteria. From the RAPD data, 12 different bacteria strains from tropics and four different strains from Antarctica were obtained. Five bacteria strains from tropics and five strains from Antarctica bacteria including one non-mannanase producing bacterium with fungi-liked characteristic were later identified based on their 16S rDNA gene sequences. Tropical bacteria were amplified using primers BSF 8/20 and BSR 1541/20 while Antarctic bacteria with primers FrvF and FrvR. 16S rDNA of two of terrestrial bacteria and three of the Antarctica bacteria were cloned onto pCR 2.1-TOPO vector and sequenced. Tropical bacteria that produce mannanase were identified as *Streptomyces sp.* and *Paenibacillus amylolyticus*. Mannanase producing bacteria that isolated from Antarctic bacteria were identified as *Arthrobacter sp.* and *Arthrobacter oxydans*. Additionally, a non-mannanase producing bacterium was identified as *Bacillus cereus*.

PENGSKRINAN AKTIVITI MANNANASE DARIPADA BAKTERIA DARI SAMPEL TANAH DAN AIR YANG DIDAPATI DARI MAITRI, ANTARCTICA DAN SABAH.

ABSTRAK

Intisari kelapa sawit (PKC) merupakan ramaun yang penting dalam formula makanan bagi haiwan perternakan. Namun demikian, ia mengandungi kandungan hemicellulosa (mannan) yang tinggi menyebabkan ia sukar dimakan oleh haiwan. Selain itu, mannan juga merupakan agen anti-khasiat yang menghalang khasiatnya diserap sepenuhnya oleh haiwan. Mannan boleh diurai oleh enzim mannanase untuk meningkatkan khasiat. Mannanase dihasilkan oleh tumbuh-tumbuhan, fungi dan bakteria. Objektif kami adalah untuk membuat pengskrinan dan mengenalpasti bacteria dari Maitri, Antarctica dan Sabah yang menghasilkan mannanase. Bakteria telah diasingkan dan dikulturkan dalam pelbagai jenis agar. Bakteria yang telah diasingkan akan melalui satu pengskrinan untuk mannanase dengan mengunakan 0.15% guar gum atau locust bean gum yang telah dilabelkan dengan Remazol Brilliant Blue (RBB) bersama dengan estrak yis, polipepton, ammonia nitrat, kalium fosfat dan magnesium klorida. Lingkaran cahaya (halo) akan terbentuk jika kehadiran aktiviti mannanase. Bakteria yang menunjukkan aktiviti mannanase yang positif akan diasingkan dengan menggunakan RAPD-PCR dengan sembilan primer (A10, A11, A13, A17, E1, E3, E4, E5, and E6). Didapati primer E3 dan E5 menunjukkan data yang sesuai untuk analisis. Data RAPD menunjukkan terdapat 12 jenis bakteria yang berlainan dari Sabah manakala empat jenis bacteria dari Antarctica. Lima jenis bakteria dari Sabah dan lima jenis bakteria dari Antarctica termasuk satu bakteria yang mengandungi ciri-ciri seperti kelihatan fungi telah dikenalpasti dengan menggunakan gen yang mengekodkan 16S rDNA. Bakteria dari Sabah telah dijalankan PCR yang menggunakan primer BSF 8/20 dan BSR 1541/20 manakala bakteria dari Antarctica menggunakan FrvF dan FrvR. Gen 16S rDNA daripada dua bakteria dari Sabah dan tiga dari Antarctica telah diklonkan ke dalam plasmid pCR 2.1-TOPO dan disequence. Bakteria dari Sabah yang menghasilkan mannanase dikenalpasti sebagai *Streptomyces sp.* dan *Paenibacillus amylolyticus*. Bakteria yang menghasilkan mannanase yang diasingkan dari Antarctica dikenalpasti sebagai *Arthrobacter sp.* dan *Arthrobacter oxydans*. Bakteria yang kelihatan seperti fungi dikenalpasti sebagai *Bacillus cereus*.

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LIST OF SYMBOLS

α	alpha
β	beta
°C	degree Celsius
g	gram
µg	micro gram
µl	micro liter
bp	base pairs
kb	kilo base pairs
ml	milliliter
min	minutes
mM	milli Molar
mm	millimeter
pmol	pico mol
s	seconds
Sq km	square kilometer
V	volt
w/v	weight per volume
BLAST	Basic Local Alignment Search Tool
DNA	deoxyribonucleic acid
dNTP	2'-dexoyribonucleoside-5'-triphospates
EDTA	ethylenediaminetetraacetic acid
PCR	polymerase chain reaction

RAPD	randomly amplified polymorphic DNA
RBB	Remazol Brilliant Blue R
rDNA	ribosomal deoxyribonucleic acid
RNA	ribonucleic acid
rRNA	ribosomal ribonucleic acid
rpm	rotation per minute
TAE	tris-acetate-EDTA
TBE	tris-borate-EDTA
tRNA	transfer ribonucleic acid
SDS	sodium lauryl sulfate
SST	Sekolah Sains dan Teknologi
U	unit

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CHAPTER 1

INTRODUCTION

1.1 BACKGROUND

Malaysia is one of the largest suppliers of palm kernel oil. Palm kernel cake is obtained from the kernel of the palm oil seed which has been squeezed to extract the crude palm oil content (Sabu *et al.*, 2005). This by-product consists mainly of nut and shell and is available in abundance. Palm kernel cake is dry and gritty and is not readily accepted by all types of stock. It contains high fibers, and consists of the dietary factors which affect the voluntary intake and entero-hepatic function of the animal (Salam Abdullah *et al.*, 1997). As an ingredient in mixed feeds, its unpalatability is of less importance. Nevertheless PKC is mixed with other nutrients and used as animal feed. PKC is now well-established as a major feed for ruminants in Malaysia.

Recent researches show that PKC is an important ingredient for the formulation of animal feed but it has a high percentage of hemicellulose and cellulose contents that hardly consumed by nonruminant animals (Ong *et al.*, 2004).

Hemicelluloses are the second most abundant polysaccharide in nature and are found in plant cell walls as linkers between lignin and cellulose (Ethier *et al.*, 1998). The polysaccharide must be removed through pretreatment in order to increase the absorption of the nutrients by the animals (Ong *et al.*, 2004). One of the abundant polysaccharide in PKC is mannan which is a hemicellulose. Mannan is an antinutritional fiber in feed (Wu *et al.*, 2005). One of the methods to remove the mannan is using the mannanase producing microorganisms. Mannanases are a hemicellulase (a polysaccharide-hydrolyzing enzyme) which is involved in cell wall disassembly and the weakening of plant tissues by degrading mannan polymers in the cell walls to mannose, a reducing sugar (Filichkin *et al.*, 2004). Mannanases are usually produced by the plants, bacteria, and fungi.

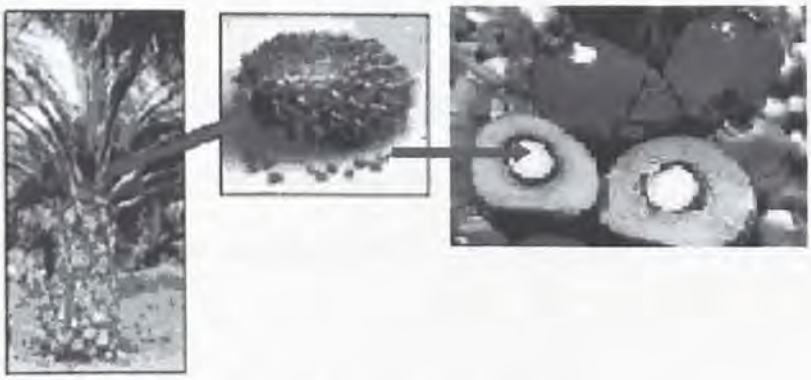

Figure 1.1 Palm kernels.

The PKC is mixed with the mannanase producing bacteria in a solid state fermenter so that the bacteria can use the mannan as a substrate for growth. These types of bacteria can degrade the mannan by producing mannanase enzyme so that not only the nutrients in the PKC can be easily absorbed by the cattle and poultry but also increase the protein contents. Moreover, the prices of the PKC which do not contain of mannan fetch a higher price.

The major problem of culturing the mannanase producing bacteria in the solid state fermenter with the PKC that has to be concerned is many species of bacteria can grow well in the PKC which has a high content of nutrients for the growth of bacteria. These bacteria can compete with mannanase producing bacteria for other nutrients or produce antimicrobial substances that inhibit their growth. It is important to eliminate other bacteria from our mannanase producing bacteria so that our bacteria can grow well in the fermenter and degrade the mannan to produce PKC with less content of mannan. We need to find a mannanase producing bacteria which have high or low temperature optima which are not favourable, the other strains of bacteria to grow and thus eliminating competitions.

1.2 OBJECTIVES

The aims of this study are: (1) To screen for microbes that produce the mannanase from extreme condition such as from the Antarctica and from tropical soil. (2) To isolate and identify microorganisms from Sabah and Antarctic with potential to remove mannan from PKC.

CHAPTER 2

LITERATURE REVIEW

2.1 MANNAN

2.1.1 Sources

Mannan is a polysaccharide which found abundantly in various seeds and beans and a major component of the hemicellulose fraction in soft woods. Mannans also found in most yeast either as major glycoprotein components of the cell envelope or as extracellular products (Sutherland, 2002). Mannans are also the major constituents group of hemicellulose in the cell wall of the angiosperms. Mannan is also an important structural component of some marine algae and terrestrial plants such as ivory nuts and coffee beans (Tamaru *et al.*, 1995).

2.1.2 Function

Mannans are vital structural components of plant cell walls and in this role they are not plant storage products. However, in some tissues there is extensive production of some

mannan to the inside of the primary cell wall to provide an available store of carbohydrates. It functions as a mechanical resistance and enlargement that occur during germination (Ethier *et al.*, 1998). It also functions as storage of carbohydrates in the bulbs and endosperm of some plants (Stoll *et al.*, 1999). The mannan that found in the plant seeds are served as energy reserve carbon source mobilized in the process of germination (Puchart *et al.*, 2004).

2.1.3 Structure

Mannan (Figure 2.1) consists of a backbone β-1,4-linked mannose units such as the palm kernel cake (Akita *et al.*, 2004). It is also a linear polymer of mannose and glucose or of mannose only (Stoll *et al.*, 1999). These polysaccharides are also usually found in various seeds and beans. Mannans are principal hemicelluloses in soft woods, accounting for up to 25% of the dry weight, are O-acetylgalactoglucomannans in which the backbone comprises mannose and glucose in the ratio 3:1; the glucose residues may be distributed randomly (Stoll *et al.*, 1999). Galactose monomers are linked α-1,6 to some of the mannose residues; some 2- and 3-hydroxyls of the mannose residues and, to a lesser extent, of the glucose residues, are acetylated (Stoll *et al.*, 1999).

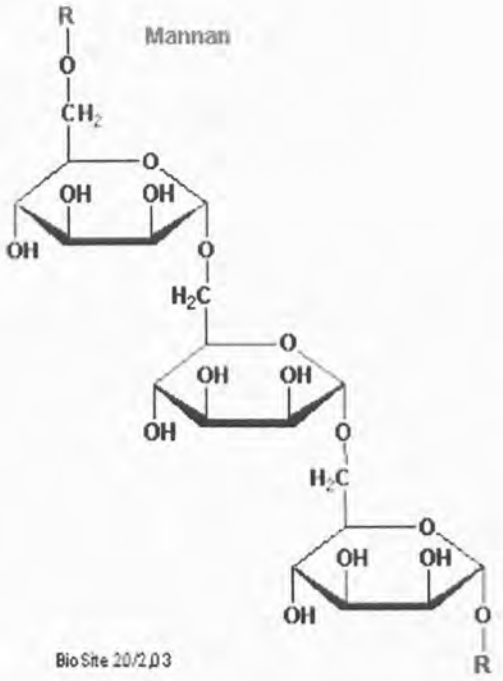

Figure 2.1 Basic structure of mannan

Most mannans are galactomannans (Figure 2.2) or (galacto-) glucomannans. The galactomannans are widespread in the endosperm of seeds, particularly from the Family Leguminosae, where they are occur as extracellular material. The structure is based on single α-(1,6)-D-galactopyranosyl residues attached directly to the mannan backbone. (Galacto-) glucomannans are major neutral noncellulosic polysaccharides of secondary cell walls. They occur mainly in gymnosperms. The glucomannans dominate in coniferous woods but many other plant families, especially the Leguminosae, contain galactoglucomannans.

The backbone structure is unique with two different sugar residues, D-glucose and D-mannose, being present although both have of β-(1,4)-linkages. The D-galactopyranose residues are present as side-chains, linked by α-(1,6) bonds to D-mannose residues. The sugar residues in the backbone also contain a significant proportion of O-acetyl groups. Since the ratio of D-mannose to D-glucose is always lower than one, it follows that two or

more contiguous D-monnose residues can occur (Morrison, 2002). The polymeric backbone of these polysaccharides is hydrolyzed by endo-β-1,4-mannanases, whereas the side chains of galactomannan are removed by α-galactosidases. The mannooligosaccharides generated by these enzymes are further hydrolyzed by β-mannosidases, which are exo-acting glycoside hydrolases that catalyze the removal of the nonreducing end β-D-mannose (Dias *et al.*, 2004).

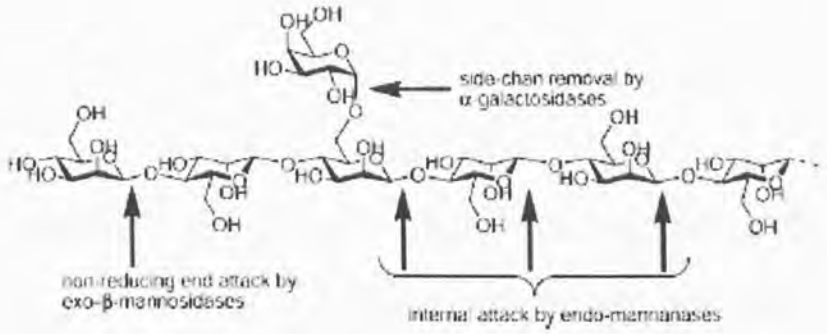

Figure 2.2 Schematic galactomannan structure. The nonreducing end of guar gum is shown schematically. The polymannose chain (b-linkage) is substituted every 2 residues by a galactose molecule (a-linkage). The hydroxy groups are shown as thick bars in their correct equatorial or axial positions. The arrows represent the glycosidic links recognized by β-mannanase, β-mannosidase, and α-galactosidase.

2.2 MANNANASES

Mannanases are produced by plants, fungi and bacteria (Stoll *et al.*, 1999). Recently, researchers have shown their interest in hemicellulase from these microorganisms not only the academic reasons but also because of their possible applicability in feed and food industry and pulp and paper industries (Stalbrand *et al.*, 1995). For example, β-mannanase is employed for preparation of mannooligosaccharides used as non-nutritional food addictives for selective growth of human-beneficial intestinal microflora