MANNANASE PRODUCTION BY *Aspergillus niger* IBRL F16.A4 USING PALM KERNEL CAKE AS SUBSTRATE

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Nukilan untuk :

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Kerana setia bersama,

Kerana

tanpa kalian semua tiada terdaya insan kerdil ini meneruskan perjuangan

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LIST OF SYMBOLS

α	alpha
β	beta
%	percentage
&	and
p	para
^{o}C	degree Celsius
±	plus minus
=	equal
+	plus
-	minus

LIST OF ABBREVIATION

AA	amino acid
AD	acid detergent
ADF	acid detergent fiber
$C_2H_5OCH_2CH_2OH$	ethyleneglycol monoethylether
$CH_3COONa.3H_2O$	sodium acetate
CMC	carboxymenthyl cellulose
CTAB	centrytrimethyl ammonium bromide
DNS	dinitrosalicylic acid
FTCC	Food Technology Culture Collection
g/l	gram per liter
HIKS	hampas isirung kelapa sawit
IBRL	Industrial Biotechnology Research Laboratory
kDA	kilo Dalton
KH_2PO_4	calcium phosphate crystal
M	Molar
ME	metabolisable energy
MJ	metabolisable joule
mM	millimolar
$MgSO_4.7H_2O$	magnesium sulfate heptahydrate
mg/g	milligram per gram
Min	minute
$Na_2B_4O_7.10H_2O$	sodium borate
$Na_2HPO_4.12H_2O$	disodium phosphate
NDF	neutral detergent fiber
nm	manometer
NMR	^{13}C Nuclear Magnetic Resonance

NSP	non-starch polysaccharides
OD	optical density
PABA	*p* – aminobenzoic acid
PDA	potato dextrose agar
PKC	palm kernel cake
PKM	palm kernel meal
P >	probability more than
P <	probability less than
rpm	rotation per minute
SUK-1	a fungal strain obtained from Universiti Kebangsaan Malaysia (UKM)
U/g	unit per gram
UV	ultraviolet
µl	microliter
v/v	volume per volume
vvm	volume per volume per minute
w/v	weight per volume
X	multiply

PENGHASILAN ENZIM MANNANASE OLEH *Aspergillus niger* IBRL F16.A4 MENGGUNAKAN HAMPAS ISIRUNG KELAPA SAWIT SEBAGAI SUBSTRAT

ABSTRAK

Kajian ini bertujuan untuk menentukan faktor yang mempengaruhi penghasilan enzim mannanase oleh kulat terpilih, *Aspergillus niger* IBRL F16.A4 menggunakan hampas isirung kelapa sawit (HIKS) sebagai sumber karbon. Hampas isirung kelapa sawit (HIKS) merupakan sisa buangan industri pertanian yang diperolehi selepas pengekstrakan minyak daripada buah kelapa sawit. HIKS digunakan secara meluas dalam industri makanan ternakan tetapi penggunaannya agak terbatas kerana berpunca daripada kandungan fiber yang tinggi dan protein yang rendah. Mannan meliputi hampir 60% daripada keseluruhan kandungan fiber pada HIKS. Melalui penguraian mannan dengan menggunakan mikroorganisma dapat menghasilkan HIKS yang lebih sesuai untuk digunakan sebagai makanan ternakan.

HIKS digunakan sebagai substrat dalam fermentasi separa pepejal secara sekelompok oleh pencilan tempatan dan dikaji kebolehannya untuk penghasilan enzim mannanase serta penghasilan glukosamina dan mannosa. Pencilan yang berpotensi dipencilkan daripada pelbagai sumber seperti tanah gambut, batang kelapa sawit yang telah reput serta hampas isirung kelapa sawit itu sendiri. Sumber ini diambil secara rambang di Ladang United Plantation (UP), Jenderata, 36400 Hutan Melintang, Perak, Malaysia.

Pencilan-pencilan ini disaring berdasarkan pada zon penjernihan pada media agar yang bersifat pemilih mengandungi azo-karob-galaktomannan. Tiga puluh enam pencilan dipencilkan dan disaring berdasarkan agar bersifat pemilih. Hanya tiga belas pencilan dipilih berdasarkan kepada penghasilan

zon penjernihan dalam lingkungan 3.42 ± 0.02 hingga 6.44 ± 0.66 mm. Aktiviti-aktiviti mikrob pencilan ini dinilai semula pada media kelalang goncangan yang mengandungi 10 g/L HIKS.

Penghasil enzim yang terbaik ialah pencilan IBRL F16.A4 dengan aktiviti enzim mannanase spesifik 17.82 ± 0.05 U/mg dengan penghasilan glukosamina 9.84 ± 0.11 mg/g dan mannosa 9.54 ± 0.06 mg/g selepas 48 jam pengkulturan. Kulat penghasil enzim pengurai mannan dikenalpasti menggunakan pemerhatian secara mikroskopik. Pencilan IBRL F16.A4 dikenalpasti sebagai *Aspergillus niger* IBRL F16.A4 melalui pemerhatian yang dibuat menggunakan Mikroskop Pengimbas Elektron.

Kulat *Aspergillus niger* IBRL F16.A4 digunakan untuk meningkatkan penghasilan enzim mannanase melalui penambahbaikan media pengkulturan. Fermentasi separa pepejal secara sekelompok dilakukan melalui sistem kelalang goncangan. Dengan menggunakan medium pengkulturan yang ditambahbaik (g/L) : hampas isirung kelapa sawit, 10; $MgSO_4.7H_2O$, 0.5; $CaCl_2.7H_2O$, 1.0; ekstrak yis, 3.0; pepton, 8; NH_4NO_3, 7 dan sebanyak 0.1% (v/v) larutan logam surih pada suhu, 35 ± 2°C dan kadar goncangan, 150 rpm, sebanyak 41.12 ± 0.32 U/mg enzim mannanase spesifik dihasilkan selepas 48 jam pengkulturan. Glucosamina dan mannosa turut dihasilkan iaitu sebanyak 11.87 ± 0.47 mg/g dan 16.06 ± 0.41 mg/g, masing-masing. Walau bagaimanapun, penambahan larutan vitamin tidak menunjukkan sebarang peningkatan dalam penghasilan enzim mannanase.

Pengkulturan di dalam tangki teraduk dilakukan dengan menggunakan keadaan yang ditambahbaik pada suhu, 35 ± 1°C; kadar pengadukan, 150 rpm dan pengudaraan, 1.5 vvm selama 42 jam pengkulturan. Didapati terdapat peningkatan aktiviti enzim mannanase spesifik 48.64 ± 0.12 U/mg serta glucosamina dan mannosa 16.26 ± 0.15 mg/g dan 18.73 ± 0.43 mg/g berbanding dengan menggunakan sistem kelalang goncangan. Ini disebabkan kekangan pengadukan dan pengudaraan pada sistem kelalang goncangan menyebabkan masa pengkulturan yang lebih panjang diperlukan.

Penggunaan pengadukan dan pengudaraan didapati membantu dalam meningkatkan pencampuran fasa cecair-pepejal di dalam sistem fermenter.

Peningkatan kuantiti hampas isirung kelapa sawit sehingga 40 g/L boleh meningkatkan penghasilan enzim mannanase. Aktiviti enzim mannanase spesifik yang maksimum sebanyak 55.13 ± 0.46 U/g pada 40 g/L hampas isirung kelapa sawit yang digunakan. Walau bagaimanapun, peningkatan kepekatan hampas isirung kelapa sawit sebanyak 50 g/L menyebabkan penurunan kadar penghasilan enzim mannanase. Ini disebabkan peningkatan kepekatan medium pengkulturan. Memandangkan satu medium yang murah perlu disediakan, formulasi medium minimum direkabentuk. Namun begitu, medium yang ditambahbaik masih didapati menghasilkan aktiviti enzim mannanase spesifik dan pertumbuhan sel yang maksimum.

MANNANASE PRODUCTION BY *Aspergillus niger* IBRL F16.A4 USING PALM KERNEL CAKE AS SUBSTRATE

ABSTRACT

The aim of present study was to determine the factors influencing the mannanase production by locally isolated strain, *Aspergillus niger* IBRL F16.A4 using palm kernel cake as a carbon source. Palm kernel cake (PKC) is an agro-industrial by-product obtained after extraction of oil palm from oil palm seeds. PKC was used widely in the animal feed industry but has limited used in poultry feed due to its high fiber and low protein contents. Almost 60% of the fiber content is a mannan. By degradation of mannan using microbial its seems suitable to use as poultry feed.

PKC was used as a substrate in semi solid fermentation (SmF) by locally isolated strains and their feasibility for mannanase enzyme, glucosamine and mannose production. The potential isolates were obtained from various sources such as peat soils, rotten oil palm trunks and raw PKC. The sources were randomly obtained from Ladang United Plantation (UP), Jenderata, 36400 Hutan Melintang, Perak, Malaysia.

The isolates were screened based on the clearing zone method on selective agar media containing Azo-carob-galactomannan as substrate. Thirty six isolates have been screened based on selective media. Only, thirteen isolates in which the clear zone ranging 3.42 ± 0.02 to 6.44 ± 0.66 mm were used for further analysis. All the isolates were re-valued in shake flasks system.

The best enzymes producer was isolate IBRL F16.A4 with specific enzyme mannanase acitivity of 17.82 ±0.05 U/mg with production of glucosamine 9.84 ± 0.11 mg/g and mannose 9.54 ± 0.06 mg/g after 48 hr of incubations, respectively. The mannan degrading enzyme producer was identified using

microscopic. Under the microscopic view, isolate IBRL F16.A4 was identified as *Aspergillus niger* IBRL F16.A4.

The fungal, *Aspergillus niger* IBRL F16.A4 was used for improve the mannase production by improvement of medium culture. Semi solid batch fermentation (SmF) experiment was carried out in shake flasks system. Using the improved medium composition of (g/L) : palm kernel cake, 10; $MgSO_4.7H_2O$, 0.5; $CaCl_2.7H_2O$, 1.0; yeast extract, 3.0; peptone, 8; NH_4NO_3, 7 and 0.1% (v/v) of trace metal supplementation at 35 ± 1°C and 150 rpm of agitation speed, the specific enzyme mannanase activity of about 41.12 ± 0.32 U/mg was achieved after 48 hr of cultivation. About 11.87 ± 0.47 mg/g and 16.06 ± 0.41 mg/g of glucosamine and mannose, respectively were obtained. The supplementation of vitamin in the medium did not enhance the enzyme production.

The cultivation in stirred fermenter was carried out using the improved conditions consisting of pH, 5.0; temperature, 35 ± 1°C; agitation speed, 150 rpm and aeration rate, 1.5 vvm for 42 hr of cultivation. The specific enzyme mannanase activity, glucosamine and mannose obtained were about 48.64 ± 0.12 U/mg, 16.26 ± 0.15 mg/g and 18.73 ± 0.43 mg/g, respectively compared with shake flasks system. This is due to an eeration and agitation limitation resulted in a longer cultivation time in the shake flask system. The application of agitation and aeration was observed to enhance the gas-liquid transfer and mixing in the fermenter.

The increase in the amount of palm kernel cake used was observed to enhance the enzyme activity. The maximum specific enzyme mannanase activity was shown to be about 55.13 ± 0.46 U/mg using about 40 g/L of palm kernel cake used. Nevertheless, larger amount of palm kernel cake to about 50 g/L ultimately decreased the mannanase production due to highly viscosity of medium. Due to the

need for cost-effective media, a minimum medium formulation was designed. However, the improved medium was still observed to produce the maximum mannanase activity and cell growth.

CHAPTER ONE

INTRODUCTION

1.1 Scenario of the poultry feed industries in Malaysia

The largest single cost item in the poultry industry is the feed. Feed predominantly composes of plants materials mainly cereals and vegetable proteins. Much of these cannot be fully digested and utilized especially by monogastric animals such as chickens. This is because the cereals have large proportion of their energy content packed up in the form of non-starch polysaccharides (NSP), more commonly known as fiber, which monogastric animals are not able to digest.

Maize has a low amount of soluble NSP and is considered to be an ideal cereal as feed. It is used widely in places where it is easily available and the cost can be justified. However, its availability and cost is not favorable for the poultry production in Malaysia. Although, the poultry industry in Malaysia can be considered as modern, highly competitive and successfully, but the industry totally depends on imported feedstuffs.

The high price of imported feed ingredients such as maize and soybean has resulted in high costs of poultry production. According to report published in the New Straits Time on the 17th January, 2008, prices of animal feed increased up to 15 times in the past two years. Malaysia has to import more than 80% of the total feed ingredients. Imports of raw materials for animal feed amounted to

nearly RM 5.2 billions in 1999, and by the year 2008, maize quantitatively constituted about 78% of the ingredient. By the year 2008, Malaysia required more than 5 million tones of animal feed of which, 3.7 million tones was for non – ruminants and the other 1.3 million tones was for ruminants (Loh, 2002).

As a result the country is less competitive in the poultry world market. The importation of large quantities of grain for stock feeding has resulted in great lost of foreign exchange and this has become a major concern to the poultry industries. Attempts to produce home – grown feed crops, particularly maize and soybean, have not been successful. Under the current situation, the production of feed grain is technically feasible but not an economically attractive venture. For these reasons, other feed resources have to be considered.

Some industrial by-products or even agro-waste on provision that the fibrous wall can be enzymatically degraded, can help to counter the effect of spiraling cost of imported corn and be used as a substitute for corn as animal feed. One of the examples is the palm kernel cake (PKC). Malaysia produces about 1.1 million tones of PKC per year which justifies its utilization feed for poultry, thus able to save the cost of imported feed (Loh, 2002). This PKC can be used to substitute for at least 20% of the imported feed mainly corn.

PKC is a by-product derived from the oil palm fruit. It is a heterogeneous residue from the extraction of oil palm kernel. The residue is either called palm kernel cake (PKC) or palm kernel meal (PKM) depending on the oil extraction process. PKC is a cake residue from expeller (press) extraction and PKM is a loose bran-like particulate material from the solvent extraction process. The residue is always contaminated with small amounts of shell fragments and the testa. Thus, the

composition of PKC does not purely reflect the kernel although it forms a major constituent of the by-product. Typically, PKC contains about 90% of dry matter, 20.4 MJ kg^{-1} gross energy, 16 –19% crude protein and 0.8 – 6.0% ether extract. Fiber content has been analysed by two methods, proximate analysis method or neutral detergent and acid detergent fiber according to Van Soest (1982). The crude fiber content of PKC ranges from 13 to 15.7% while neutral detergent fiber (NDF) and acid detergent fiber (ADF) were about 52% and 31.7%, respectively (Van Soest, 1982; Panigrahi and Powel, 1991). The fiber content of PKC may be influenced by the variety of the oil palm tree, the region in which it grows, and the method used in the processing of the fruits (Panigrahi and Powell, 1991). The ash content are quite consistent at about 4.0%. The high content of phosphorus (0.40 – 0.79%) as compared to calcium (0.25 – 0.29%) a calcium : phosphorus imbalance for large inclusion rates of PKC in animal rations (Yusuf *et al.,* 1989).

From the fiber data, it appears that the by-product is comprised mainly of cell wall. This is the component that is responsible for the low digestibility of PKC by poultry. Yeong (1985) reported that PKC had a ME value of 6.2 MJ kg^{-1}, whereas values of 11.7, 11.1 and 12.55 MJ kg^{-1} were reported by Onwudike (1986), Wong and Zahari (1997) and Lawal *et al.* (2010) respectively. In establishing the potential of PKC for inclusion in broiler chick's diets, Panigrahi and Powell (1991) indicated that the material had approximately 8.35 MJ kg^{-1} ME. The ME value of PKC was considered very low compared to 15.0 and 13.7 MJ kg^{-1} for maize and wheat, respectively (Lawal *et al.,* 2010). In assessing this problem and to gain a better understanding of palm kernel characteristics in relation to its nutritive improvement, overall characterization down to the structural components with emphasis on the cell wall component must be extensively examined.

There were several methods available for cell wall preparation specifically for structural studies (Selvendran *et al.,* 1987; Fry, 1988 and Brett and Waldron, 1990). However, the low solubility of protein in PKC (Onuora and King, 1985) requires further deproteinising of the mineral by phenol acetic acid water (Selvendran *et al.,* 1987).

^{13}C Nuclear Magnetic Resonance (NMR), both solution and solid state, and High Performance Liquid Chromatography (HPLC) were used in characterizing the cell wall of PKC. It was confirmed that the major components of PKC were mannan and cellulose to a lesser extent (Jaafar and Jarvis, 1992). These two polysaccharides represented 69% or 95% of the total cell wall or the total non-starch polysaccharides (NSP), respectively. The results of hydrolysis and HPLC analysis revealed that mannan comprised of 57.8% and cellulose 11.6% of the total cell wall. A small amount of 3.7% of the total cell wall of xylan was detected (Jaafar and Jarvis, 1992).

The mannan of PKC is hard and crystalline, with a degree of low substitution of galactosyl residue compared to the seed of galactomannans. As a result, the mannan is insoluble and resistant to enzymatic degradation. Due to its fibrous nature, its use in monogastric diets is not favorable. To digest the fiber, enzymes produce by microorganims could be added. The removal of the fiber will liberate the starch and protein which masked the cell structure and this can lead to increase in the metabolisable energy and protein utilization by the poultry.

A saccharification process has to be carried out to hydrolyse the fiber (polysaccharide) to simple sugar (mannose) so that the poultry can digest it. Microorganisms grown on the PKC could be isolated and tested for its ability to produce the enzyme mannanase. The mannanase can be extracted and tested on the PKC for use as the poultry feed.

Many researches have been done on the bioconversion of mannan to simple sugar using the copra mannan, guar galactomannan and carob galactomannan (Ademark *et al.,* 1998; Tse and Chinshus 2004; Sumitra *et al.,* 2005; Sabu *et al.,* 2005; Illuyemi *et al.,* 2006). However the research on the PKC mannan is still at its early stage and limited information is available.

The enzyme industry is becoming important due to the rapid development seen primarily over the past four decades in the field of biotechnology. Enzymes found in nature have been used since ancient times in the production of food product, such as cheese, sourdough, beer, wine and vinegar (Nout, 1992; Moo-Young and Chisti, 1994; Jordening, 2004). In Malaysia, enzymes are used in the preparation of traditional food product such as tempe, tapai and soya sauce (kicap). All of these processes relied on either enzyme produced by spontaneously growing microorganisms or enzymes which are added to the processes.

The development of fermentation process during the later part of the century aimed specifically at the production of enzymes using selected microbial strains on a large scale. This development allows enzymes to be one of the industrial products and processes, for instance, the use of enzymes in the poultry industry has become a common practice in improving the nutritive value of diets.

Numerous publications have indicated the importance of enzymes, particularly when certain indigestible and viscous compounds are present in the diets. This condition coupled with advances in microbial technology, has improved our understanding of enzymes and their target substrates. Many studies on carbohydrate related enzymes have focused on its applications in the preparation of cereal (wheat) and legume (soybean) based diets, which are mainly used in many developed

countries. A great success has been achieved in using enzymes such β-glucanase, xylanase and β-mannanase on these feedstuffs (Jackson *et al.,* 2004).

The main nutritional problem of PKC is either physically or physiologically is to mainly have the content of indigestible fiber. Recent findings have shown that problems related to high indigestion of fibrous materials can be overcomed by enzymatic treatment which has been performed with many other feedstuffs (Kim *et al.,* 2003). From the composition of carbohydrates in PKC, it appears that three main enzymes are needed to improve the nutritional quality of palm kernel cake, namely mannanase, celullase and α - galactosidase to digest mannan, cellulose and the α – galactosidic side chains, respectively. The use of these enzymes will help to accelerate hydrolytic processes of isolated mannan – based polysaccharides and cellulose (Balasubramaniam, 1976) and the use of these enzymes may improve the digestion of PKC which is used as feed for poultry. Therefore, research on the production of these enzymes by potential microbes must continue to ensure that it can be applied for the production of economical, local animal feed preparation.

1.2 Research scope

In enzyme production, various research work focus on the use of solid state and submerged fermentation systems. However, in the present work a semi solid fermentation was considered for mannanase production.

Mannanase can be useful in several processes in the food, feed, as well as in the pulp and paper industries. Despite having high practical potentialities, the use of the mannanase is still limited due to the low yield and high production costs. A scale-up strategy must be considered for bulk production with high enzyme yield. Substrate is one of the main factor affecting the production cost.

The use of PKC as a substrate for enzyme production will minimize the cost of production as PKC is available in competitive pricing. Screening of potential microorganisms can be carried-out to identify the high mannanase producers based on quantitative and qualitative analyses. The optimization of the mannanase production was performed using the step-wise method on various physico-chemical parameters in a shake flask system. The effects of carbon, nitrogen, mineral salts, vitamin and trace metals supplementation in shake flask were studied for the optimization of mannanase production. The effects of physical conditions were also studied using on a fermenter system which include the incubation temperature, agitation speed and aeration rate on mannanase production by the potential isolate.

1.3 Research objectives

In order to find the best local fungal mannanase producers and also to enhance the mannanase production by the selected fungal isolate (*Aspergillus niger* IBRL F16.A4) the objectives of this study are :

1. To isolate and identify the local fungal mannanase producers.
2. To improve the medium compositions for the mannanase production in semi solid fermentation system using a shake flask system.
3. To improve the physical conditions for enzyme production in a stirred-tank fermenter system.

CHAPTER TWO

LITERATURE REVIEW

2.1 Palm kernel cake

Palm kernel cake (PKC) is a byproduct from the extraction of oil from oil palm (*Elaeis guineensis*) kernel. It is normally contaminated with small amounts of shell fragments and the testa. Thus, the composition of PKC does not purely reflect upon the kernel although it forms a major constituent of the byproduct.

PKC is valuable in supplying both energy and protein. Its chemical composition and nutritive value were similar to those of wheat bran and rice bran (Mishashige *et al.,* 1987). The PKC would possibly be used as substituted feed for poultry if polysaccharides are enzymically solubilised prior to feeding (Daud and Jarvis, 1991). This is because to digest the polysaccharide which is basically mannan to simple sugar, animals need the enzymes released by bacteria and protozoa which are present in the large intestines of the monogastric animal and the ruminants (Mc Donald *et al.,* 1988).

Yeong (1985) has conducted a trial on laying chickens fed with mixed PKC with maize and found that there were no significant differences in feed intake and body weight gain in birds fed with PKC

from 5 to 20%. However, the feed rations were significantly poorer when PKC levels were above 20% in the diets.

Dae and Morrison (1975) have reported that mannan is present in most member of the Palmae family. Mannan is also present in the endosperm of other palms such coconut (Monro *et al.,* 1985). Research done by Daud and Jarvis (1992), indicated that the endosperm tissues of a number of palm species contained significant quantities of β 1.4 linked D - mannopyranose. The present of mannan in the oil palm kernel is evidenced by Nuclear Magnetic Resonance (NMR) spectroscopy. The alkaline extract is examined directly in solution-contained galactomannans with a low degree of galactose substitution. About 50% of the PKC consisted of mannan. However, cellulose and small quantities of branched galactomannans are associated with the linear mannan and contaminating lignified shells contained xylans (Desterhoft *et al.,* 1991 and 1992).

The β-mannan is an important structural component of cell walls of many terrestrial and aquatic plants. Galactomannans are present in plants as stock polysaccharides (Araki and Kitamikado, 1982; Veerappa and Sirigeri, 2002). Moreira and Filho (2008) distinguished mannan with more than 5% galactose residues as galactomannans. These linear mannan and a small amount of galactomannan component cannot be depolymerised in the digestive tract of the poultry as mannan is hard and insoluble.

If the enzymic depolymerization of the mannan could be achieved, it would release the monomer mannose, with a small amount of galactose, and possibly render the cellulose degradable by

cellulase at the same time. Mannose has been reported to be absorbed and metabolized by birds (Oluwafemi, 2008), although not as readily as glucose.

Palm kernel polysaccharides are apparently hydrolysed in the rumen (Mishashige *et al.,* 1987; Shibata and Osman, 1988) and during germination (Alang *et al.,* 1988), and therefore enzymatic degradation is possible. To hydrolyse the mannan and galactomannan to simple sugars, it needs an enzyme, mannanase, an extracellular enzyme that could be isolated from the valuable microorganisms.

Emi (1972) and Hashimoto and Fukumoto (1996) showd that mannanase has been detected from bacteria, fungi and higher plants. Several β-mannanases have been isolated and purified from *Bacillus subtilis* (Emi, 1972), *Trichoderma reesei* (Tenkanen *et al.,* 1997) and *Tyromces palustris* (Ishihara and Shimizu, 1980). Similarly, *Streptomyces sp.* (Takahashi *et al.,* 1984) and *Trichoderma harziarmum* (Torri *et al.,* 1990) a plant patogen could also produce mannanase.

This mannanase is able to hydrolyse gluco-, galacto- and galactoglumannans to monomers. The mannanase differed in their ability to hydrolyze oligomers and to transglycosylate mannotetraose and mannose, glucose, galactose or mannitol yielding mixed oligosaccharides (Daud and Jarvis, 1992).

2.2 Status of PKC production

Malaysia is the world's largest palm oil producer with oil palm plantation covered an area of 1.28 million hectares, although Indonesia is catching up closely in term of land acreage of oil palm plantation. PKC is a byproduct that obtained from oil palm extraction. As the oil palm production

increases, so as the production of PKC. The annual production of PKC is estimated to be around 1.3 million tons in Malaysia alone in 1998 (Porla Palm Oil Statistics, 1998).

About 90 – 95% of the PKC was exported yearly to European Union (EU) as concentrate for dairy cattle until the mid nineties. However, since the abolishment of subsidies for the cattle and dairy industry, the EU has stopped importing the PKC from Malaysia and has made the byproduct a glut.

An industrial byproduct or agro-waste, like the PKC could be enzymically altered to suit the poultry feed. With these reasons, PKC was chosen to be utilised as poultry diets.

2.2.1 Oil palm and its by-products

The oil palm (*Elaeis guineensis* Jacq) a monocotyledonous plant, which is widely native to West Africa, belongs to the family Palmae, order Palmales and genus *Elaeis*. The name of the genus *Elaeis* derived from Greek word *'elaion'*, meaning oil. The specific name *guineensis* indicates its origin in the Guinea Coast. *Elaeis guineesis* is one of the largest palm species. It has a stem that can reach a height of 25 – 35 m, topped by 35 to 60 pinnate leaves. An oil palm has an economically productive life of 20 – 30 years, and replanting is usually carried out after about 24 years. Almost 6 months after pollination, a fruit bunch weighing 15 to 20 kg and consisting of approximately 1,000 to 1,500 fruit is produced. The shape and the size of the fruit vary considerably. The fruit is about 2.5 – 5 cm in length and 2.5 cm in diameter and weigh about 3 – 30g (Pushkar *et al.,* 2003). The mature fruit is a feed oranged-red drupe containing pulp (mesocarp), shell (endocarp) and kernel (endosperm) as illustrated in Figure 2.1. The fibrous mesocarp is rich in oil and is yellowish-orange in color, due to its high carotene content.

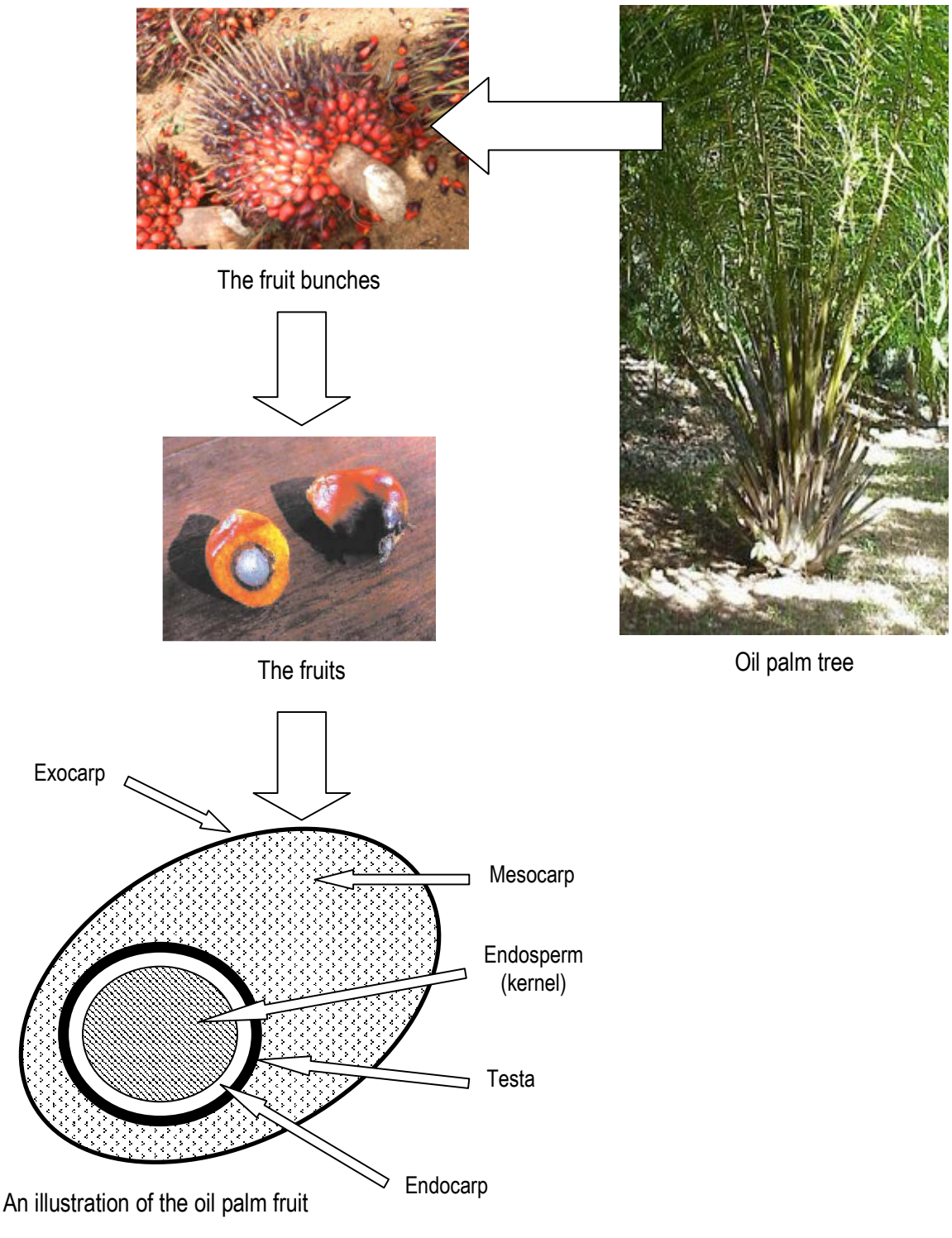

Figure 2.1 : The oil palm tree and its fruits
(http://www.startribune.com/newsgraphics/35269809.html)

Upon harvesting, oil palm fruits which are produced in bunches called fresh fruit bunches (FFB), are transported to a palm oil mill where the fruits are sterilised, stripped off bunches, and crushed to extract the crude palm oil. The crude palm oil collected in a tank contains impurities, which are made up of both oil-soluble and oil-insoluble non-triglycerides. The oil-insoluble impurities such as fruit fibers, free moisture, and nut shells can later be removed through a purification process. In the meantime, the nut shells are further processed to separate the kernels from the shells. At the end of the milling process, crude palm oil and palm kernels are produced (Figure 2.2).

Besides producing palm oil, the by-product consists mainly of shell, palm kernel cake (PKC), palm oil sludge (POS), palm pressed fiber (PPF) and empty fruits bunches (EPF). Fronds from pruning are constantly generated in the plant plantations, and these are mainly used in inter row mulching. An even larger quantity of waste material, in the form of oil palm stems as well as fronds, is produced in the plantations during replanting. Finding appropriate applications for these by-products should become more important economically as well as environmentally. Table 2.1, shows some present uses of oil palm by-products.

Recent trend show that valuable products can be produced from various biofibers of oil palm by-products. Examples of such products are oil palm component plastics composites, oil palm component rubber composites, sheet molding compounds, and pulp and paper. Since the chemical composition of oil palm is similar to that of wood, these wastes could be turned into new raw materials with expanding potential.

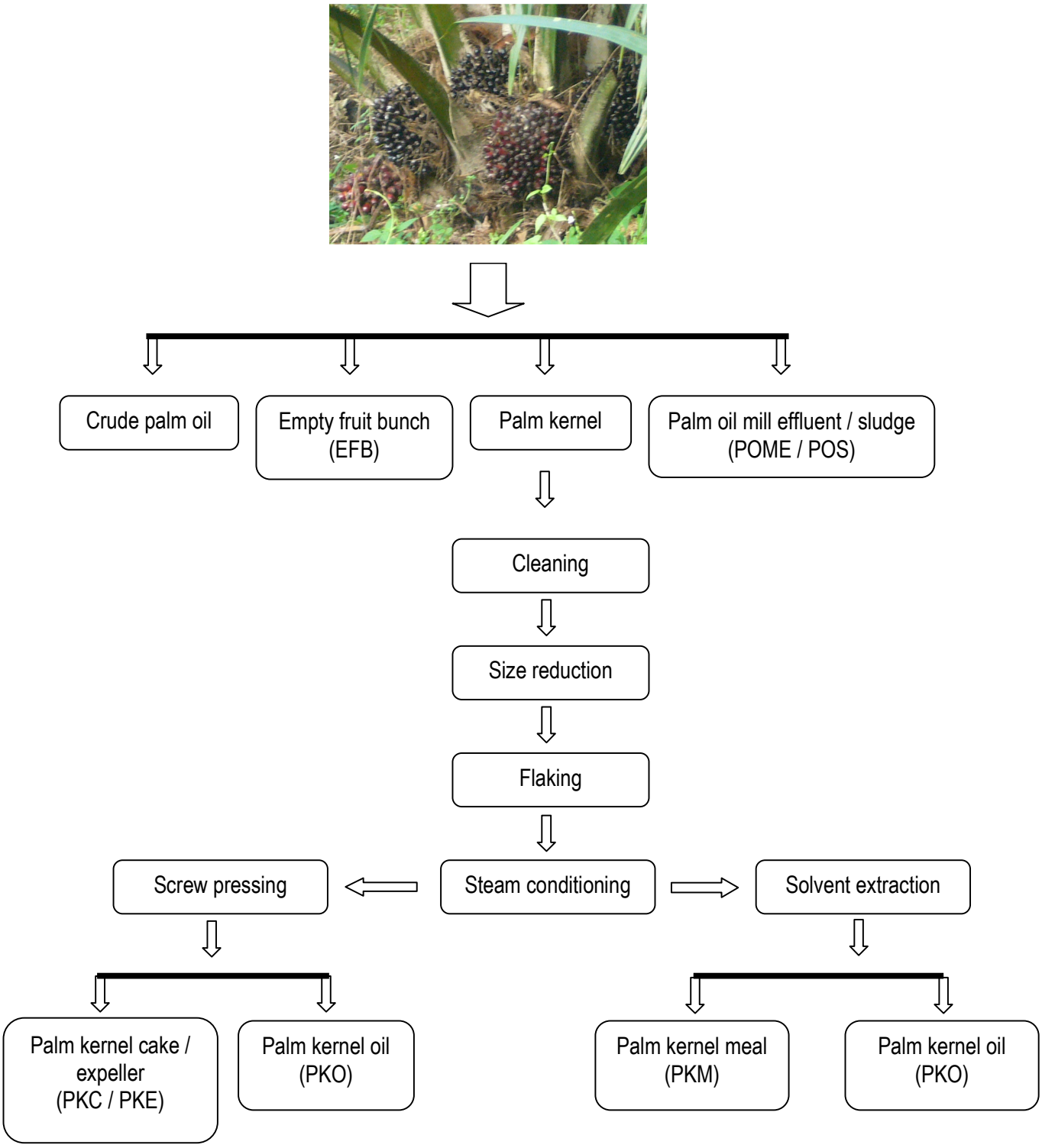

Figure 2.2 : Palm oil processing steps and its by-product (Hishamudin, 2000)

Table 2.1 : Some uses of oil palm by products

BY-PRODUCT	USES
Palm kernel cake (PKC)	• Animal feed (ruminants) (Chin, 2002; Wan and Alimon, 2004; Alimon, 2004) • Broiler feed (Osman and Ramlah, 2006; Soltan, 2009) • Supplement for dairy cattle (Chin, 2001) • Aquaculture feed (Saurin *et al.,* 2008)
Palm kernel shells (PKS)	• Biomass fuel (Mohammad, 2007) • Concrete (Johnson, 2010).
Empty fruit bunches (EFB)	• Boiler fuel (Mohammad, 2007) • Oil palm mulching (Christopher *et al.,* 2010) • Substrate for mushroom cultivation (Ravi Menon *et al.,* 2003; Amal *et al.,* 2008) • Fuel application (Abdullah *et al.,* 2011)
Palm oil sludge (POS)	• Fish feed (Mahmud *et al.,* 2011) • Fertiliser (Parveen *et al.,* 2010) • Biogas (Chotwattanasak and Puetpaiboon, 2011) • Compost (Dayana *et al.,* 2011)
Fronds	• Mulching (Faridah, 2001)

2.2.2 Nutrient composition of palm kernel cake

Palm kernel cake meal is the major byproduct of palm kernel oil extraction. It is a useful source of protein and energy for livestock. However, the composition varies significantly especially in its oil and fiber contents. Such differences in composition are due to the oil palm types that exist in different geographical regions, the extent of oil extraction from palm kernel and also to the methods of processing used in producing the PKC (Onwudike, 1986). Palm kernel cake generally contains 17 – 21% protein, 10 – 17% crude fiber, 4 – 5% ash and ether extract of 0.7 – 9.0% depending on the efficiency of oil extraction from the kernel (Nwokolo *et al.,* 1976; Davendra, 1978; Hutagalung

et al., 1986; Onwudike, 1986). The apparent metabolisable energy (AME) for PKC has been reported to range from 2,008 to 2,999 kcal/kg (Nwokolo *et.al*., 1976; Ngoupayou, 1984; Onwudike, 1986). However, as low as 1,482 kcal AME/kg were reported by Yeong (1985). The protein is of relatively good quality, but its high crude fiber content may affect the availability of amino acids and the digestibility of PKC. Some of the amino acid profile of PKC are listed on Table 2.2.

Table 2.2 : Crude protein and amino acid composition of palm kernel cake (PKC, % dry matter)

	References		
	Onwudike, (1986)	Yeong, (1985)	Nwokolo *et al.,* (1976)
Origin of PKC	Nigeria	Malaysia	Nigeria
Crude protein	19.2	16.06	21.3
Amino acid			
Arginine	2.65 (13.80) [1]	2.18 (13.57)	2.68 (12.58)
Histidine	0.42 (2.19)	0.29 (1.81)	0.41 (1.92)
Isoleucine	0.62 (3.23)	0.62 (3.86)	0.60 (2.82)
Leucine	1.20 (6.25)	1.11 (6.91)	1.23 (5.77)
Lysine	0.68 (3.54)	0.59 (3.67)	0.69 (3.24)
Methionine	0.32 (1.67)	0.30 (1.87)	0.47 (2.21)
Cystine	-	0.20 (1.25)	-
Phenylalanine	0.74 (3.85)	0.73 (4.55)	0.82 (3.85)
Tyrosine	0.53 (2.76)	0.38 (2.37)	0.58 (2.72)
Threonine	0.68(3.54)	0.55 (3.42)	0.66 (3.10)
Valine	0.88 (4.58)	0.93 (5.79)	0.43 (2.02)
Aspartic acid	1.72 (8.96)	1.55 (9.65)	1.69 (7.93)
Glutamic acid	4.01 (20.89)	3.15 (19.61)	3.62 (17.00)
Proline	0.62 (3.23)	0.63 (3.92)	0.50 (2.35)
Serine	0.92 (4.79)	0.69 (4.30)	0.90 (4.23)
Glycine	0.92 (4.79)	0.83 (5.17)	0.91 (4.27)
Tryptophan	-	0.17 (1.06)	-
Alanine	0.76 (3.96)	0.92 (5.73)	0.81(3.80)

[1]*Value in brackets is % of crude protein*

There seem to be some differences in the amino acid content among samples from the different countries. There is good agreement between samples from the same country (Nigeria). However, the concentrations of amino acid in Malaysian PKC seem to be lower than the concentration of amino acid for the Nigerian PKC. In general, PKC is deficient in lysine and sulfur containing amino acid. The data on the digestibility of amino acid in PKC for poultry are very limited. Only a limited number of researchers (Nwokolo *et al.,* 1976; Yeong and Murkherjee, 1983 and Onwudike, 1986) have reported the digestibility of the amino acid from PKC (Table 2.3). The digestibility of AA in PKC averaged 83.8% and 84.5% according to Onwudike (1986) and Nwokolo *et al.,* (1976). However, research conducted in Malaysia (Yeong and Murkherjee, 1983) reported a low value of 64.4%. The reason for the low digestibility of amino acid in PKC from Malaysia is not clear.

Palm kernel cake contains a relatively high amount of minerals, particularly calcium, phosphorus and iron (Nwokolo *et al.,* 1976). However, the availability of most minerals is poor. The availability of calcium, phosphorus, mangnesium, zinc and copper was reported as 68.6, 70.8, 56.4, 45.7, 13.9 and 44.7, respectively (Nwokolo *et al.,* 1976). In another study, Nwokolo and Bragg (1977) found that PKC contained 1.42% phytic acid and half of the total phosphorus was in the form of phosphorus phytate. The study also conducted that phytic acid reduced the availability of calcium, phosphorus, magnesium and zinc, whereas the crude fiber content depressed the availability of all minerals tested. The ratio of calcium to phosphorus in PKC is reported to be more favorable than in other oilseed meals (McDonalds *et al.,* 1988).

Table 2.3: Digestibility of palm kernel cake (PKC) amino acids (%)

References	Onwudike, (1986)	Yeong and Mukherjee, (1983)	Nwokolo *et al.,* (1976)
Origin of PKC	Nigeria	Malaysia	Nigeria
Amino acids (%)			
Arginine	92.7	87.0	93.2
Histidine	88.7	66.8	90.1
Isoleucine	87.5	64.9	86.1
Leucine	90.6	66.7	88.5
Lysine	88.9	58.6	90.0
Methionine	92.1	72.1	91.0
Phenylalanine	91.6	70.4	90.5
Tyrosine	89.9	65.7	85.0
Threonine	85.3	60.7	86.5
Valine	66.7	62.8	68.4
Aspartic acid	85.3	64.4	87.6
Glutamic acid	88.6	74.4	90.1
Proline	64.2	55.0	68.0
Serine	85.4	65.0	88.7
Glycine	52.1	25.8	63.3
Alanine	83.0	67.7	85.5
Overall mean	83.3	64.4	84.5

2.2.3 Non-starch polysaccharides in palm kernel cake

Although starch is the most researched and economically the most important seed storage polysaccharide, it is by no means the only polymeric carbohydrate stored in seeds. There are seeds of many plant species that contain little or no starch, but are nevertheless rich in other forms of polysaccharides reserves. The special groups of non-starch polysaccharides are stored outside the plasmalemma, and were categorized collectively as cell wall storage polysaccharides (Meier

and Reid, 1992). Mannan type cell wall storage polysaccharides are all based on a linear β-1,4-linked chain or 'backbone'. They may be subdivided into the 'pure' mannans, the glucomannans in which some of the D-mannose residues in the backbone are replaced by D-glucose and the galactomannans in which the backbone carries α-1,6-D-galactosyl substituents. According to Meier and Reid (1992), the mannan-group polysaccharides of seeds are major reserve substances only in endosperms, as opposed to storage cotyledons. 'Pure' mannans may be defined to include those polysaccharides that contain less than 10% non-mannose sugar residues. Pure mannans form the major part of the endosperm (kernel) of many palm seeds. They take the form of massive wall thickenings in the endosperm and are clearly the molecular basis of the palm kernel's characteristic hardness (Meier and Reid, 1992).

A researcher group of the Agricultural University of Netherlands have characterized the polysaccharides components of PKC (Desterhoft *et al.,* 1991; Desterhoft *et al.,* 1992). Palm kernel cake was found to contain mannans, cellulose and xylans, with the major part of the mannans originating from the endosperm (kernel) and xylans being almost exclusively located in the endocarp (shell). Palm kernel cake was found to contain negligible amounts of starch (1g/kg) and some of the protein was not digested even after the Pronase treatment, implying that the residue protein was either structurally bound in the cell wall or present as inaccessible cytoplasmic material. There are about 726 g of cell wall materials per kg of PKC containing approximately 7.3% protein, 17.5% lignin, 5% ash and 74.6% non-starch polysaccharides (Desterhoft *et al.,* 1991). Further analysis showed that mannose (from mannan) made up about 57.1% of the cell wall material, which means that PKC contained about 41.45% mannose in this particular study. Further study confirmed that major polysaccharides in PKC are linear mannans with very low galactose

substitution (78% of total non-starch polysaccharides), followed by cellulose (12%), and small amounts of (4-O-methyl)–glucoronoxylans and arabinoxylans (3% each) (Desterhoft *et al.,* 1992). Daud and Jarvis (1992) also showed that the linear β(1,4)-D-mannan was the major component of PKC cell wall non-starch polysaccharides, and that the mannans of PKC appeared to be highly crystalline, in keeping with their insolubility.

2.2.4 Mannan

2.2.4.1 Structure of mannan

Mannan-containing polysaccharides are a major component of the hemicellulose fraction in both hardwoods and softwoods as well as in the endosperm of many leguminous seeds and some mature seeds of non-leguminous plants. Hemicelluloses are usually associated with the cellulose and lignin in plant cell wall (Aroujo and Ward, 1990; Perez *et al.,* 2002)

The β-D–mannans are linear extended ribbon like molecules consisting of (1 – 4) – link β-D mannopyranosyl residue (Painter, 1983). Mannan molecules differ, both within and between plants. For instance, mannan A (alkali-soluble) from ivory nut has a DP of about 20, whereas mannan B (alkali-insoluble) has a DP of 80 (Meier and Reid, 1992). In nature, pure mannan is very rare.

In ivory nut walls, the mannan occurs as both an alkali-insoluble fibrillar component (mannan II) and alkali-soluble granular encrusting component (mannan I) (Chanzy *et al.,* 1982, 1984, 1987). Like cellulose, the mannan displays crystalline polymorphism and can undergo mannan I – mannan II transformation which depends on chain length (Chanzy *et al.,* 1984). The mannan chain conformation closely resembles that of cellulose, although in contrast to cellulose, it does not have

a precise 2 – fold screw axis. It allows intramolecular hydrogen bonding (Nieduszynski and Marchessault, 1972).

The polymers are almost always branched with galactose chains of various length at an α (1, 6) bond (Brett and Waldron, 1990). Aspinall (1959) distinguished mannans with more than 5% galactose residues as galactomannans. McCleary and Matheson (1986) deduced that, in an aqueous solution of the polymer, α - D – galactosyl stubs, when separated by no, or an even number of D – mannosyl residues, lie on opposite sides of the main chain, and that those separated by an odd number of D – mannosyl residue lie on the same side of the chain as illustrated in Figure 2.3. Galactopyranosyl substitution of the mannan chain results in enhanced solubility. The solubility properties depend on the mannose : galactose ratios within the range 1.0 – 5.25. Solution properties also depend on the distribution of the galactopyranosy substituents on the mannan backbone which is irregular to random (McCleary and Matheson, 1986).

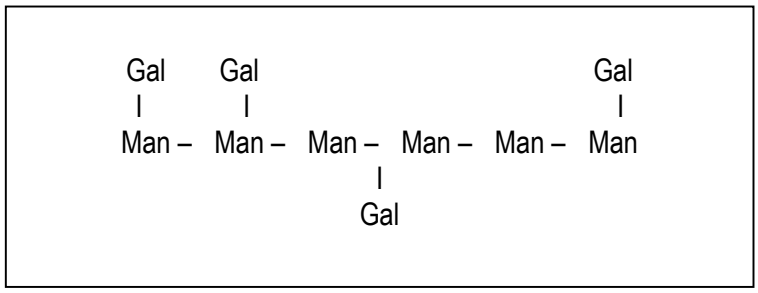

Figure 2.3: Structure of mannan with galactose branching

i. Hydrolysis of Mannan

Mannan is the main component in PKC, therefore mannan is to be degraded or converted into a mannose for effective digestion of monogastric tract. Hydrolysis of mannan required an extracellular enzyme. The β-mannanase enzyme is use to hydrolysed the β - 1,4 – mannanase

lingkages in the polymer chain of mannan to produce mannose (Civas *et al.,* 1984; Standbrand *et al.,* 1993)

ii. Significant of β-mannanase enzyme

β-mannanase enzyme has found several industrial applications. They were employed for preparation of mannooligasaccharides used as non – nutritional food additives for selective growth of human beneficial intestinal microflora (bifido – bacteria and lactobacilli) (Akino *et al.,* 1987; Mia *et al.,* 1998). The positive effect of β-mannanase in liquefaction and extraction of fruit β - mannanase is well known (Hashimoto and Fukumoto, 1996). Decrease of viscosity of storage galactomannan is the purpose of their application in coffee bean extraction (Roswitha *et al.,* 2001). The coffee preparations obtained in conjunction with β - mannanase show better volatile aroma, taste properties and visual appearance of the final drink (Hashimoto and Fukumoto, 1996).

The main future industrial application of mannanases may be seen in pulp and paper industry. Extraction of lignin can be improved by pretreatment of the pulp with β-mannanase alone (Gubitz *et al.,* 1996; Suurnakki *et al.,* 2003) or in combination with cellulase–free xylanase (Buchert *et al.,* 1993; Gubitz *et al.,* 1996). This lead to important savings on bleaching chemicals and to a reduction of the amount of ecologically hazardous wastes. Since the bleaching process is performed at higher temperature and under alkaline conditions, there is a strong demand for β-mannanase with properties meeting the conditions of industrial applications. However, the application of β-mannanase in this work is in the case enhancement of the digestibility of palm kernel cake for poultry feed.

2.3 Previous studies of PKC

The utilisation of PKC in poultry diets was first reported by Temperton and Dudley (1940). They conducted a trial on laying chickens and found that PKC was a satisfactory substitute for wheat middling in layer diets. The use of PKC in broiler rations was continued by several researchers (Nwokole and Bragg, 1977; Armas and Chicco, 1977). Hence, the possible utilization of PKC a protein and energy source in monogastric animals was reported (Babatunde *et al.,* 1975; Nwokolo *et al.,* 1976; Armas and Chicco, 1977; Yeong, 1980; Yeong *et al.,* 1981).

However, in many years of research, it is reported that chickens fed with more than 20% PKC in ration depressed the growth (Armas and Chicco, 1977; Yeong *et al.,* 1981). Yeong (1980) reported that ME values were 1484 kcal/kg for 20% substitution and 1146 kcal/kg for 40%. This is due to the presence of a large amount of mannan in PKC and the high fibrous nature of PKC causes the low digestibility of PKC by monogastric animals such as poultry (Yeong *et al.,* 1981). Increased level of PKC in the poultry diets resulted in the increased level of dietary crude fiber which exerts its effect on the carcass composition by inhibition on fat deposition (Fetuga *et al.,* 1977).

Therefore, it is reported that a pre-treatment is necessary on PKC before PKC is use as poultry feed (Daud and Jarvis, 1991). The PKC will undergo a saccharification process by using enzyme to saccharify polysaccharides into digestible sugar that will finally absorb by animals for metabolic action. Daud (1996) reported the use of enzyme supplement on the feed and indicates that enzyme supplementation improves the nutritive value of PKC. It also indicates that more specific is the enzyme, resulted in better improvement. A beneficial effect on the health of chickens has been reported (Oyofo *et al.,* 1989; Deloach *et al.,* 1990) and attributed to the inhibition of the binding of *Salmonella spp.* to the gut wall.

2.3.1 Influence of fiber on digestion

Large insoluble molecules in the feed must be broken down to simpler molecules before they can pass through the mucous membrane of the alimentary canal into the blood and lymph. For instance, polysaccharides are broken down into simple sugar, protein into amino acids, and lipids into free fatty acids. The breakdown process is termed 'digestion'. In this respect, the diet of farm animals that normally consists of plant and plant products is seldom completely digested.

The digestibility of a feed is closely related to its chemical composition. The fiber fraction of a feed has a great influence on its digestibility, and both the amount and the chemical composition of the fiber are important (McDonald, 1988).

It was found to be quite effective in distinguishing fractions of cell wall and cell contents. For forages of graminaceous origin Haris (1970) has illustrated the concept with respect to the ruminants as in Table 2.4.

Table 2.4: A realistic system for portioning nutrients of foods and feeds in PKC

CELL WALLS			CELL CONTENT
Non – nutritive matter	Partially nutritive matter		Nutritive matter
Lignin and Acid Insoluble ash	Cellulose	Hemi - cellulose	Soluble carbohydrate, protein, ether extract, soluble ash

Plant materials are divided into cell walls and cell contents. The cell walls include partially nutritive matter and non-nutritive matter. The partially nutritive matter in grasses and cereals consists of cell wall carbohydrates, cellulose and hemicellulose, and is digested only by enzymes produced by microorganisms within the digestive tract. The non-nutritive matter includes lignin and acid insoluble ash that is mainly comprised of silica. These constituents have no known nutritive value for animals. For monogastric animals the whole cell walls are not digestible. Thus, it is clear that digestibility if any feedstuff is influenced by the nature of its cell wall structure.

The cell contents include the nutritive matter that is digested by enzymes secreted by the digestive system, or is otherwise soluble enough for absorption. This includes soluble carbohydrate, protein, lipids (ether extract) and soluble ash.

Van Soest (1982) outlined an alternative approach to crude fiber by using residues from extraction with acid or neutral detergent solution for fiber analysis. Neutral detergent leaves 'fiber' that often corresponds fairly closely to the total cell wall content of the material analysed; it gives low values for this in dicot crop materials, when the walls are richer in pectic substances. 'Acid detergent fiber; is similar in composition to crude fiber, but retains all the lignin, part of the pectic substances and hemicelluloses. As such this method is suitable for a rapid method for forage analysis (Van Soest, 1982).

PKC contains 52% neutral detergent fiber and 31.7% acid detergent fiber with only 15% in crude fiber. The composition shows as wide differenced suggesting that chemical methods of fiber determination do not work with PKC. Dae and Morrison (1975) reported that as 'seeds of most members of the Palmae are known to contain mannans', it suggests that mannan is present in oil