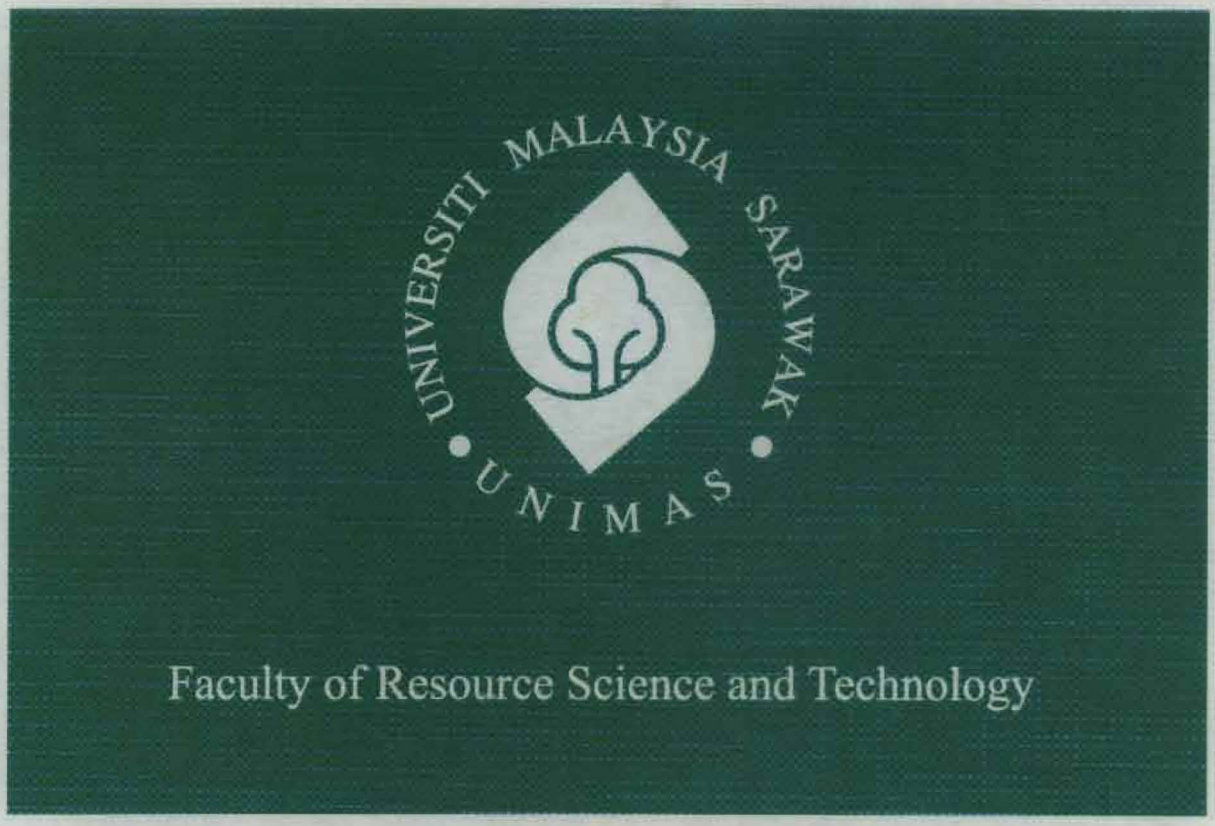

PRODUCTION OF BACTERIAL PECTINASE USING BANANA PEELS AGRICULTURAL WASTE AS SUBSTRATE

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PRODUCTION OF BACTERIAL PECTINASE USING AGRICULTURAL WASTE AS SUBSTRATE

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This project is submitted in partial fulfilment of the requirements for the degree of Bachelor of Science with Honours (Resource Biotechnology)

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Statement of Originality

The work described in this Final Year Project, entitled

"Production of Bacterial Pectinase using Agricultural Waste as Substrate"

is to best of the author's knowledge that of the author except where due reference is made

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% w/w	weight percent
%	percent
°C	degree Celcius
µl	microliter
µmol	micromol
16S rDNA	16S ribosomal DNA
1kb DNA ladder	1 kilobase DNA ladder
ANOVA	Analysis of Variance
BLAST	Basic Local Alignment Search Tool
bp	basepair
C	carbon
$CaCl_2$	calcium chloride
cm	centimeter
CTAB	cetyl trimethyl ammonium bromide
dH_2O	distilled water
DNSA	dinitrosalicylic acid reagents
dNTPs	deoxynucleotide triphosphates
EDTA	ethylene diamine tetracetic acid
EtBr	ethidium bromide
g	gram
g/L	gram per liter
H_2O_2	hydrogen peroxide
M	molar
$MgCl_2$	magnesium chloride
min	minute
ml	milliliter
mm	millimeter
mM	millimolar
MR-VP	Methyl red-Voges Proskauer
NaCl	sodium chloride
NaOH	sodium hydroxide

nm	nanometer
O_2	oxygen
PCR	polymerase chain reaction
PGA	polygalacturonic acid
PPB 1	Pectinase-producing bacteria 1
PPB 2	Pectinase-producing bacteria 2
rpm	revolutions per minute
SDS	sodium dodecyl sulfate
SIM agar	Sulfate Indole Motility agar
SmF	submerged fermentation
SSF	solid state fermentation
TAE buffer	tris acetate EDTA buffer
TE buffer	tris-EDTA buffer
U	unit of enzyme
UV	ultraviolet
V	volt
w/v	weight to volume
YEP medium	yeast extract pectin medium
α	alpha

Production of Bacterial Pectinase using Banana Peels Agricultural Waste as Substrate

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ABSTRACT

Bacterial pectinases have emerged as important industrial enzymes with wide-ranging applications recently. In this study, two bacterial strains obtained from orange peels indicated their ability to produce pectinase from qualitative and quantitative screening using iodine-potassium iodide and DNS assay method, respectively. Morphological, biochemical and molecular characteristics using 16S rDNA analysis were done to identify the both strains and their identities are confirmed to be *Bacillus subtilis* and *Bacillus* sp. cp-h24. Production of pectinase from both bacteria strains using banana peels agricultural waste as substrate under submerged fermentation has been studied. Higher polygalacturonase activity produced by *Bacillus subtilis* (13.03 U/ml) and *Bacillus* sp. cp-h24 (21.18 U/ml) confirmed that banana peels can be a better replacement to commercial pectin (9.785 U/ml and 14.14 U/ml, respectively). Optimization studies of the growth medium showed that *Bacillus subtilis* produced higher polygalacturonase activity with 10% w/v banana peels (109.8 U/ml), at 24 hours fermentation (52.61 U/ml), pH 10 (33.55 U/ml) and temperature 37°C (53.38 U/ml) while *Bacillus* sp. cp-h24 produced higher polygalacturonase activity with 5% w/v banana peels (91.28 U/ml), at 24 hours fermentation (52.33 U/ml), pH 7 (38.27 U/ml) and temperature 37°C (49.66 U/ml). The isolation of pectinase-producing bacteria from agricultural waste and also the use of agricultural waste to produce maximum pectinase suggest strategic ways to reduce the production cost in enzymes commercialization.

Key words: Pectinase(s), *Bacillus*, production, banana peels agricultural waste

ABSTRAK

Baru-baru ini, pektinase yang diperoleh daripada bakteria telah muncul sebagai enzim yang penting dalam industri dengan mempunyai pelbagai aplikasi. Dalam kajian ini, dua jenis bakteria yang didapati daripada kulit oren telah menunjukkan keupayaan dalam menghasilkan pektinase melalui pemilihan secara kualitatif menggunakan larutan kalium iodida dan melalui pemilihan secara kuantitatif dengan menggunakan kaedah ujian DNS. Pencirian melalui kriteria morfologi, biokimia dan molekular melalui analisis 16S rDNA telah dilakukan untuk mengenal pasti kedua-dua jenis bakteria dan identiti mereka telah dikenal pasti sebagai Bacillus subtilis dan Bacillus sp. cp-h24. Penghasilan pektinase daripada kedua-dua bakteria menggunakan kulit pisang sebagai substrat semasa fermentasi perendaman telah dikaji. Aktiviti poligalakturonase yang lebih tinggi telah dihasilkan oleh Bacillus subtilis (13.03 U/ml) dan Bacillus sp. cp-h24 (21.18 U/ml)) telah membuktikan kulit pisang boleh menjadi pengganti pektin komersial (9.785U/ml dan 14.14 U/ml) yang lebih baik. Kajian pengoptimuman media pertumbuhan bakteria telah menunjukkan Bacillus subtilis menghasilkan aktiviti poligalakturonase lebih tinggi dengan 10% w/v kulit pisang (109.8 U/ml), pada 24 jam fermentasi (52.61 U/ml), pH 10 (33.55 U/ml) dan suhu 37°C (53.38 U/ml) manakala Bacillus sp. cp-h24 menghasilkan aktiviti poligalakturonase lebih tinggi dengan 5% w/v kulit pisang (91.28 U/ml), pada 24 jam fermentasi (52.33 U/ml), pH 7(38.27 U/ml) dan suhu 37°C (49.66 U/ml). Pengasingan bakteria yang menghasilkan pektinase daripada bahan buangan dan juga penggunaan bahan buangan untuk memaksimumkan penghasilan pektinase merupakan antara cara yang strategik untuk mengurangkan kos penghasilan dalam pengkomersialan enzim.

Kata kunci: Pektinase, Bacillus, penghasilan, kulit pisang

1.0 INTRODUCTION

The biotechnological potential of pectinases produced from bacteria has drawn a great deal of attention for use as biocatalyst in a variety of industrial processes recently. Their all-embracing applications in degumming and retting of plant fibers, pretreatment of pectic wastewater from fruit and vegetable processing units (Tewari *et al.,* 2005), degrading the skin of black pepper to white pepper (Gopinathan and Manilal, 2005), coffee and tea leaf fermentation, vegetable oil extraction and virus purification (Yadav *et al.,* 2008) have make these enzymes become the upcoming enzymes of the commercial sector. Besides, pectinases also have been used in paper and pulp industry, poultry feed additives, alcoholic beverages and food industries, wood preservation, plant pathology, and in protoplast fusion technology (Gummadi and Panda, 2003).

Considering the great significance with tremendous potential to offer to industry, one can conclude that the strategic ways to produce maximum pectinase with low cost production should be studied. This is because the major impediments to the exploitation of commercial enzymes are their yield, stability, specificity, and the cost of production. Application of agricultural waste as carbon sources in enzyme production processes certainly reduces the cost of production and at the same time helps in preventing environmental pollution (Silva *et al.,* 2002). Besides, agricultural wastes especially those which contain residues of pectin are cheap pectinase-producing bacteria providers too. The isolation of pectinase-producing bacteria from agricultural waste, thus would be one of the another strategic way to minimize the cost of production.

Therefore, the objectives of this study are:

1. To screen and isolate pectinase-producing bacteria from pectin residues-containing agricultural waste.
2. To identify and characterize the isolated pectinase-producing bacteria according to morphological, biochemical and molecular characteristics.
3. To produce pectinase from the bacteria isolated using banana peels agricultural waste as substrate under submerged fermentation.
4. To optimize the growth medium of the bacteria isolated for maximum pectinase production.

2.0 LITERATURE REVIEW

2.1 Banana Peels

Banana is the general term embracing a number of species or hybrids in the genus *Musa* of the family Musaceae. In Malaysia, bananas are the second largest cultivated fruit crop after durian and they are available throughout the year. Generally, they are used as a source of food in either raw or in processed form.

Bananas are rich in potassium, riboflavin, niacin, and dietary fiber. They also contain vitamins A and C, B6, some calcium, iron, and magnesium. Because of their sweetness, they have a high energy value and delicious taste, making them being chosen as one of preferred source of food for human (Bananas, n.d). This condition actually will lead to the increased of banana peels and this definitely will contribute to environmental pollution. One method to solve this problem is to utilize them for the conversion into useful byproduct. One of valuable byproduct that can be obtained from banana peels is pectin (Madhav and Pushpalata, 2002).

It has been reported that banana peels contained 0.62% w/w of pectin (Walter, 1991). In other reports, pectin content in banana peels is 0.7-1.2% w/w (Jayani *et al.,* 2005). Even though the amount of pectin in banana peels is not very high compared to pectin from other fruit wastes such as apple and orange, this amount however is among the highest pectin content in fruit waste of other local fruit such as papaya, mango and pineapple (Walter, 1991). This makes pectin of banana peels are preferable to be utilized as a source of substrate for pectinases. Stage of banana ripeness also can be contributed to the pectin

content in banana peels. According to Emaga *et al.* (2008), stage 5 of banana ripeness where the colour of banana fruit is more yellow than green has given the greatest yield of pectin.

2.2 Pectin

Pectin and other pectic substances such as protopectin, pectic acid and pectinic acid are a group of complex plant polysaccharide that exists mostly in middle lamella and primary cell wall of plants (Tewari *et al.,* 2005). Hence, they form important natural substrates for pectinases. They constitute about one third of the cell-wall dry substance of dicotyledonous and some monocotyledonous plants (Walter, 1991). These substances are believed to contribute both to the adhesion between cells and to the mechanical strength of the cell, behaving in the manner of stabilized gels. They also involved in the interaction between plant hosts and their pathogens (Alkorta *et al.,* 1998).

The principal constituent present in pectin polysaccharides is D-galacturonic acid units which act as a backbone (**Figure 2.1**). They joined in chains by means of α-(1→4) glycosidic linkages. The carboxyl groups of galacturonic acid are partially esterified by methyl groups and partially or completely neutralized by sodium, potassium or ammonium ions. Some of the hydroxyl group on C_2 and C_3 may be acetylated. 2-4% of rhamnose units are inserted into the main uronide chain and they are joined to the reducing end of the uronide by (1→2) linkages and the nonreducing end of the next uronide unit by (1→4) bonds (Jayani *et al.,* 2005). Often, arabinan, galactan, or arabinogalactan side chains are linked (1→4) to the rhamnose. In the side chains, the arabinose units have (1→5) linkages while galactoses are mutually joined mainly by (1→4) linkages, but (1→3) and (1→6)

linkages also occur. Other sugars, such as D-glucuronic acid, L-fucose, D-glucose, D-mannose, and D-xylose are also sometimes found in side chains (Walter, 1991).

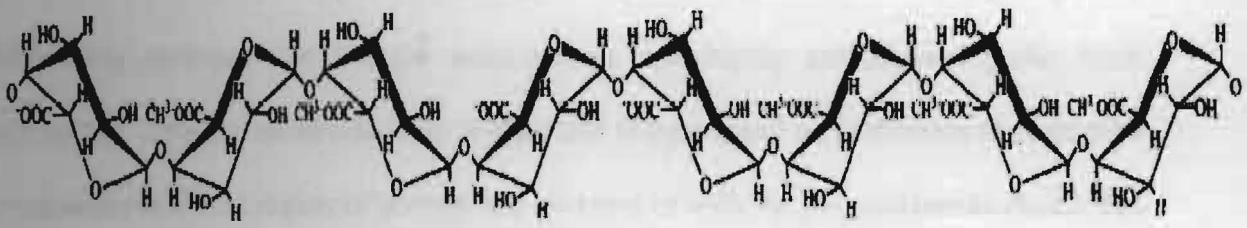

Figure 2.1 Structure of the pectin molecule. Only one chain of the major component of pectins-galacturonic acid partially methylesterified is represented here. Side chains of galactose, arabinose, xylose and other sugar residues are not included in the figure. Taken from Alkorta *et al.,* (1999).

2.3 Pectinases

Pectinases are a group of heterogenous enzyme that hydrolyzes the pectin and other pectic substances. The classification of pectinases is based on their attack on the galacturonan backbone of the pectic substance molecule. Thus, pectinases are broadly classified into three types. They are de-esterifying enzymes (pectinesterases), depolymerizing enzymes (hydrolases and lyases) and protopectinases (Alkorta *et al.,* 1998).

Pectinesterases catalyze de-esterification of the methoxyl group of pectin which then forming the pectic acid. On the other hand, the depolymerases split the α (1→4) glycosidic bonds between galacturonic monomers in pectic substances either by hydrolysis (hydrolases) or by β-elimination (lyases). They have been subdivided into groups by criteria of whether their preference for pectic acid or pectin as substrate and whether breakdown starts from within the polymer or from the end of polymer. Enzymes belonged to hydrolases group are endopolygalacturonase (polygalacturonase), exopolygalacturonase (polygalacturonase), endopolymethylgalacturonase (pectin hydrolase) and

exopolymethylgalacturonase (pectin hydrolase) whereas lyases are endopolygalacturonate lyase (pectate lyase), exopolygalacturonate lyase (pectate lyase), endopolymethylgalacturonate lyase (pectin lyase) and exopolymethylgalacturonate lyase (pectin lyase). Another type of pectinase is protopectinases which is protopectin-solubilizing enzymes that liberate water-soluble and highly polymerized pectin from protopectin. They are subdivided into A-type and B-type based on preference to react with polygalacturonic acid region of protopectin (A-type) or with the polysaccharide chains that connect polygalacturonic acid chain and cell wall constituents (B-type) (Alkorta *et al.*, 1999).

2.4 Pectinase-producing Bacteria

Pectinases can be produced by a large number of microorganisms such as bacteria (Kapoor *et al.*, 2000) and fungi (Yadav *et al.*, 2008). Apart from that, some animals (Shen *et al.*, 1996) and plants (Payasi *et al.*, 2006) are have also been reported to produce this group of enzyme. Generally, earlier researches focused on fungi such as *Aspergillus*, *Sclerotium*, *Penicillium* and *Rhizopus* on their ability to produce pectinases especially *Aspergillus niger* because this strain posses GRAS (Generally Regarded As Safe) status, so that metabolites produced by this strain can be safely used (Gummadi and Panda, 2003). In addition, pectinases from fungi are also widely used because they have acidic pH and this is very close to the pH of many fruit juices prepared in the juicemaking industry. However, such pH is not suitable for other preparations in which pH values are close to neutral and alkali. Furthermore, fungal enzyme has relatively low temperature stability (Soares *et al.*, 1999). Therefore, recently researchers are more interested to focus on pectinase from bacteria as a lot of pectinase-producing bacteria have been successfully isolated included *Pseudomonas*,

Bacillus, Erwinia and *Clostridium*. In contrast to fungi, bacteria like *Bacillus licheniformis* are predominately produced alkaline pectinases and were thermostable because of their ability to retain 100% activity after 2 hours of incubation at 65°C used to have been reported (Singh *et al.,* 1999). Besides, a novel, alkaline and thermostable polygalacturonase has been produced from an environmental isolate *Bacillus* sp. MG-cp-2 in which that polygalacturonase is not only active, stable under highly alkaline conditions and has broad range of pH (7.0-12.0), but also exhibits a broad range of thermostability at temperatures up to 80°C, also tolerance to metal ions and surface-active agents (Kapoor *et al.,* 2000).

Sunnotel and Nigam (2002) have successfully examined the production of different pectinolytic activities in five alkalophilic soil bacteria under submerged fermentation by using pectin as substrate. The result has shown that maximal activity of alkaline pectin lyase and polygalacturonase have been produced by all the five bacteria. In addition, both activities were higher than most other microorganisms reported in literature. Kashyap *et al.* (2000) also has reported that *Bacillus* sp. DT7 has produced higher yield of pectinase compared to most other microbes such as *Aspergillus* and *Penicillium*. Better yield along with the advantages of its alkalophilic and thermotolerant properties have indicated that this organism has the potential to be used at commercial level for degumming of natural fibers, in pretreatment of waste water from fruit-juice processing industry and also many other industrial applications. Soares *et al.* (1999) also reported that activities of polygalacturonase produced by *Bacillus* sp. strains were higher than those produced by *Aspergillus niger* and *Tubercularia vulgaris*. The other bacteria that has the same properties and has been successfully isolated is *Bacillus* sp. KSM-P443 (Koboyashi *et al.,* 1999)

In a lot more other reports, bacteria have been admitted and justified as one of the great producer of pectinases. For example, *Bacillus subtilis* IFO 12113 (Sakai and Ozaki, 1988) and *Bacillus subtilis* IFO 3134 (Sakai and Sakamoto, 1990) have been reported to produce B-type protopectinase. On the other hands, pectate lyase have been reported to be produced by a wide range of bacteria such as *Bacteroides thetaiotaomicron* (McCarthy *et al.,* 1985), *Erwinia carotovora* (Kotoujansky, 1987), *Amycolata* sp. (Bruhlmann, 1995) *Pseudomonas syringae* pv. *Glycinea* (Margo *et al.,* 1994), and E*rwinia chrysanthemi* (Favey *et al.,* 1992). Pectinesterase also have been reported to be produced by bacteria such as *Erwinia chrysanthemi* B341 (Pitkanen *et al.,* 1992), *Lachnospira pectinoschiza* (Cornick *et al.,* 1994), *Pseudomonas solanacearum* (Schell *et al.,* 1994) and *Lactobacillus lactis* subsp. *Cremoris* (Karam and Belarbi, 1995).

2.5 Applications of Bacterial Pectinase

Bacterial pectinases have great importance in industrial processes such as degumming and retting of natural fibers (Kashyap *et al.,* 2000). The application of pectinase in these processes not only reduces the overall cost, but also helps to conserve energy and preserve the environment (Kapoor *et al.,* 2000) as the chemical retting and degumming treatment is polluting, toxic and non-biodegradable. Retting and degumming process using pectinases presents an environmental friendly and economic alternative to the problem (Jayani *et al.,* 2005).

In retting process, pectinases have been used in retting of flax to separate the fibers and eliminate the pectins since pectinases are believed to play a lead role in fiber processing, with 40% of the dryweight of plant cambium being pectin (Kapoor *et al.,* 2000). Yadav *et al.* (2008) have been reported that pectin lyases produced from *Aspergillus flavus* have the

ability in retting the *Crotolaria juncea* sticks or known as sunn hemp only in 24 hours. Interestingly, the retting process is done with the absence of EDTA, a chemical which usually use to enhance the retting efficiency.

In degumming process, pectinases are usually used in degumming of plant bast fibers (Jayani *et al.,* 2005). The fibers contain gummy materials consisting mainly of pectin and hemicelluloses which have to be removed before being use in textile making (Kapoor *et al.,* 2001). A study by Kapoor *et al.* (2001) has shown that alkaline and thermostable polygalacturonase produced from *Bacillus* MG-cp-2 has the ability in degumming of *Boehmeria nivea* (ramie) and *Crotolaria juncea* (sunn hemp) bast fibers by 37% and 56% respectively only in 11 hours with the help of a few chemicals. The process of degumming is enhanced by its stability in high temperature and pH, which is the pre-requisite for an efficient degumming process. Besides, culture supernatants contained pectate lyase produced from *Amycolata* sp. has also been reported to effectively reduce the gum content of ramie fibers by 30% only in 15 hours (Bruhlmann *et al.,* 2000).

One other appreciative application of bacterial pectinase that also should be given more attention is the potential of pectinase in reducing the time needed to degrade the skin of black pepper and green pepper to white pepper. Considering that white pepper is more preferred than black or green pepper in some country such as Europe, Japan and America because of its charming colour, suitability to use in all food preparations, more starch-containing, less microbial load and contaminant-free (Gopinathan and Manilal, 2005), it best to conclude that white pepper has a very high commercial value. Thus, the great ability of pectinase regarding this matter should not be ignored.

Pectinases also has the potential to be applied in more other applications such as in waste water treatment and in coffee and tea fermentation. Vegetable food processing industries release pectin, containing waste waters as by-products. Pretreatment of these wastewaters with pectinolytic enzymes facilitates removal of pectinaceous material and renders it suitable for decomposition by activated sludge treatment (Hoondal *et al.,* 2000). In coffee and tea fermentation on the other hands, pectinase treatment accelerates tea fermentation and also destroys the foam forming property of instant tea powders by destroying pectins (Carr, 1985). They are also used in coffee fermentation to remove mucilaginous coat from coffee beans.

Pectinases also are believed can help in improvement of chromaticity and stability of red wines in alcoholic beverages industry. Pectinases added to macerated fruits before the addition of wine yeast in the process of producing red wine resulted in improved visual characteristic (colour and turbidity) as compared to the untreated wines. Enzymatically treated red wines presented chromatic characteristics, which are considered better than the control wines. These wines also showed greater stability as compared to the control (Revilla and Ganzalez-san jose, 2003). In paper making industry, pectinase can depolymerize pectins and subsequently lower the cationic demand of pectin solutions and the filtrate from peroxide bleaching (Viikari *et al.,* 2001).

2.6 Production of Pectinase using Agricultural Waste

Evidence showed that pectinases are inducible and they can be produced with the presence of different carbon sources (Aguilar and Huitron, 1987; Maldonado *et al.,* 1989; Friedrich *et al.,* 1994; Nair *et al.,* 1995; Nair and Panda, 1997). In the course of time, numerous

reports have appeared on the different strategies for maximizing the production of pectinases with the reduction of production cost. Soares *et al.,* (1999) reported on production of polygalacturonase from *Bacillus* sp. under solid state fermentation (SSF) and submerged fermentation (SmF) by using wheat bran as the substrate and the results shown that the wheat bran can be used as a good substrate replacing the commercial pectin. Kashyap *et al.,* (2002) also has enhanced the production of pectinase by *Bacillus* sp. DT7 by using a few types of natural solid substrates such as wheat bran, rice bran and apple pomace under SSF. His report also shows the same results with Soares *et al.,* (1999) and the wheat bran has been the prime producer among all the substrates that have been used.

Furthermore, Kapoor *et al.,* (2000) also has revealed that complex polysaccharides such as agricultural wastes and agro-industrial by-products are the better stimulant for the polygalacturonase production from *Bacillus* sp. MG cp-2 which has the properties of thermo-alkali stable. The use of wheat bran, sunflower seed cake, rice bran, orange peel and guar gum was enhanced the polygalacturonase production to a significant extent when substituted individually in place of commercial citrus pectin. On the other hands, Bayoumi *et al.,* (2008) used potato peels for cost-effective production of the pectinase. *Solanum tuberosum* peels and *Solanum melanogena* peels have been used as the substrates to produce pectinase from *Bacillus firmus* under solid state fermentation and those cheap substrates have shown to be the great substrates.

Besides that, Silva *et al.,* (2002) have produced pectinase from *Penicillium Viridicatum* RFC3 under solid state fermentation using many types of agricultural wastes and agro-industrial by-products such as orange bagasse, sugar cane bagasse, wheat bran, banana and mango peels and corn teguments. The results showed that all those agricultural wastes and

agro-industrial by-products are suitable to be used as carbon source for *Penicillium Viridicatum* as they produce polygalacturonase and pectin lyase after 2 to 12 days of fermentation.