EXTRACELLULAR PECTINASE PRODUCTION BY *Aspergillus niger* HFM 8 THROUGH SOLID SUBSTRATE FERMENTATION USING POMELO PEELS AS A SUBSTRATE

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by

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TABLE OF CONTENTS

CHAPTER 5 : DISCUSSION

CHAPTER 6 : CONCLUSION AND RECOMMENDATION

APPENDICES

LIST OF PUBLICATIONS

LIST OF TABLES

LIST OF FIGURES

LIST OF PLATES

LIST OF SYMBOLS AND ABBREVIATION

%	Percent
±	Plus minus
^{o}C	Degree Celcius
α	Alpha
β	Beta
®	Registered Trade Mark
µ	Micro
Δ	Delta
A	Absorbance
g	Gram
L	Litre
mg	Milligram
ml	Millilitre
mm	Milimeter
cm	Centimeter
µg	Microgram
µmol	Micromol

M	Molar
nm	Nanometer
v/v	Volume over volume
w/v	Weight over volume
U	Unit of activity
U/ml	Unit of activity per millilitre enzyme solution
U/g	Unit of activity per gram substrate
V	Volt
g/L	Gram per litre
g/mol	Gram per mol
bp	Base pair
R_f	Relative mobility
kDa	Kilo Dalton
SDS	Sodium dodecyl sulphate
SDS-PAGE	Sodium dodecyl sulphate polyacrylamide gel electrophoresis
SEM	Scanning Electron Microscope
CMC	Carboxymethylcellulose
rpm	Revolution per minute

PENGHASILAN PEKTINASE EKSTRASEL OLEH *Aspergillus niger* HFM 8 MELALUI FERMENTASI SUBSTRAT PEPEJAL MENGGUNAKAN KULIT LIMAU BALI SEBAGAI SUBSTRAT

ABSTRAK

Penimbunan kulit limau bali (*Citrus grandis*) sebagai sisa industri tani setelah pemprosesan buah-buahan sewajarnya dieksploitasi sepenuhnya dengan bantuan mikroorganisma untuk menghasilkan produk lain yang mempunyai nilai komersil seperti enzim. Kulit limau bali telah dibuktikan mengandungi pektin pada kadar yang tinggi dan berperanan sebagai penggalak untuk penghasilan pektinase oleh mikroorganisma melalui kaedah fermentasi substrat pepejal (SSF). Pencilan HFM 8 telah dipilih sebagai kulat penghasil pektinase yang terbaik dengan aktiviti maksimum sebanyak 112.79 ± 17.2 U/g substrat setelah dua kali proses penyaringan melibatkan penyaringan primer dan penyaringan sekunder. Pencilan ini dikenalpasti sebagai *Aspergillus niger* HFM 8 setelah melakukan proses identifikasi berdasarkan sifat-sifat koloni dan juga secara molekul. Penambahbaikan keadaan fizikal dan kimia adalah ditekankan dalam kajian ini dengan objektif untuk meningkatkan produktiviti pektinase oleh mikroorganisma tersebut. Hasilnya, peningkatan pektinase sebanyak 48.82% telah berjaya dihasilkan oleh *Aspergillus niger* HFM 8 apabila ditumbuhkan di dalam sistem fermentasi keadaan pepejal menggunakan kelalang di dalam keadaan fizikal dan kimia yang telah dioptimumkan iaitu dengan menggunakan substrat bersaiz 0.75 mm, kadar kelembapan sebanyak 60% (i/b) (menggunakan air suling steril pada pH 5.0), saiz inokulum sebanyak 1 x 10^4 bilangan spora /ml, pengeraman pada suhu bilik (30 ± 2^oC), penambahan sumber nitrogen iaitu 0.1% (b/b) urea, pengeraman tanpa kesan pengadukan dan penggunaan

larutan penimbal asetat (pH 4.5, 0.1 M) untuk pengekstrakan pektinase. Penghasilan pektinase oleh *Aspergillus niger* HFM 8 telah dilakukan pada skala besar dengan menggunakan sistem dulang. Dalam keadaan fizikal yang telah dioptimumkan iaitu dengan ketebalan substrat sebanyak 1.0 cm bersamaan 60 g serbuk kulit limau bali (saiz 0.75 mm), 40% (i/b) kelembapan (air suling steril pada pH 5.0), saiz inokulum sebanyak 1 x 10^6 bilangan spora/ml, pengeraman pada suhu bilik (30 ± 2^oC), penambahan 0.1% (b/b) urea sebagai sumber nitrogen tambahan, kesan pengadukan sekali pada setiap 24 jam dan penggunaan larutan penimbal asetat (pH 4.5, 0.1M) untuk pengekstrakan pektinase, didapati produktiviti pektinase telah meningkat sebanyak 23.00%. Secara perbandingan, penghasilan enzim pektinase telah meningkat sebanyak 191.41% daripada pemfermentasian di dalam sistem kelalang ke sistem dulang yang membuktikan potensi *Aspergillus niger* HFM 8 untuk menghasilkan lebih banyak pektinase pada skala industri. Penulenan enzim seterusnya dijalankan dengan melaksanakan kaedah kromatografi pada dua peringkat melibatkan penukaran ion (DEAE Sephadex) dikuti penapisan gel (Sephadex G-100). Prosedur SDS-PAGE mengesahkan bahawa berat molekul pektinase tulen ini adalah 55.37 kDa. Enzim ini bertindak-balas secara optimum pada 40^oC dan pH 4.0 di samping stabil pada suhu 35^oC ke 45^oC dalam julat pH 3.5 ke pH 4.0. Pektinase tulen ini didapati bertindak-balas sangat spesifik terhadap pektin tanpa melibatkan peranan agen pelarut atau ion logam untuk aktiviti yang optimum. Sebaliknya, $FeCl_3$ dan $AgNO_3$ didapati telah merencatkan tindak-balas pektinase. Secara jelasnya, kulit limau bali terbukti dapat diaplikasikan untuk penghasilan pektinase secara komersil melalui kaedah fermentasi substrat pepejal di mana enzim pektinase yang dihasilkan berpotensi untuk digunakan dalam industri pengekstrakan dan penjernihan jus buah-buahan.

EXTRACELLULAR PECTINASE PRODUCTION BY *Aspergillus niger* HFM 8 THROUGH SOLID SUBSTRATE FERMENTATION USING POMELO PEELS AS A SUBSTRATE

ABSTRACT

The accumulation of pomelo peels (*Citrus grandis*) as the agro-industrial residues after the fruit processing and manufacturing should be maximally exploited for the microbial bioconversion of the detrimental wastes into a value-added by product such as enzymes. Pomelo peels are authenticated as rich in pectin, acting as the main inducer for pectinase enzyme production by microorganism through solid substrate fermentation system (SSF). Isolate HFM 8 revealed as the best fungal pectinase producer after the implementation of double steps screening processes (primary and secondary screenings) with maximal activity of 112.79 ± 17.2 U/g substrate. Based on the phenotypic features and molecular identification, this novel isolate was denoted as *Aspergillus niger* HFM 8. The improvement of physicochemical cultural conditions was emphasized in this current research with an attempt to enhance the pectinase productivity by the microorganism. An augmentation of 48.82% on pectinase yield by *Aspergillus niger* HFM 8 in a shake flask system was recorded after being cultivated in an enhanced physicochemical condition constituted of 0.75 mm particle size of substrate, 60% (v/w) of initial moisture (sterilized distilled water, pH 5.0), inoculum size of 1 x 10^4 spores/ml, incubation in room temperature (30 ± 2^oC), inclusion of 0.1% (w/w) urea as the external nitrogen source, no mixing effect (static condition during fermentation) and utilization of acetate buffer (pH 4.5, 0.1 M) as the extracting solvent during the

enzyme recovery. A large scale pectinase production by *Aspergillus niger* HFM 8 was then conducted employing a tray system. Under the optimized physical conditions comprised of 1.0 cm substrate depth equalized to 60 g pomelo peels substrate (0.75 mm of particle size), 40% (v/w) of initial moisture (sterilized distilled water, pH 5.0), inoculum size of 1×10^6 spores/ml, incubation in room temperature (30 ± 2^oC), inclusion of 0.1% (w/w) urea as the external nitrogen source, inclusion of mixing effect once for every 24 hours interval and utilization of acetate buffer (pH 4.5, 0.1 M) as the extracting solvent during the enzyme recovery, an amount of 23.00% increment on pectinase productivity was detected. Comparatively, the fungal pectinase production boosted up to 191.41% of increment from a shake flask system to a tray system indicating the potentiality of *Aspergillus niger* HFM 8 to be industrially employed particularly in pectinase production. Further purification of the crude pectinase was conducted by practising double steps chromatography constituted of anion exchange (DEAE Sephadex) followed by gel filtration (Sephadex G-100). SDS-PAGE procedure determined the molecular weight of the purified pectinase as 55.37 kDa. This enzyme was found optimal at 40^oC and pH 4.0 whereby the catalytic reaction was stable between 35^oC to 45^oC and in condition of pH 3.5 to pH 4.0. The purified pectinase was highly specific on pectin as the substrate whilst no solvents or metal ions found enhancing the catalytic reaction. Inversely, $FeCl_3$ and $AgNO_3$ prohibit the pectinase reaction. Apparently, pomelo peels are markedly applicable for commercial pectinase production employing a solid substrate fermentation system whereby the produced enzyme is highly applicable to be incorporated into the fruit juice extraction and clarification process.

CHAPTER 1

INTRODUCTION

1.1 General introduction on pectinase research

Pectinase is one of the forthcoming enzymes at the moment which constitutes 25% of the global food enzyme market (Jayani *et al*., 2005). This group of heterogenous enzymes catalyze the degradation of pectic substances in plant structures such as pectin, pectic acid or oligo-D-galacturonate in different mode of actions including transelimination and hydrolysis (Favela-Torres *et al*., 2006; Jayani *et al*., 2010 ; Heidar *et al*., 2011). In nature, pectinase is mainly found in plant tissue to facilitate the cell wall extension and softening some tissues during the maturation of part of the plant such as fruits. In addition, pectinase also catalyze the decomposition of plant residues and contribute to the carbon cycle and ecosystem stabilization. During the phytopathogenic process, pectinase is secreted copiously by microorganisms to infect the host plant (Siddiqui *et al*., 2012). For industrial sector, pectinase is vastly applied in fruit juice extraction and clarification, textile and fiber processing and manufacturing, coffee and tea fermentations, industrial pectic wastewater treatment, paper making, purification of plant virus, poultry feed production and oil extraction (Saito *et al*., 2004; Sharma and Satyanarayana, 2006; Siddiqui *et al*., 2012). Furthermore, this novel enzyme is also utilized in the extraction of specific plant bio-compounds from raspberry residues, tomatoes, grape skin and also apple skin (Neagu *et al*., 2012). Undeniably, pectinase is a profitable enzyme which has enormous significant contributions to the mankind.

In order to accomplish the increasing worldwide demand, industrial scale production of pectinase enzyme has been generated either through solid substrate fermentation (SSF) or submerged fermentation (SmF) (Neagu *et al*., 2012). It has been proven that pectinase is an inducible enzyme specifically with the visibility of pectic substances as the substrate (Reddy and Sreeramulu, 2012). In solid substrate fermentation, the microorganisms grow together with the metabolites production occur on the surface of the solid substrate in near absence of free water (low water activity) (Heidar *et al*., 2011) whilst in submerged fermentation, the microorganisms grow in free flowing liquid substrate such as broth and the metabolites are secreted into the liquid medium (Subramaniyam and Vimala, 2012). Comparatively, in producing bioactive compound such as pectinase enzyme, fungi is more applicable in solid substrate fermentation rather than submerged fermentation which is more suitable for bacteria and yeast due to the high water activity (a_w). Low water activity in solid substrate fermentation generally mimicing the nature environment favoured by fungi to grow and produce various metabolites. Furthermore, the fungal metabolism is enhanced since the bacterial contamination is prohibited due to the low water activity in solid substrate fermentation (Favela-Torres *et al*., 2006).

Several studies conducted have significantly proven that the volume of pectinase yielded through solid substrate fermentation is higher compared to submerged fermentation (Favela-Torres *et al*., 2006). Moreover, the cost of production through solid substrate fermentation is cheaper due to the exploitation of agro-industrial residues as substrates and the pectinase generated has better physicochemical properties, contradictory to submerged fermentation. In fact, the application of agro wastes as the main carbon source in solid substrate fermentation

is an effective solution in solving detrimental problem arises due to the waste disposal management. For example, every year in Egypt, large amount of orange peels are generated as residues after the oranges canning process by the manufacturing factories (Abdel-Mohsen, 1996). According to Mamma *et al*. (2008), about 35 000 tons (dry weight) of citrus peels are generated every year in Greece. In Spain, around 250 millions kilograms of grape pomace which consists of seed, stem and skin are produced by the wine cellars (Chatterjee *et al*., 2013). While in Karnataka, India, approximately, 3.1 tons/hectare husk of pulses and 14.3 tons/hectare deseeded sunflower head are being generated every year respectively after the processing of sunflower oilseed. All of these agro-industrial residues are rich in pectin and applicable as the pectin-containing substrate to be utilized in the microbial pectinase production through solid substrate fermentation. In Malaysia, approximately 1.2 million tonnes of agro-wastes are being accumulated each year, especially from the palm oil and oil palm industries.

In order to produce pectinase enzyme, different types of microorganism including fungi, bacteria and yeasts are incorporated into the fermentation system. According to Favela-Torres *et al*. (2006), approximately 30 genera of filamentous fungi, bacteria and yeast which are frequently employed in producing pectinase such as *Erwinia, Rhizopus, Penicillium, Fusarium, Aspergillus, Bacillus, Saccharomyces* and *Kluyveromyces.* These microorganisms must be non-pathogenic, non-toxic and generally do not produce antibiotics which are the important characteristics of GRAS (Generally regarded as safe) microorganism. One of the most frequently utilized microorganisms in fungal pectinase production is *Aspergillus niger* which is proclaimed possessing the GRAS status (Reddy and Sreeramulu, 2012).

Technically, commercial enzyme with enviable biochemical and physico-chemical properties with low cost production has been the focus of researchers currently (Silva *et al.*, 2002). It is undeniable that the utilization of agro industrial residues as pectin-rich substrate in pectinase production through solid substrate fermentation is promising a low cost and eco-friendly enzyme industry (Suresh and Viruthagiri, 2010). However, further research is crucial in screening the best substrate to be incorporated into the fermentation system and its suitability for the microbial growth and enzyme production.

1.2 Rational of study

Pomelo (*Citrus grandis*) is massively cultivated in Asia especially in Malaysia, China, Southern Japan, Vietnam, Indonesia and Thailand (Hameed *et al*., 2007). Commonly in Malaysia, during the trimming stage, some of the immature pomelo fruits are removed to improve the quality of the others and disposed to the environment subsequently. This scenario will only lead to the detrimental pollution problem to the community (Saikaew *et al*., 2009). Evidenced by research conducted by Norziah *et al*. (2000) and Burana-Osot *et al*. (2010), pomelo peel is rich in pectin and applicable in pectinase production through solid substrate fermentation employing fungal strains. Since the biotechnological research on pectinase production using pomelo peel is still scanty (Foo and Hameed, 2011), this vigorous study is implemented to maximize its potential as the main substrate in fermentation system to generate commercialized pectinase enzyme employing local fungal isolate.

1.3 Objective of research

With an aim to use pomelo peels as a substrate for pectinase production by a local fungal isolate via solid substrate fermentation system, these objectives are employed;

i. To identify and screen the best fungal pectinase producer based on its capability to produce extracellular pectinase qualitatively and quantitatively.

ii. To optimize the physical and chemical conditions for a maximal pectinase production by the selected fungal isolate in a shake flask system and a tray system.

iii. To purify and characterize the purified pectinase

CHAPTER 2

LITERATURE REVIEW

2.1 Growth of agricultural industry worldwide

With an aim to stimulate the agricultural growth, combating poverty and hunger for human worldwide and at the same time to encourage environmental sustainability, agricultural sector is believed as the efficient tool.

In United States Of America, agriculture is the major industry with 2.2 millions farms which dominate an area of 922 million acres in 2007. Corn, tomatoes, potatoes, peanuts and sunflower seeds are among the major crops being cultivated. Meanwhile, in China, over 300 millions farmers are employed in agricultural industry which endowed this country as the first place in worldwide farm output. Primarily, China is the main producer of rice, wheat, sorghum, potatoes, peanuts, tea, millet, barley, oilseed and cotton. Besides, India ranks as the second largest contributor of farm output after China. According to FAO (Food And Agriculture Organization) 2010 Worldwide Statistics, India is the largest supplier of many fresh fruits and vegetables, spices, milk, jute, millet and castor oil seeds. India is also the second largest producer of wheat and rice and also ranked as one of the five largest contributors of 80% of agricultural products including coffee and cotton in 2010 (FAO, 2012).

The same scenario is also taking place in Malaysia where agricultural sector plays an imperative role to the economy, providing 16% of employment to the local citizen. Rubber, palm oil and cocoa are among the foremost commercial crops being cultivated locally. In 1999, about 10.55 million matric tons of palm oil was generated which ranked Malaysia as the world's largest producer. Besides, paddy, banana, coconut, durian, pineapple, rambutan and other local fruits and vegetables are also cultivated for domestic market (Encyclopedia Of The Nation, 2013).

In future, it is assumed that the expansion of agricultural sector will be boosting up throughout the year in order to accomplish the worldwide population's demand. Nonetheless, the disposal management of agro-industrial residues remains as problematic issue to be solved by the developing countries.

2.2 Agro-industrial residues as a detrimental issue

Despite of soaring development depicted by the global agricultural sector yearly, the accumulation of assorted disposed agro-industrial residues remain a major concern to the public and the ecosystem as well. According to Agamuthu (2009), around 998 million tonnes of agricultural wastes were produced around the world. While in Malaysia, approximately 1.2 million tonnes of agro wastes were being disposed every year. Table 2.1 shows the agricultural wastes generated in Asian countries as reported by Agamuthu (2009). The table also demonstrates the predicted increasing numbers of agricultural wastes in the year of 2025.

Table 2.1 : Agricultural waste generation in Asia

Country In Asia	Agricultural Waste Generation (kg/capita/day)	Projected Agricultural Waste Generation In 2025 (kg/capita/day)
Brunei	0.099	0.143
Cambodia	0.078	0.165
Indonesia	0.114	0.150
Laos	0.083	0.135
Malaysia	0.122	0.210
Myanmar	0.068	0.128
Philipines	0.078	0.120
Singapore	0.165	0.165
Thailand	0.096	0.225
Vietnam	0.092	0.150
Nepal	0.060	0.090
Bangladesh	0.040	0.090

Adapted from : Agamuthu (2009)

Commonly, for agro-industrial waste management in developing countries, crop residues, animal garbages, forest litter and grasses are being burnt as fuel which leads to problematic air pollution. In general, the main source for these horrendous agro industrial wastes comes from the processed animal products and crops. Brewer's waste, maize milling by-product, molasses, oilseed cakes and bagasse are among the common wastes produced by the agro-industrial processing sector. While for the crop residues such as straw, stem, peel, shell, leaves, husk, seed, pulp, lint and also stubble which are derived from cereals (which include rice, maize or corn, millet, barley, wheat and sorghum), legumes (soy bean and tomatoes), jute, groundnut, cotton, coffee, cocoa, tea, olive, fruits (banana, mango, cashew) and palm oil are also defined as agricultural waste (Pandey *et al*., 2009).

Undeniably, managing agro-industrial waste as one of the solid waste is a big challenge for a developing country around the world. Without an effective step, the accumulation of these residues will only lead to the public health hazard and detrimental pollution to the environment (Hwa, 2007). Apparently, converting the 'waste' into 'wealth' through the utilization of agro-industrial residues in profitable enzyme production is one of the practical steps in encountering such problem.

2.3 Utilization of agro industrial residues in pectinase production

Abundance of agro-industrial waste as by-product in agricultural sector (around 3.5 billion tonnes/year) is promising a low cost enzyme yield especially in microbial pectinase production employing solid substrate fermentation. These renewable residues can be utilized as source of energy for microbial growth during fermentation and also as carbon supply during the synthesis of biomass and other products (Jacob, 2009). In addition, these agro industrial residues also serve as an anchorage for the cells and provide sufficient nutrients for the microbial growth. However, for certain nutrients that are available in sub-optimal concentration or even absent, the substrate should be supplemented externally (Pandey *et al.*, 1999). In consideration of agro-industrial residues' suitability and availability to be exploited as the substrate in solid substrate fermentation, the problem regarding the pollution as a result to its accumulation and disposal method should be overcome effectively (Hoondal *et al*., 2002).

In nature, variety of substrates come from agro-industrial wastes which are applicable in producing profitable value added products especially pectinase enzyme through solid substrate fermentation. However, the number and type of potential residues varies between the crops and districts in the world (Cohen and Hadar, 2001). Some potentially utilized residues in pectinase production through solid substrate fermentation using various microorganisms are recorded in Table 2.2

Table 2.2 Consumption of assorted agro industrial residues in pectinase production through solid substrate fermentation (SSF)

Agro Industrial Residue	Microorganism	Reference
Sugar cane bagasse	*Aspergillus niger*	Jacob (2009)
	Aspergillus awamori	Suresh and Viruthagiri (2011)
	Penicillium sp.	Jacob (2009)
	Aspergillus niger CH4	Solis-Pereyra *et al.* (1996)
	Moniliella sp. SB9	Martin *et al*. (2004)
	Penicillium sp EGC5	Martin *et al*. (2004)
Monosodium glutamate waste water	*Aspergillus niger*	Bai *et al*. (2004)
Sugar beet pulp	*Aspergillus niger*	Bai *et al.* (2004)
	Bacillus gibsonii S2	Li *et al.* (2005)
	Penicillium oxalicum	Neagu *et al.* (2012)
Wheat bran	*Aspergillus carbonarius*	Jacob (2009)
	Aspergillus niger	Castilho *et al.* (2000) ; Heidar *et al.* (2011)
	Aspergillus sojae	Heerd *et al.* (2012)
	Thermoascus aurantiacus	Jacob (2009)
	Bacillus sp. DT7	Kashyap *et al.* (2003)
	Penicillium sp.	Jacob (2009) ; Heidar *et al*. (2011)
	Streptomyces sp. RCK-SC	Jacob (2009)
	Penicillium viridicatum RFC3	Silva *et al.* (2005)
	Streptomyces lydicus	Jacob (2009)
	Bacillus pumilus dcsr1	Sharma and Satyanarayana (2012)
	Alternaria alternata	Faten and Abeer (2013)

Table 2.2 Continue

Agro Industrial Residue	Microorganism	Reference
Rice bran	*Aspergillus niger*	Suresh and Viruthagiri (2011)
	Aspergillus awamori	Suresh *et al.* (2009)
Orange bagasse	*Thermoascus aurantiacus*	Jacob (2009)
	Penicillium viridicatum RFC3	Silva *et al.* (2005)
	Moniliella sp. SB9	Martin *et al*. (2004)
	Penicillium sp EGC5	Martin *et al*. (2004)
Apple bagasse	*Penicillium* sp	Heidar *et al.* (2011)
	Aspergillus niger	Heidar *et al.* (2011)
Orange pulp	*Fusarium moniliforme*	Jacob (2009)
Orange peel	*Aspergillus niger*	Vasanthi and Meenakshisundaram (2012)
Coffee pulp	*Aspergillus niger*	Loera *et al*. (1999)
	Aspergillus niger C28B25	Antier *et al.* (1993)
	Aspergillus niger V22 B35	Boccas *et al.* (1994)
Coffe mucilage	*Erwinia herbicola*	Avallone *et al.* (2002)
	Lactobacillus brevis	Avallone *et al.* (2002)
Grape pomace	*Aspergillus awamori*	Botella *et al*. (2005)
Grape peel	*Saccharomyces cerevisiae* CECT 11783	Arévalo-Villena *et al.* (2011)
Soy bran	*Aspergillus niger*	Castilho *et al.* (2000)
Citrus peel	*Aspergillus niger*	Dhillon *et al.* (2004)
Citrus waste	*Aspergillus foetidus*	Garzȯn (1992)
	Aspergillus niger F3	Rodríguez-Fernàndez *et al.* (2012)
Corn cobs	*Alternaria alternata*	Faten and Abeer (2013)

2.4 Pomelo peel as a potential substrate for pectinase production

Pomelo or its scientific name *Citrus grandis* (better known in Malaysia as limau bali or limau kedang) is the largest citrus fruit with average diameter of 30 centimetres and sometime weighs until 10 kilograms. Generally, it possesses very thick rind with soft texture but yet still easy to peel off (Hameed *et al*., 2007). This citrus fruit is also known as "shaddock" and believed as an ancient species to the genus of *Citrus.* It is classified in family of Rutaceae, sub-family Aurantioidae, tribe Citreae and sub-tribe Citrinae (Paudyal and Haq, 2008).

Pomelo is cultivated immensely in Malaysia, Indonesia, Thailand, China, Fiji, India and Japan. Currently, this large citrus fruit is also grown widely in Caribbean and United States Of America especially in California and Florida. It is believed that pomelo or "shaddock" was introduced to the West Indies (Barbados) from the Dutch East Indies by Captain Shaddock and latterly being spread to the other tropical countries (Hameed *et al*., 2007).

Commonly, the juicy flesh of pomelo which is rich in Vitamin C, B1, B2, B12 and protein is eaten raw or being extracted for commercial purpose. Meanwhile, its thick rind is utilized widely in sweet manufacturing industry (covered with sugar) and also in marmalade production (Hameed *et al*., 2007). The flowers, seeds and fruits are also employed in medication and the flower itself is applied in perfumery extraction. Meanwhile, the thick peel is extracted as the source of pectin and dietary

fiber (Saikaew *et al*., 2009 ; Burana-Osot *et al.,* 2010). According to the statistics provided by FAO (Food And Agriculture Organization), the total production of pomelo and grapefruit amounted about 5.05 million tonnes which constituted 6.2% of citrus fruit production worldwide in 1994 while in Thailand itself, 11% of its exported fruit comes from pomelo (Paudyal and Haq, 2008).

In Malaysia, pomelo is principally cultivated in Johor, Kedah, Melaka and Perak with approximately 1895 hectares of pomelo are cultivated commercially (Foo and Hameed, 2011). Generally, pomelo is produced throughout the year with its peak seasons between January to February and also from August to September respectively. In order to ensure the well growth and quality assertion of certain amount of pomelos, fruits that are unsuitable to be commercialized in fresh market and also the immature fruits are eradicated from the trees (Norziah *et al*., 2000). Due to the high consumption of pomelo in food manufacturing industry together with the disposal of immature fruits during the trimming stage, this circumstance leads to a severe pollution to the community and the environment as well (Hameed *et al*., 2007).

As an efficient solution in managing the waste, pomelo peel as part of the fruit could be employed as a low cost substrate in producing pectinolytic enzymes as a value added by-product through solid substrate fermentation. According to Norziah *et al.* (2000), pomelo peel is a good source of pectin which 20.8% of its total extraction is constituted by pectin after being extracted through acetone precipitation utilizing sodium hexametaphosphate. Yuxiang *et al*. (2012) also reported that

20.98% of pomelo peel was constituted of pectin after being extracted under the optimum condition determined by Response Surface Methodology (RSM). In addition, the raw material for the production of commercialized pectin nowadays is mainly contributed by residues from fruit juice manufacturing such as citrus fruits and apple pomace (Hoondal *et al.*, 2002). This chemical component plays a vital role in inducing the pectinase yielded by microorganisms during the fermentation period (Jacob, 2009; Reddy and Sreeramulu, 2012). However, the exact potential of pomelo peel as the raw material in pectinase production through microbial fermentation is still yet undiscovered since less research was reported by the previous studies.

2.5 Pectic substances

Pectic substances are a complex, high molecular weight, acidic and negatively charged biomacromolecule (Jayani *et al*., 2005; Bhardwaj, 2010) which constituted of 17 different monosaccharides and at least seven different polysaccharides (Jacob, 2009). In plant cell wall, pectic substances and hemicelluloses act as the cementing agent for celluloses microfibrils in order to provide strength and stability to the structure of cell wall (Geetha *et al.*, 2012). The structure of primary cell wall in most flowering plant is displayed in Figure 2.1. Generally, pectic substances are ubiquitous to Plant Kingdom and copiously found in the lamella between the primary cell walls of adjacent young plant cells in form of calcium pectate and magnesium pectate which are responsible for the cell cohesion (Jayani *et al*., 2005). In young and developing cell wall, pectic substances are

synthesized in the Golgi apparatus from UDP-D-galacturonic acid during the early stage of plant growth (Hoondal *et al*., 2002).

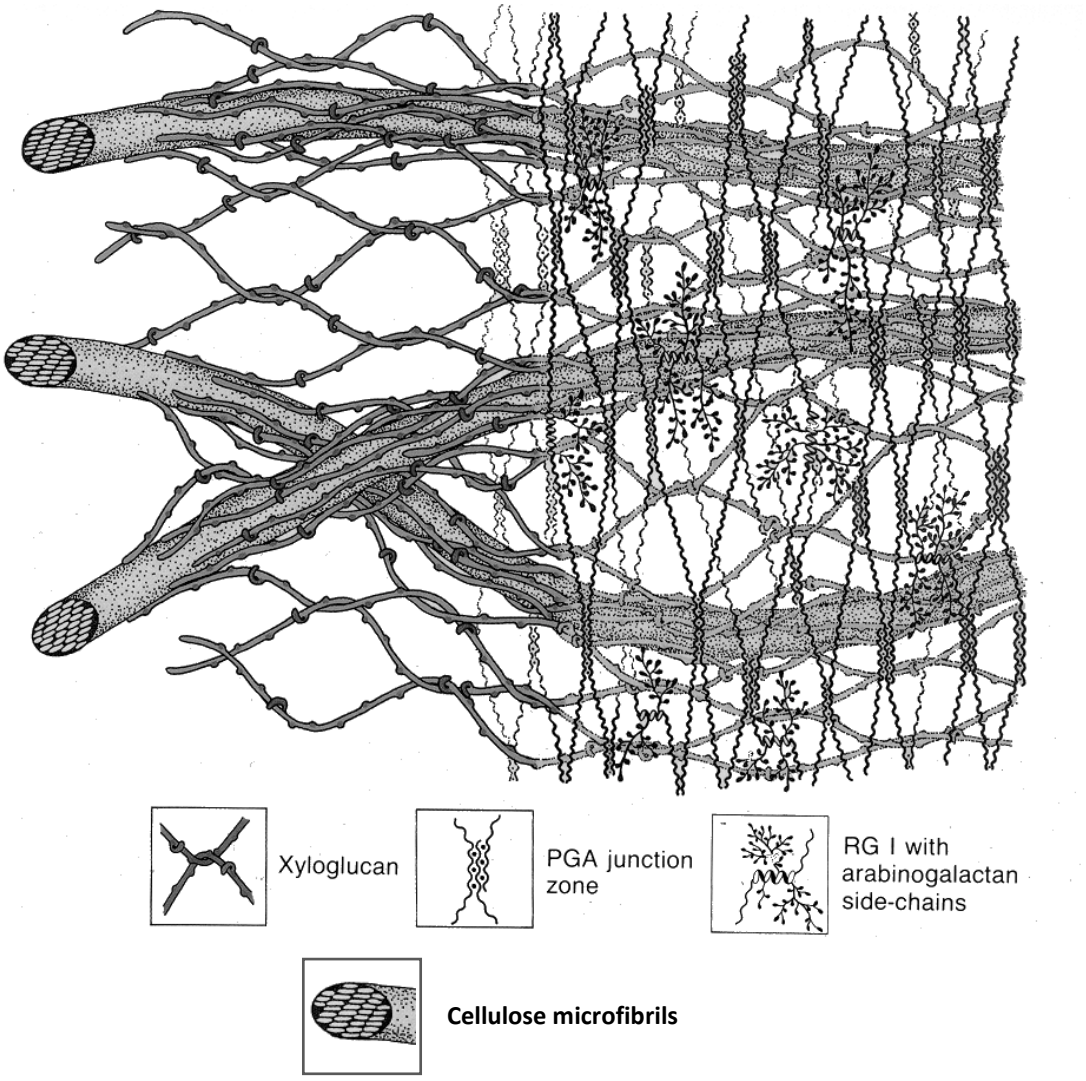

Figure 2.1 Primary cell wall structure in most flowering plants

(Adapted from Parenicova, 2000)

The cellulose microfibrils are interweaved with xyloglucan polymers, and this structure is entrenched in a matrix of pectic polysaccharides, polygalacturonic acid (PGA) and rhamnogalacturonan (RG).

2.5.1 Classification of pectic substances

According to Kashyap *et al*. (2001), pectic substances are classified based on the modification type of its backbone chain. Hence, different type of pectinolytic enzymes will react accordingly to this variety of pectic substances depending on its specificity and mode of action (Yadav *et al*., 2009). The details of pectic substances are further elaborated in the following description.

2.5.1.1 Protopectin

Protopectin is the "parents" of the pectic substances. The hydrolysis of protopectin yield pectin or pectinic acid (Kashyap *et al*., 2001; Jayani *et al*., 2005). This kind of pectic substances is a water–insoluble component and mainly found in the plant tissue predominantly in the middle lamella between the plant cells. The insolubility of this protopectin is due to its large molecular weight, salt bonding between the carboxyl group and also the ester bond structure between the carboxylic group of protopectin or the hydroxyl group of other constituents in the cell wall (Hoondal *et al*., 2002).

2.5.1.2 Pectic acid

According to Jayani *et al*. (2005), the soluble polymer of galacturonans and basically free from the methoxyl groups is called as pectic acid. The salt of pectic acid which is either neutral or acidic is known as pectates (Kashyap *et al*., 2001; Hoondal *et al*., 2002).

2.5.1.3 Pectinic acid

Pectinic acid is composed of polygalacturonan chain that consists of more than 75% of methylated galacturonate units. The salt of pectinic acid which is either neutral or acidic is known as pectinates (Jayani *et al*., 2005). Pectinic acid alone has a typical property in forming gel with acid or sugar under suitable condition. In addition, gel can also be formed with certain metallic ion or other compounds such as calcium salts if the pectinic acid is low in methoxyl content (Hoondal *et al*., 2002).

2.5.1.4 Pectin

Pectin is also known as polymethyl galacturonate. It is a soluble polymeric material which confers rigidity to the cell wall structure after binding together with cellulose (Jayani *et al*., 2005). Generally, 75% of the carboxyl groups of the galacturonate monomer are esterified with methanol (Hoondal *et al*., 2002).

Previous research had reported that pectin is copiously found in plants including fruits and vegetables (Bhardwaj, 2010; Chaudhri and Suneetha, 2012). Approximately, 0.5% to 4% of total plant fresh weight was constituted of pectin (Jayani *et al*., 2005). According to Jacob (2009), pectic substances play an imperative role in the plant cell growth and cell differentiation during the development of the plant tissue. New materials will be laid down and the old materials will be removed or degraded by the enzymes. While in an immature fruit, the pectin binds to the cellulose microfibrils which contribute to the stability and rigidity of the cell wall. However, during the ripening stage, the main backbone chain or the side chain of pectin is changing due to the naturally occurring pectinolytic enzymes reaction (Martin *et al*., 2004). Consequently, the rigid cell wall is softened since the pectin becomes more soluble resulting from the natural chain modification when the fruit turns to ripe (Kashyap *et al*., 2001). The estimation of pectin content in assorted fruits and vegetables are different to each other as described in Table 2.3.

Table 2.3 : Percentage of pectin in different fruits and vegetables

Fruit or vegetable	Tissue	Pectic substances (%)
Apple	Fresh	0.6 – 1.6
Banana	Fresh	0.7 – 1.2
Peach	Fresh	0.1 – 0.9
Strawberry	Fresh	0.6 – 0.7
Cherry	Fresh	0.2 – 0.5
Pea	Fresh	0.9 – 1.4
Carrot	Dry matter	6.9 -8.6
Orange pulp	Dry matter	12.4 – 28.0
Potato	Dry matter	1.8 – 3.3
Tomato	Dry matter	2.4 – 4.6
Sugar beet pulp	Dry matter	10.0 – 30.0

(Adapted from Jayani *et al*., 2005)

2.5.2 Structure of pectin

2.5.2.1 Primary structure of pectin

According to Chaudhry and Suneetha (2012), chemically, pectic substances are a complex colloidal acid polysaccharides with a long unbranched galacturonic acid backbone chain and linked together by α, 1-4 linkages (Figure 2.2). This is the basic unit of pectic substances which is also known as homogalacturonan (HG). Galacturonic acid which is the monomer of this polysaccharide (Reddy and Sreeramulu, 2012) can be methyl esterified at C-6 and the hydroxyl group at C-2 or C-3 can be acetylated.

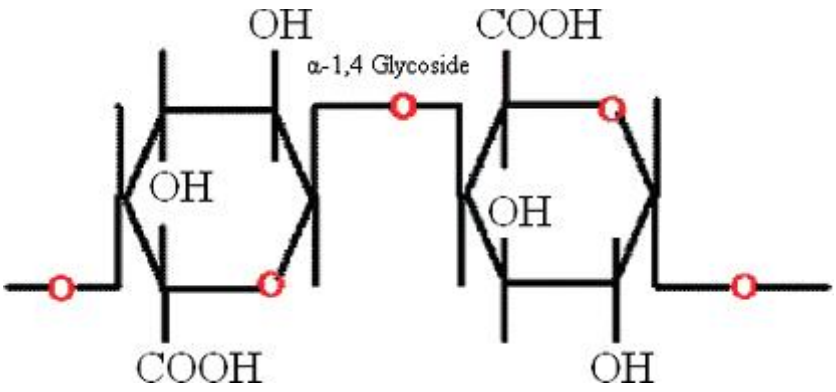

Figure 2.2 : Primary structure of pectin (adapted from Jacob, 2009)

2.5.2.2 Secondary structure of pectin

The secondary structure of pectin (Figure 2.3) is composed of two main regions which are "smooth region" (homogalacturonan) and "hairy regions" (rhamnogalacturonan I and II) (Pedrolli *et al*., 2009). Homogalacturonan constitutes the backbone chain of pectin, composed of α, 1-4 linked D-galacturonic acid residues. Rhamnogalacturonan I is a highly branched region which comprised of a huge number of side chain α, 1-2 linked L-rhamnopyranose residues. Meanwhile, the more complex Rhamnogalacturonan II is predominantly found in primary cell wall of plants and plays a significant role as a signal molecule in the development of plant cell wall. This biomacromolecule form a very flexible, extended and curve (worm like) structure in solution (Yadav *et al*., 2009)

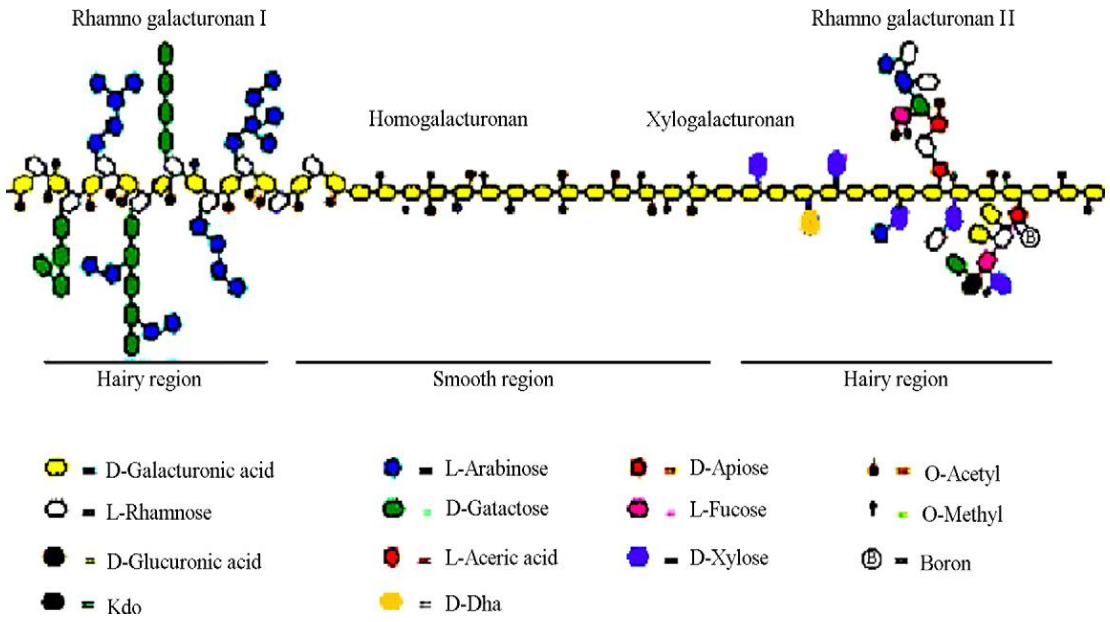

Figure 2.3 : Secondary structure of pectin (adapted from Yadav *et al*. (2009)

2.6 Pectinase

Pectinase is a kind of heterogeneous enzyme and occasionally known as pectic enzyme or pectinolytic enzyme. This enzymatic constituent exhibits pectin-containing substances degradation which is mainly found in plant structures. This inducible enzyme can be produced extensively either through solid substrate fermentation (SSF) or submerged fermentation (SmF) (Solis-Pereyra *et al*., 1993; Maldonado and Saad, 1998; Geetha *et al*., 2012) employing various species of saprophytic microorganism including fungi, bacteria and yeast (Jacob, 2009; Janani *et al*., 2011).

Historically, in 1930, the profitable potential of pectinase enzyme was principally applied in the home made preparation of fruit juices and wines. Thirty years later, in 1960s, the understanding of chemical nature and enzymatic reaction catalysed by pectinase in plant tissue was basically improved. With this awareness, research on the application of pectinase enzyme for commercial purpose was implemented extensively which in turn, endowed pectinase as one of the impending enzymes in commercial sector nowadays. Recent statistics on industrial enzyme sales worldwide in 1995 noted that $75 millions of total sales came from pectinase enzyme (Kashyap *et al*., 2001). Meanwhile, according to Jayani *et al*. (2005), almost 25% of global food enzyme sales was contributed by pectinase enzyme currently.

In nature, pectinase is produced by plants, insects, nematodes, protozoan (Pedrolli *et al*., 2009) and also by saprophytic microorganism including bacteria, fungi, actinomycetes and yeast (Jacob, 2009; Bhardwaj, 2010). Inside the plant tissue, this pectinolytic enzyme plays an essential role in supporting the extension of cell wall, facilitating the penetration of pollen tube (Blanco *et al*., 1999) and softening the plant tissue in mature or ripening fruits through the alteration of pectin backbone chain (Jayani *et al*., 2005). Oranges and tomatoes are believed as plants that possess considerable amount of pectinase enzyme inside the plant tissues.

Besides, pectinase is also found in insect such as the rice weevil (*Sitophilus oryzae*). The endosymbiotic bacteria inside the body of this insect are assumed as the genetic source of pectinase which plays a significant role in plant – insect interaction. The rice weevil feeds on seeds of cereals containing pectin. Thus, pectinase in this insect is functioning as an exclusive digestive system to degrade the pectin (Shen *et al*., 1996).

In the other hand, microbial pectinase yielded by bacteria, fungi and yeast is also important especially during the phytopathologic process, plant and microbe symbiosis interaction and also in the natural decomposition process of decaying plant residues. Ultimately, the decomposition of these plant materials, catalyzed by microbial pectinase contributes to the natural carbon cycle in the ecosystem (Pedrolli *et al*., 2009).