Faculty of Resource Science and Technology

Production of Crude Pectinase from Aspergillus versicolor under Solid State Fermentation (SSF) Using Different Agro Wastes as Substrates

Muhammad Syukran Bin Sazali

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Pusat Khidmat Maklumat Akadem
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Muhammad Syukran bin Sazali (35021)

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

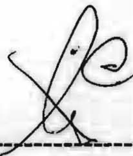
Supervisor: Ms. Rosmawati Saat
Co-supervisor: AP Dr. Awang Ahmad Sallehin Awang Husaini

Resource Biotechnology Programme
Department of Molecular Biology

Faculty of Resource Science and Technology
Universiti Malaysia Sarawak
2015

DECLARATION

I hereby declare that the study entitled "Production of Crude Pectinase from *Aspergillus versicolor* under Solid Substrate Fermentation (SSF) Using Different Agro Wastes as Substrates" submitted to the Faculty of Resources science and Technology, Universiti Malaysia Sarawak (UNIMAS) is my original work and that all the sources that I have quote referred to have been acknowledged by means of complete references. It has been submitted and shall not be submitted in any form to any institution or other university.



Muhammad Syukran bin Sazali
Resource Biotechnology
Department of Molecular Biology
Faculty of Resource Science and Technology
Universiti Malaysia Sarawak

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LIST OF ABBREVIATIONS

PDA	Potato Dextrose Agar
PE	Pectin esterase
PG	Polygalacturonase
PL	Pectin Lyase
SmF	Submerged Fermentation
SSF	Solid State Fermentation
%	Percent
cm	Centimetre
ml	Milimetre
mg	Milligram
µl	Microlitre
°C	Degree Celsius
g	Gram
nm	Nano metre

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Muhammad Syukran bin Sazali

Resource Biotechnology Programme
Department of Molecular Biology
Faculty of Resource Science and Technology
Universiti Malaysia Sarawak

ABSTRACT

In Malaysia, there are enormous agricultural residues that have been produced especially from the large plantation. Most of these wastes are degraded naturally on the soil while others are burnt which can cause pollution to the environment. Nowadays, there is a lot of biotechnological potential used to take advantages from the wastes. One of the famous techniques is a solid state fermentation which can be used to produce fuel, chemicals, enzymes and food. In this study, *Aspergillus versicolor* was used to produce pectinase enzyme in degrading pectin in the cell wall of the substrates and three SSF parameters were studied; time of incubation, initial moisture content of substrate and incubation temperature. The optimum condition for pectinase activity by using rice husk as substrate was 2 days of incubation time, 60% of moisture content and 25 °C. Meanwhile, the optimum condition for banana peel and pineapple peel as substrate was 70% moisture content and 30 °C, but they had different incubation time which was 4 days and 6 days, respectively. To conclude, the highest pectinase activity is using banana peel as SSF substrate, followed by pineapple peel and rice husk. The enzyme was extracted and assayed by using DNS method in order to determine the total amount of reducing sugar produced.

Key words: *Aspergillus versicolor*, agriculture residues, pectinase, solid state fermentation

ABSTRAK

Di Malaysia, terdapat banyak sisa pertanian yang telah dihasilkan terutamanya daripada penanaman berskala besar. Kebanyakan sisa-sisa pertanian hanya dibiarkan terurai secara semulajadi di atas tanah sementara sebahagiannya dibakar dan ini boleh menyebabkan pencemaran kepada alam sekitar. Pada masa kini, terdapat banyak potensi bioteknologi untuk memanfaatkan sisa-sisa pertanian tersebut. Salah satu daripada kaedah yang terkenal ialah fermentasi berkeadaan pepejal yang boleh digunakan untuk menghasilkan bahan bakar, bahan kimia, enzim and makanan. Dalam kajian ini, Aspergillus versicolor *telah digunakan untuk menghasilkan enzim pektinase didalam proses penguraian pectin yang berlaku didalam sel dinding substrat dan tiga parameter fermentasi berkeadaan pepejal telah dikaji; masa pengeraman, kandungan kelembapan awal substrat dan suhu pengeraman. Keadaan optima untuk aktiviti pektinase dengan menggunakan sekam padi sebagai substrat ialah 2 hari pengeraman, kandungan kelembapan 60% dan 25 °C. Sementara itu, keadaan optima untuk kulit pisang dan nenas sebagai substrat ialah kandungan kelembapan 70% dan 30 °C, tetapi kedua-dua substrat ini mempunyai masa pengeraman yang berbeza iaitu kulit pisang selama 4 hari dan kulit nanas selama 6 hari. Kesimpulannya, aktiviti pektinase yang paling tinggi adalah dihasilkan dengan menggunakan kulit pisang sebagai substrat fermentasi berkeadaan pepejal, diikuti oleh kulit nanas dan sekam padi. Enzim ini diekstrak dan dianalisa menggunakan kaedah DNS untuk menentukan jumlah gula penurun.*

Kata kunci: Aspergillus versicolor, *sisa-sisa pertanian, pektinase, fermentasi berkeadaan pepejal*

1.0 INTRODUCTION

Nowadays, pectinases are widely used in many commercial applications especially in the preparation of wines and fruit juices, since they can degrade the long and complex molecules called pectin that consist as structural polysaccharides in the middle lamella and the primary cell walls of young plant cells (Kashyap *et al.*, 2001). Through this process, the production of juice extract from the fruits can be increased by softens the cell wall. Besides, pectinases also have been used commercially in the textile industries. The enzymes are commonly used in many industrial applications because it provides an economically viable alternative and environmentally friendly (Priya & Sashi, 2014). Also, pectinase has the potential in degumming and retting of fiber crops and pretreatment of pectic wastewater from fruit juice industries. In addition, this biological degradation will help to solve some of the pollution problems caused by their accumulation. Chemical processes which are polluting the environment should be decrease and replace by the pollution-free processes involving microorganisms and enzymes. In addition, recently there has been an awareness of the effects of pollution that influencing both industry and government. This biological degradation will help to solve some of the pollution problems caused by the accumulation of agro wastes.

The two major sources of pectinase are plant and microorganism (Priya & Sashi, 2014). Microorganism groups that have the potential to synthesize pectinase, include bacteria and fungi. Filamentous fungi such as *Aspergillus sp.* is abundant in nature and live in a different type of habitats because their ability to colonise a wide variety of substrates (Fomicheva *et al.*, 2006). Filamentous fungi has many advantages as enzymes producers since some of the species are recognized as GRAS (Generally Regarded As Safe) strain and yield extracellular products which

can be easily recovered from fermented medium (Mrudula & Anitharaj, 2011). Agro wastes can be used as substrates to utilise the renewable agricultural and industrial wastes while overcome the problem of disposal wastes to the environment.

There are many biotechnological processes that can be used in reutilisation process such as solid state fermentation (SSF) and submerged fermentation (SmF). SSF is defined as the fermentation involving solid in the absence of free water but the substrate must contain sufficient moisture to support the growth and metabolism of the microorganisms (Botella *et al.*, 2007). There are several bioprocesses have been developed for the utilisation of agro-wastes product as a raw material for the production of bulk chemical products by SSF (Pandey *et al.*, 2000). The examples are, the production of cellulase (Bansal *et al.*, 2012), xylanase (Botella *et al.*, 2007) and ethanol (Sarkar *et al.*, 2012).

The purpose of this study is to produce pectinase from *A. versicolor* by using agro wastes as substrate in Solid State Fermentation (SFF). *Aspergillus versicolor* have been cultured and used to degrade agro wastes which are rice husk, banana peel and pineapple peel and utilize it as a raw material to produce enzymes. In order to get the higher activity of pectinase, three SSF parameters were studied which are temperature, initial moisture content and time of incubation. The aim of this study is to produce the crude pectinase from *A. versicolor* under solid state fermentation using agro wastes as substrates. In order to achieve this aim, the specific objectives are:

1) to discover the effectiveness of *A. versicolor* as an inoculum for pectinase production.
2) to identify the suitable agro wastes used as a substrate in SSF to produce high activity of pectinase.
3) to determine the optimal SSF conditions for the production of pectinase.

2.0 LITERATURE REVIEW

2.1 Pectinases

Pectinase is an enzyme that capable in breaking down pectin; a polysaccharide substrate, found in the cell wall of plants (Kashyap *et al.*, 2001). They are phytopathogenic substances that can be produced by microorganism, plant and animal (Chaudhri & Suneetha, 2012). Although they can be produced from different sources, microorganism has been used widely to produce this enzyme. Pectinase produced by microorganism is more favourable than plant and animal because of their cheap production, easier gene manipulation, faster product recovery and free from harmful substances (Chaudhri & Suneetha, 2012). The industrial pectinase is not consists of one type of enzyme but usually a complex mixture of enzymes such as pectin methyl esterases (PE), polygalacturonases (PG) and pectin lyases (PL) which capable in breaking down a variety of bonds in difference pectin molecules (Waites *et al.*, 2001). One of the vital enzyme used in industry is a polygalacturonases which capable in splitting polygalacturonic acid into monogalacturonic acid by opening glycosidic linkages (Khan *et al.*, 2012). Polygalacturonases can act in an endo- or exo- mode where endo-PG catalyse random cleavage of substrate while exo-PG catalyse hydrolytic cleavage at substrate nonreducing end (Pedrolli *et al.*, 2009). Meanwhile, pectin lyases cleaves glycosidic linkages preferentially on high esterified pectin, producing unsaturated methyloligogalacturonates and pectin methyl esterases catalyses deesterification of the methyoxyl group of pectin forming pectic acid and methanol (Pedrolli *et al.*, 2009).

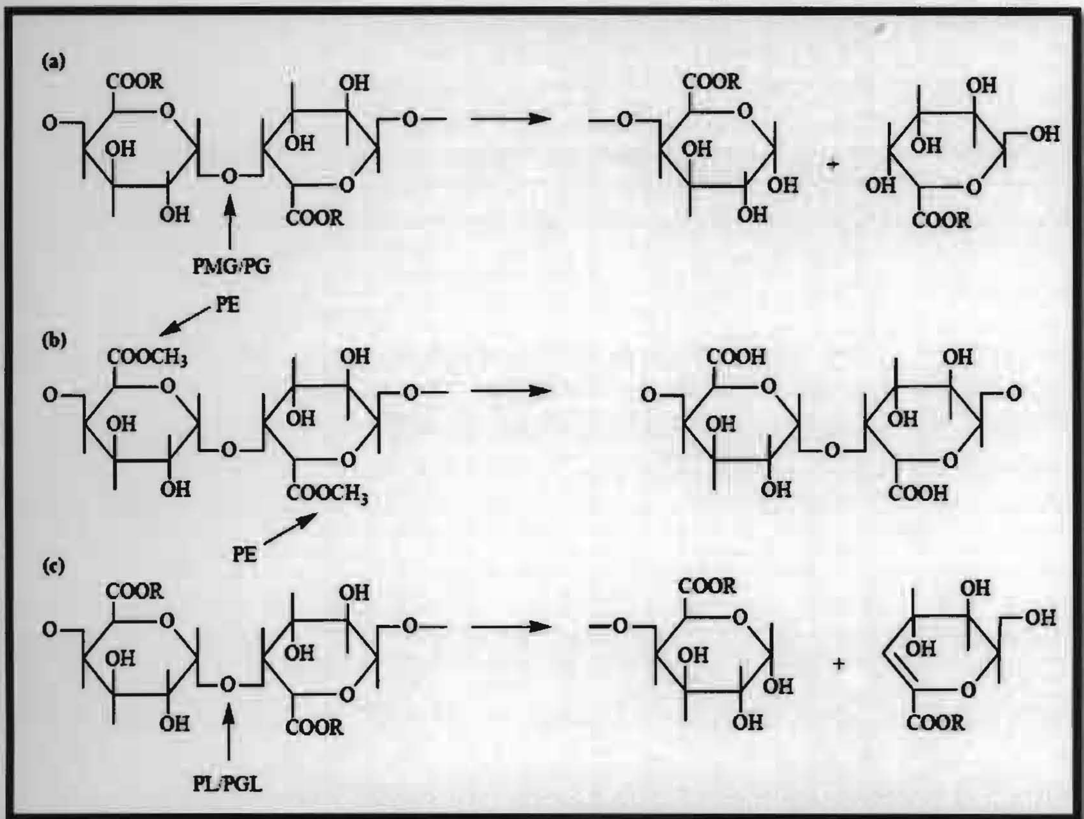

Figure 2.1: The specific bond where each pectinase enzyme attacks. (Pedrolli *et al.*, 2009.)

Furthermore, pectinases also can be divided into two broad groups that differ in their mechanisms of action because of the variety in the structure of the pectin which are depolymerizing and demethoxylating enzymes (Mrudula & Anitharaj, 2011). Depolymerizing enzymes which are polygalacturonase and pectin lyase break α-1, 4-linkages in the principal pectin chain while demethoxylating enzyme which is pectin esterase estrifies pectin to form pectic acid by removing methoxyl residues (Mrudula & Anitharaj, 2011).

Pectic substances are glycosidic macromolecules with high molecular weight and form the major components of the middle lamella and primary plant cell wall (Priya & Sashi, 2014).

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Chemically, pectic substances are complex colloidal acid polysaccharides, with backbone of galacturonic acid residues linked by α (1-4) linkages while the side chains of the pectin molecule consist of L-rhamnose, arabinose, galactose and xylose (Kashyap *et al.*, 2001). The main chain of pectin; carboxyl groups is partially methyl esterified 1,4 – D-glacturonan and known as pectic acid. Demethylated pectin can be partially or completely neutralized by sodium, potassium or ammonium ions. Based on the type of modifications of the backbone chain, pectic substances are classified into protopectin, pectic acid, pectinic acid and pectin (Kashyap *et al.*, 2001; Priya & Sashi, 2014). Protopectin is a water-insoluble pectic substance found in plant tissues and under the restricted hydrolysis will produce pectin or pectinic acid. Pectic acids are the galacturonans that contain negligible amounts of methoxyl groups while pectinic acids are the galacturonans with various amounts of methoxyl groups. Last type of pectic substance is pectins which contain the mixture of widely differing compositions containing pectinic acid as the major component.

Pectinase had been detected on sugar beet pectin, apple pectin, polygalacturonic acid and soybean flour (de Vries & Visser, 2001). The sources of enzyme pectinase are plant and microorganisms but microbial source are normally used because it is environmental friendly and help in developing economic sectors. Many industrial sectors will be benefited from this activity, from farmers to consumers that use the enzyme products. Applications of pectinase have become very important by the classification of two different pectinases which are acidic pectinases and alkaline pectinases. Acidic pectic enzymes usually from fungal sources are used in fruit juice industries and wine making while alkaline pectic enzymes usually from bacterial sources are used in the degumming and retting of fiber crops and also pretreatment of pectic wastewater from fruit juice industries (Kashyap *et al.*, 2001).

2.2 *Aspergillus versicolor*

A. versicolor is a member of the genus *Aspergillus*. The genus *Aspergillus* which is placed under the division of Ascomycetes (Pandey *et al.*, 2000), is a group of filamentous fungi which today consists of more than 200 species that produce enzymes (Soares *et al.*, 2012). Filamentous fungi were chosen because of their ability to produce extracellular enzymes in the higher amounts which are several tens of grams per litre but they also have the weaknesses such as time consuming (Aro *et al.*, 2005). Specifically, *A. versicolor* has a rough surface of conidia where 2.5 to 3.5 μm in size (Fomicheva *et al.*, 2006). Besides, their vesicles were 10 to 15 μm in diameter with various types of shape and also have two-layered sterigmas (Fomicheva *et al.*, 2006). The genus is divided into subgenera and sections, formerly called groups, based on colony colour and morphology. *A. versicolor* was placed under subgenus nidulantes (Peterson *et al.*, 2008). *A. versicolor* can survive in natural environments under unfavourable conditions for growth for a long time which up to 2 months in the hypersaline Dead Sea water (Kis-Papo *et al.*, 2003). Even though, there are several strains of *A. versicolor* that originate from different temperature regimes, their optimum temperature are the same which is between 25°C and 27°C. These fungi also easily found in damp indoor environment but they also are placed under toxigenic fungi that potentially to produce hepatotoxic and carcinogenic sterigmatocystin (Engelhart *et al.*, 2002). This inhalation of indoor air and ingestion of house dust by the children can lead to serious toxicity in their body.

2.3 Solid State Fermentation (SSF)

Solid-state fermentation is defined as those processes in which microbial growth and product formation occur on the surfaces of solid substrates in the near absence of free water (Botella *et al.* 2007). This technique was used since 1960 to 1970 in production of mycotoxin (Singhania *et al.*, 2009) but until today, there are still many technical problems that have not been solved. One of the disadvantages of the SSF is the difficulty to control and regulate the parameters such as temperature, pH, moisture, substrate concentration and pO_2 under limited water availability during the fermentation process (Hölker *et al.*, 2004). Instead of their weaknesses, this technique has been chosen for pectinase production because of the potential advantages such as high productivity, easy to conduct and solid state produce more concentrated product than submerged fermentations (Khan *et al.*, 2012). Moreover, SSF process also more economical than SmF because of their higher activities but low production costs (Singhania *et al.*, 2009).

Undoubtedly, SmF is used to produce almost 90% of all industrial enzymes using specifically improved and genetically modified microorganisms but almost all the enzymes produced using wild-type microorganisms are produced by SSF (Hölker *et al.*, 2004). Microorganisms such as fungi which have an ability to survive in low amount of water condition are used in this solid-state fermentation (Martin et al., 2004). Previous studies have been shown that SSF technique is used widely to produce variety of enzymes, secondary metabolites and protein-enriched food (Hölker *et al.*, 2004) and also in the development of bioprocesses such as bioremediation of hazardous compounds and biological detoxification of agro-industrial residues (Singhania *et al.*, 2009).

2.4 Agro wastes

2.4.1 Banana Peel

Based on statistic published by Statistics division of Food and Agriculture Organization of the United Nations (n.d.), the plantation of banana in Malaysia has been increased drastically where 22,135 ha in 2009 has increase to 29, 132 ha in 2011. This occasion also caused by the increasing demand for Cavendish bananas in the domestic market. Furthermore, banana also has a huge potential to be used as substrates because after harvesting the fruit, the rest of the plant would be cut down. Instead of disposing or wasting the remaining of the plant, the banana waste can be used for this fermentation. During ripening of banana fruit, softening of fruit occurs because of loosening of cell wall and texture degradation where insoluble protopectin is converted into soluble pectin (Mohapatra *et al.*, 2010). High amount of glucose, galactose, arabinose, rhamnose and xylose in banana also be influenced by the pectin molecule where those components function as their side chains (Kashyap *et al.*, 2001).

Table 2.4.1: Banana peel composition (Mohapatra *et al.*, 2010)

Composition	Percentage
Dietry fibre	43.2-49.7%
Lignin	6-12%
Pectin	10-21%
Cellulose	7.6-9.6%
Hemicelluloses	6.4–9.4%
Protein	6-9%
Crude fat	3.8-11%
Starch	3%
Galacturonic acid	Unknown

2.4.2 Pineapple peel

Other than banana, pineapple waste also has a high potential as a source for pectinase production using enzymes. In addition, high production of pineapple fruits was recorded by Malaysian Pineapple Industry Board which is 127,415 metric tonnes in 2010 and this leads to high amount of waste product (Malaysian Pineapple Industry Board, n.d.). Pineapple peel contains certain polysaccharides such as a cellulose, hemicellulose and pectic substances. The hemicelluloses consist of glucose, xylose and mannose while pectic polysaccharides include galacturonic acid, rhamnose, arabinose and galactose (Huang *et al.*, 2011). In total, arabinose, rhamnose, galactose and uronic acids in the soluble dietary fiber accounted for approximately 76.1% of the total sugar content (Huang *et al.*, 2011).

Table 2.4.2: Sugar composition of fiber-rich fractions prepared from pineapple peels (Huang *et al.*, 2011)

Sugar component	**Amount dry weight (g/100 g peel)**
Cellulosic glucose	8.33-10.7 g/100 g
Xylose	7.83-9.23 g/100 g
Uronic acid	6.83-8.26 g/100 g

2.4.3 Rice husk

Rice also the plant that regularly receives great attention in Malaysia because of their important as country's staple food. About 20% of the rice paddy is husk and usually this husk will be burnt by the farmer or discarded as a waste (Kumar *et al.*, 2012). All of this agro wastes contain high amount of cell wall polysaccharides materials that can give benefit to the users. Cell wall polysaccharides can be divided into three groups which are cellulose, hemicellulose and pectin (de Vries & Visser, 2001). The rigid structures that strengthen the plant cell wall are the pectin and hemicellulose polysaccharides combine with the aromatic polymer lignin and cellulose fibrils (de Vries & Visser, 2001). Even though, this substance can be degraded naturally, the user also can take advantages from the wastes to get profit from it. Then, the suitable technique to utilise the wastes should be done to overcome the problem and profiting the users. Environmental problems have attracted the worldwide attention in utilisation of renewable agricultural and industrial wastes. The agricultural wastes such as banana residue, pineapple waste and rice husk may give problems where some of the farmers will burn agricultural wastes and residues after harvesting their useful products. As a consequence, this activity will lead to other environmental problems such as haze and smog and also health hazards to the surrounding residents.

3.0 MATERIALS & METHODS

3.1 Method

3.1.1 Preparation of media

In this experiment, Potato Dextrose Agar (PDA) media was used to subculture *Aspergillus versicolor*. The PDA media contained (g/l): potato extract – 200 ml, dextrose – 20 grams, agar – 20 grams. Approximately, 3.9 grams of Potato Dextrose powder were suspended into 100 ml of ultrapure water. Then, the PDA powder was allowed to dissolve completely by stirring using magnetic stirrer and next, undergo autoclaving in the autoclave machine for 20 minutes at 121 °C. After slight cooling, 100 µml of ampicillin (50mg/ml) was added in.

3.1.2 Microorganisms

A. versicolor was obtained from the Molecular Genetic Laboratory, UNIMAS Fungal Collection. The isolated fungi were inoculated on Potato Dextrose Agar (PDA) medium with 50 mg/ml of ampicillin. The cultures were grown at room temperature and maintained at 4 °C before used in SSF process.

3.1.3 Substrates and pretreatment

The substrates that have been chosen to use for SSF fermentations are rice husks, banana peels and pineapple peels. Rice husks were obtained from the Molecular Genetic Laboratory and finely crushed. While, pineapple peels and banana peels were obtained from the farmer at Kota Samarahan and cut into small pieces. Next, the pineapple and banana peels were washed with tap water. Both substrates were dried in the oven at 60 °C for 72 hours (3 days) until the materials

are completely dried. The materials were then ground into fine size and stored in the tight container to be used as substrates.

The existing moisture content was calculated by weighing all three of substrates and then, the substrates were dried by keeping them in an oven until the constant mass was recorded. Each substrate was done in duplicate to get the average of dry basis sample. For the standard, 1 g of substrates was used instead of 5 g to decrease the time taken for the total moisture content in the substrate to evaporate. The existing moisture content for each 1 g of substrates was calculated by using formula below (example can be seen in **Appendix A**):

$$\textbf{Moisture content dry basis} = \frac{Initial\ weight - Final\ weight}{Final\ weight} \times 100\%$$

3.1.4 Solid State Fermentation and enzyme extraction

Solid state fermentation was performed in duplicate of 250 ml Erlenmeyer flasks contained 5 g of rice husk and distilled water that acts as moistening agent. The distilled water was added into the substrate until it reached 70% of initial moisture content (calculations are shown in **Appendix B**) in solid state medium for appropriate growth of fungus for the production of maximum enzyme. 3 discs (1 cm x 1 cm) cut from the periphery of actively growing colonies of 72 h-old culture of *A. versicolor* on PDA medium were added and incubated at 30 °C for 6 days under a static condition. The procedure was repeated using banana peel and pineapple peel as substrate. After 6 days, the enzyme was harvested by mixing the entire substrate with 20 ml of sodium acetate buffer and agitated the flask on a rotary shaker at 120 rpm with a contact time of 30 minutes at room temperature. The biomass was separated by filtration through muslin cloth and collected extracts were centrifuged at 6000 rpm for 20 minutes at 4 °C (Mrudula &

Anitharaj, 2011). Lastly, the supernatant collected was filtered using filter paper and the clear supernatant was used as the source of crude enzyme solution.

3.1.5 Pectinase assay

Pectinase activity was assayed by measuring the reducing sugars released from the action of pectinase on citrus pectin using 3, 5-dinitrosalicylic acid (DNS) reagent (Martin et al., 2004; Miller, 1959; Mrudula & Anitharaj, 2011). The method used to determine the reducing sugar of the enzyme solution is according to Mrudula and Anitharaj (2011). The reaction mixture (1 ml) consists of 0.8 ml of (1.0% w/v) citrus pectin in sodium acetate buffer (0.1 M, pH 5.8) and 0.2 ml of appropriately enzyme source. The reaction mixture was incubated at 40 °C for 30 min followed by addition of 1 ml of DNS to the tubes and the reaction was stopped by heating the tubes in boiling water bath for 15 minutes. After boiling, 0.5 ml of 40% (w/v) Rochelle salt (sodium potassium tartarate) was added to stabilise the colour reaction. Lastly, the reaction mixture was measured at absorbance of 575 nm against blank. The blank was prepared as the same as the samples, but the crude enzymes was substituted with sodium acetate buffer. The reducing sugars released by enzymatic hydrolysis were determined. One unit of enzyme activity (U) is defined as the amount of enzyme required to release 1 mmole of reducing groups per minute with galacturonic acid as standard under the assay conditions. A graph of absorbance at 575 nm versus galacturonic acid concentration for galacturonic acid standards was prepared (refer to **Appendix C**). The absorbance obtained was used to determine galacturonic acid concentration that had been released during the assay by referring to galacturonic acid standard curve. The concentration of galacturonic acid was needed to calculate enzyme activity (example can be seen in **Appendix D**).

3.1.6 Optimisation of SSF parameters

3.1.6.1 Effect of time of incubation on SSF

The effect of time of incubation on SSF was determined by the usage of different period of time at 30 °C (Mrudula & Anitharaj, 2011). The crude enzyme was collected at an intervals of 2 days; day 2 (48 hours), day 4 (96 hours), day 6 (144 hours) and day 8 (192 hours) and day 10 (240 hours).

3.1.6.2 Effect of temperature on SSF

The effect of temperature on SSF is determined by the usage of different temperature to incubate the fermentation. In this experiment, different temperatures were used which are 25 °C, 30 °C, 35 °C and 40 °C as incubation temperature for growth and enzyme production in 6 days (144 hours).

3.1.6.3 Effect of initial moisture content on SSF

The effect of moisture content on SSF was determined by the usage of different initial moisture content which controlled by adding the distilled water. The moisture contents used are 50%, 60%, 70% and 80%.