PRODUCTION OF FERULIC ACID FROM BANANA STEM WASTE BY USING CO-CULTURE

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PRODUCTION OF FERULIC ACID FROM BANANA STEM WASTE BY USING CO-CULTURE

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Thesis submitted in partial fulfilment of the requirements
for the award of the degree of
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SUPERVISOR'S DECLARATION

We hereby declare that we have checked this thesis and in our opinion, this thesis is adequate in terms of scope and quality for the award of the degree of Bachelor of Chemical Engineering (Biotechnology).

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STUDENT'S DECLARATION

I hereby declare that the work in this thesis is my own except for quotations and summaries which have been duly acknowledged. The thesis has not been accepted for any degree and is not concurrently submitted for award of other degree.

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Dedication

To my dedicated supervisor, Assoc. Prof. Dr. Norazwina bt Zainol,
supportive family and friends.

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I would like to thanks the following people and organisations;

- My supervisor Dr. Norazwina bt Zainol for her guidance through an effective well-arranged weekly meeting.
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- My supportive friends in helping me throughout this project.

ABSTRACT

Ferulic acid (FA) or known as (E)-3-(4-hydroxy-3-methoxy-phenyl) prop-2-enoic acid is a type of caffeic acid derivative. It is widely found in vegetables, fruits and some beverages such as beer and coffee. The abundance of these aromatic compounds in plant materials combine with known microbial transformation made them attractive for biotransformation research. Therefore production of FA by co-culture was a main focus in this study. This research study consists of two objectives. First objective was to perform growth profile for co-culture and produce FA from banana stem waste, thus test for the best producer. Second objective was to perform kinetic study of FA production by using co-culture. Possibility of using cheap materials in this research study had been proven as the co-culture was isolated from soil and banana stem waste (BSW) as the substrate. The method for this research study was divided into six steps. Firstly, substrate was prepared by collecting BSW at Gambang, Pahang, Malaysia. Then, the inoculum for pure culture was prepared to perform the fermentation process. After 24 hours of fermentation, they were ready for the analysis by using HPLC. Based on the analysis, six best producers from pure culture were determined as the best producers which are bacteria *Bacillus thuringiensis* strain NRBC101235 (A), *Brevibacillus formosus* (B), *Bacillus pumilus* SAFR-032 (C), *Bacillus cereus* strain JCM 2152 (D), *Lysinibacillus fusiformis* strain NRBC15717 (E) and *Bacillus cereus* strain ATCC14579 (F). Then, growth profiles for pure culture and co-culture were carried out by using indirect method. From the growth profile result, two sets of inoculum for co-culture were prepared and injected into substrate at their stationary phase. The co-culture was from combination of bacteria ABEF and BCDEF. Then, fermentation process began. From the analysis, the set of co-culture BCDEF was identified as the best producer. They were able to produce FA from the range of 2.0 to 3.0 mg/g. According to the previous research, the concentration of FA should be from the range of 1 to 3 mg/g. Kinetic study for co-culture BCDEF was carried out. The hyperbolic equation proposed by Monod which modified by Lawrence and McCarty was used to describe microbial growth and biodegradation process. In order to solve the equation, the values of kinetic parameters which are Ks, q_{max} and Y were calculated by using Runge-Kutta Fourth method. The values obtained were 0.9 g/L, 0.1669 mmol/cell.h and 0.8077 g/g respectively with R-squared value for substrate and biomass were 0.8538 and 0.8833 each. Hence, FA was successfully produced from banana stem waste by using co-culture.

ABSTRAK

Asid Ferulik (AF) adalah sejenis asid “caffeic derivative”. Ia boleh didapati secara meluas di dalam sayur-sayuran, buah-buahan dan minuman seperti bir dan kopi. Apabila tumbuhan dicampurkan dengan transformasi mikrob, ia menjadikan mereka sebagai bahan tarikan untuk penyelidikan biotransformasi. Oleh itu pengeluaran AF dengan menggunakan ‘co-culture’ merupakan tumpuan utama di dalam kajian ini. Kajian penyelidikan ini terdiri daripada dua objektif. Objektif pertama adalah untuk melaksanakan profil pertumbuhan ‘co-culture’ dan menghasilkan AF daripada batang pisang, dan mengenal pasti pengeluar yang terbaik. Objektif kedua adalah untuk melaksanakan kajian kinetik pengeluaran AF dengan menggunakan ‘co-culture’. Kegunaan bahan kos rendah dalam kajian penyelidikan ini telah terbukti dengan menggunakan ‘co-culture’ yang telah diambil daripada tanah dan batang pisang sebagai substrat. Kaedah di dalam kajian penyelidikan ini telah dibahagikan kepada enam langkah. Pertama, substrat telah disediakan dengan mengumpulkan batang pisang terbuang. Kemudian, inokulum bagi ‘pure culture’ telah disediakan untuk melakukan proses penapaian. Berdasarkan analisis, enam pengeluar terbaik daripada ‘pure culture’ telah ditentukan sebagai pengeluar terbaik iaitu bakteria *Bacillus thuringiensis* NRBC101235 (A), *Brevibacillus formosus* (B), *Bacillus pumilus* SAFR 032 (C), *Bacillus cereus* JCM 2152 (D), *Lysinibacillus fusiformis* (E), dan *Bacillus cereus* ATCC 14579 (F). Kemudian, profil pertumbuhan ‘pure culture’ dan ‘co-culture’ telah dijalankan dengan menggunakan kaedah tidak langsung. Dari hasil profil pertumbuhan, dua set inokulum untuk ‘co-culture’ telah disediakan dan disuntik ke dalam substrat. Kumpulan ‘co-culture’ adalah daripada gabungan bakteria ABEF dan BCDEF. Daripada analisis, ‘co-culture’ BCDEF telah dikenal pasti sebagai pengeluar yang terbaik. Mereka dapat menghasilkan AF dari lingkungan 2.0 hingga 3.0 mg/g. Menurut kajian sebelum ini, kepekatan AF harus dari lingkungan 1 hingga 3 mg/g. Kajian kinetik untuk ‘co-culture’ BCDEF telah dijalankan. Persamaan hiperbolik dicadangkan oleh Monod yang diubahsuai oleh Lawrence dan McCarty telah digunakan untuk menggambarkan pertumbuhan mikrob dan proses biodegradasi. Dalam usaha untuk menyelesaikan persamaan, nilai parameter kinetik iaitu Ks, qmax dan Y telah dikira dengan menggunakan kaedah Runge-Kutta Keempat. Nilai-nilai yang diperolehi ialah 0.9 g/L, 0.1669mmol/cell.h dan 0.8077 g/g, masing-masing dengan nilai R^2 untuk substrat dan biomass adalah 0.8538 dan 0.8833. Oleh itu, AF telah berjaya dihasilkan daripada batang pisang dengan menggunakan ‘co-culture’.

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LIST OF ABBREVIATIONS

FA	Ferulic Acid
BSW	Banana Stem Waste
HPLC	High Performance Liquid Chromatography
GDP	Gross Domestic Product

1 INTRODUCTION

1.1 Motivation and statement of problem

Agricultural is one of the sectors which contribute to Malaysia's Gross Domestic Product (GDP) (D. Bilanović et. al., 2011) . Nearly twenty four percents of Malaysia's land area composed of land which dedicated to agriculture alone. Malaysian farmers produced high quality fruits and vegetables for domestic market consumption such as durian, coconuts, bananas, pineapples and paddy. Consequence to this activity, many agricultural residues left abundant. It may cause environmental pollution. By exploiting agricultural residue, it will solve the problem facing by most of the farmers. Banana plant is the largest herbaceous flowering plant (Kumar & Kumar, 2011). In fact, banana stem waste (BSW) is one of the agricultural wastes that can be found easily in Malaysia. Moreover, it is available in large quantity after harvesting. Banana stem cell wall contain lignocelluloses materials (Cruz et. al., 2001). There are three main components of lignocelluloses which are cellulose, hemicellulose, and lignin (Ibrahim et al., 2010). Cellulose and hemicellulose can be hydrolysed with chemicals and/or enzymes to monomeric sugars, which can subsequently be converted biologically to any bio products (Hasyierah et al., 2011). Hence, it can be used as an alternative source of phenolic acid production.

Ferulic acid (FA) [(E)-3-(4-hydroxy-3-methoxy-phenyl) prop-2-enoic acid] is belongs to the family of phenolic acids (Mancuso & Santangelo, 2014). FA is a chemical compound that plays role in various biochemical processes. It grabs all the attention as a chemical with many potential applications. FA had been used for several applications especially in the food, health, cosmetic and pharmaceutical industries (Ou and Kwok, 2004). Therefore, recovery of FA from BSW becomes very important to fulfil the market demand in large scale of production. **Error! Reference source not found.**.1 below shows the chemical structure of ferulic acid.

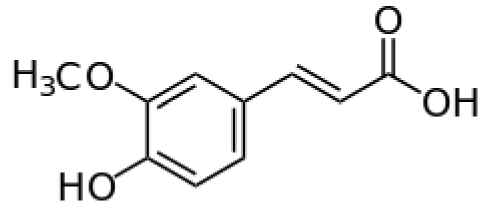

Figure 1.1: Chemical structure of ferulic acid

Fermentative production is highly recommended by using cheap by-products and waste substrates (S. Dominik and J. Anna, 2011). The main problem in producing FA is to find raw materials which are environmental friendly and cheap. Most of the materials used for FA production are sugar based feedstock. There were several researches on FA production from waste such as wheat bran, paddy straw (Hasyierah et al., 2011) and wheat straw. According to Papagianni et al (2001), chemical purity is mainly depends on the essential part in the fermentation medium especially when cheap materials are being used. So, contrast to the statement, production of FA from BSW can be produce in large scale due to low cost raw material and easy to find in Malaysia. This second largest tropical fruit cultivation has led to generate several tons of underuse by-product and waste. So, by exploiting this residue, it will reduce ecology damage due to improper agricultural management practice. This is an advantage to take the BSW as the raw material and study their FA production.

Apart from that, many researchers had been reported in releasing FA by using only a single pure culture. Sometimes the process needs more than one culture to release FA efficiently. Co-culture fermentations may result in increased yield, improved control of product qualities and the possibility of utilizing cheaper substrates. Co-cultivation of different microorganisms may also help to identify and develop new biotechnological substances. As Bertrand et al (2014) had mentioned in their findings, the co-production of enzyme by microorganisms is very important to increase the production of ferulic acid. Hence by using soil co-culture, FA production from BSW can be produce in maximal yield. Co-culture provides better biomaterial to be used under physiological condition. Hence, in this study, the best condition to produce FA was proved by using co-culture which is facultative anaerobic bacteria and BSW as the substrate.

1.2 Objectives

The following are the objectives of this research:

- o To perform growth profile for co-culture and produce FA from banana stem waste thus test for the best producer.
- o To perform kinetic study of FA production by using co-culture.

1.3 Scope of this research

In this research study, scopes function as a guideline to achieve the objectives. As FA is most abundant in plant, BSW was used as the substrate. Firstly, BSW was collected at Gambang, Pahang, Malaysia. The co-culture was isolated from the soil where soil was collected from the banana farm. In order to keep the BSW moist, it was stored in the $4^{o}C$ refrigerator. To complete the first objective, 21 sets of pure cultures were grown on the agar plate. Then, BSW and nutrient broths were prepared. Nutrient broths were prepared for the inoculum process. After 24 hours streaking and 24 hours inoculum process, pure cultures were ready for the fermentation process. Samples containing BSW and pure cultures were incubated in an incubator shaker for 24 hours at 150rpm and $35^{o}C$. On the next day, the production of FA was analyzed by using High Performance Liquid Chromatography (HPLC). Based on the data analyzed, the best producers for FA production were determined. There were six pure cultures determined. From them, two sets of co-cultures were prepared and analyzed again for the best co-culture producer. Before fermentation process for the co-culture started, growth profiles for each six types of pure cultures and two sets of co-cultures were studied. Method using was indirect method. This method using spectrophotometric measurements of the developing turbidity at the same 1 hour interval with total of 24 hours as an index of increasing cellular mass. Therefore, the optical densities (OD) of the co-culture were recorded. Identification of the best co-culture was done based on their contribution to produce FA by using BSW. Therefore, first objective was completed.

The best pure cultures were going through identification processed by using molecular method. This identification required PCR and cycle sequencing to isolate genomic DNA from bacteria. This protocol involved breaking the cells opened with a series of freeze/thaw cycles. PCR reaction was set up to amplify a region of the 16S rRNA gene. The cleaned PCR product was used as the template for a sequencing reaction was run in a thermocycler (PCR machine). The electropheragrams was viewed from the sequencing reaction and the sequence was used in a BLAST search limited to a bacterial data base. Unknown bacteria were identified by examining the top-scoring sequences from the BLAST search results. For the second objective, kinetic study of FA production from one set of co-culture which was the best producer was studied. Modified monod equation method was used in this study. BSW and biomass concentration data were collected by interval of 3 hour for total of 24 hours. Based on the data, Runge Kutta-Fourth method was used to calculate the kinetic constants which were Ks, q_{max} and Y.

1.4 Main contribution of this work

From this research study, it can enhance the knowledge about production of FA by using co-culture. The usage of co-culture is one of the best methods in the fermentation as it was able to release multi-enzyme in the process. Many researchers had been reported in FA released using only a single pure culture. However this process needs more than one pure culture to release FA in higher yield. Therefore the co-production of enzyme by microorganisms is very important to increase the production of ferulic acid (Bertrand et al., 2014). Nevertheless, co-cultivation of different microorganisms may also help to identify and develop new biotechnological substances. Besides that, degradation of FA from BSW was one of the initiatives in using cheap materials and waste as the substrate. As mentioned by Zhao S. et al (2013), even though corn bran has highest content of ferulic acid, corn bran has low value and often used for animal feed alone or in combination with corn germ cake or meal. Hence by using soil co-culture, FA production from BSW can be produce in maximal yield. In fact, the study helps to optimize the production of FA by scaling up the process. Considering the exponential increase in the number of papers on this topic that have been published last several years, the use of this method will expand rapidly and yield important and fascinating discoveries.

1.5 Organisation of this thesis

Chapter 2 provides a description of the FA, its characteristic and application. In addition, the advantages of using BSW as the substrates and soil microorganism were explained. The chapter also provides a brief discussion on growth profile of microorganism and kinetic study of FA production. Apart from that, additional information on composition of natural cellulosic feedstock also explained below. There are three composition of natural cellulosic which are lignin, cellulose and hemicellulose. A summary of the previous experimental work on FA fermentation was also presented.

Chapter 3 gives a review of the chemicals, raw materials and the methods for the fermentation process. This chapter presents the experimental setup in completing the laboratory works. It includes in performing growth profile of the microbe, fermentation process and analysis of FA by using HPLC. Lastly, kinetic study for FA production was carried out. In this microbial process, bacteria were isolated from soil and BSW as a substrate. Preliminary studies were conducted in determining the bacteria that was chosen to further with the research of FA production from co-culture. Selection of co-culture was based on the performance of the pure culture in the release of FA from BSW.

Chapter 4 is devoted on the fermentation process to determine the best producer of FA production. In this chapter, result and discussion for the experimental study were presented. There were results for fermentation process by using pure culture and co-culture and growth profile for six pure culture and two sets of co-culture. Apart from that, kinetic study result for co-culture also discussed here.

Chapter 5 draws together a summary of the thesis and recommendation which might be derived in this work.

2 LITERATURE REVIEW

2.1 Overview

This study presents a microbial fermentation study of FA production by using co-culture. There were several researchers had done their research in the production of FA. However, there are plenty of studies in the fermentation of FA by using co-culture. Most of the research studies were on the production of FA by using pure culture. Previous work on the production of FA was explained below. In addition, detail explanations about FA, soil microorganism, banana stem waste (BSW), composition of natural cellulosic feedstock, growth profile of bacteria and kinetic study by using Monod equation were explained below.

2.2 Introduction

Phenolic acids have been studied extensively due to their anti-oxidative, anti-inflammatory, and other health related properties. It had been demonstrated both in vitro and in vivo (Sarangi and Sahoo, 2009). FA was bound to the hemicellulose of plant cell walls which is ubiquitous hydroxycinnamic acid. Agricultural lignocellulosic residues are the major potential source of low-cost raw material for commercial FA production. In order to achieve it, many researchers did their study on FA production by using agricultural waste as the raw material. However, plenty of them were using co-culture to degrade the waste. Thus, this research mainly focuses on the production of FA by using banana stem waste and co-culture. There are promising studies on FA fermentation with co-cultures bacteria. The main challenge with co-culture fermentation is finding and providing the optimal environmental conditions for two different bacteria, simultaneously.

2.3 Previous work on Ferulic Acid

According to Karagöz and Özkan (2014), agricultural lignocellulosic residues are the major potential source of low-cost raw material to commercialize FA production. Therefore, most of the researchers were using agricultural material in their studies. FA is commonly found in grains such as corn, wheat, barley, rye, oats and rice (Perez et. al., 2005). Besides, it also contains in fruits, vegetables, and in a wide variety of plant tissues. In barley, rye and wheat, the levels of FA decrease with age. This is correlated with an increase in the biodegradability of the plant tissue. In corn, the concentration of FA in hulls is 2.0 to 4.0% of the dry weight. Typically, levels of FA are higher in the hulls or bran of grains.

Based on Sarangi and Sahoo (2009) research, commercial natural FA was obtained from wheat bran. Wheat bran is an important by-production of the flour industry. The bran contains some starch, protein and hemicelluloses. It also contains many phenolic acids, such as FA and vanillic acid. Besides that, FA can be produced by using rice bran. However, it is expensive due to the nutrition and γ-oryzanol it contained. Therefore, some alternative FA containing lignocellulosic materials have been investigated. One of the potential lignocellulosic materials is paddy straw (Hasyierah et al., 2011) as it is abundantly available and is currently under-utilized. For example, in Malaysia, the production of rice is reported to be 2.4 million tons/year. It was resulting into a huge production of rice-straw. Since this paddy straw is regarded as a waste, Hasyierah et al. (2011) had made an initiative to extract FA. Ferulic acid is found varying from 5 g/g in wheat bran and corn kernel, 9 g/g in sugar beet pulp, and 15−28 g/g of rice bran oil (Zhao et al., 2013). Although wheat bran is rich in FA, it is expensive due to the nutrition and γ-oryzanol it contained.

There are also researches of FA production by different microorganism. For example, production of FA from clove oil by *Pseudomonas fluorescens* E118. *Pseudomonas fluorescens* E118 contains abundant eugenol-degrading microorganisms. It is a clove-oil-tolerant strain, accumulated 6.1g/L FA under optimized culture conditions with the intermittent addition of eugenol (Musatto et. al., 2007). When the bacterium was applied to FA production, 5.8g/L FA was produced with the intermittent addition of clove oil. Since clove oil is much cheaper than eugenol, FA production from clove oil by the bacterium was promising for the industrial production of FA (Furukawa et al., 2003).

There was also release of FA from agroindustrial by-products by the cell wall-degrading enzymes produced by *Aspergillus niger* I-1472 (Bonnin et al., 2002). *Aspergillus niger* I-1472 was grown on sugar beet pulp to produce cell wall polysaccharide-degrading enzymes, including feruloyl esterases. These enzymes were tested to release FA from sugar beet pulp (Ferreira et al., 2007), maize bran, or autoclaved maize bran. They were efficient as the commercial mixture to release FA from sugar beet pulp (Ferreira et al., 2007).

As a result, they were much more efficient to release FA from maize bran after autoclaving pretreatment, as 95% of FA ester was solubilized. Scientific databases provide only limited knowledge on ferulic acid esterases (FAEs) produced by bacteria, especially by FA bacteria (Wang et al. 2004). Donaghy et al. (1998) screened 80 Bacillus-type strains and 50 gram positive bacteria (*Enterococcus, Lactobacillus, Lactococcus, Leuconostoc, Pediococcus* and *Propionibacterium*) in agar-plate assay. The highest FAE activities were seen in *Lactobacillus fermentum* and *Bacillus subtilis* strains (McMurrough et. al., 1996)

2.4 Ferulic Acid

Ferulic acid is a derivative of cinnamic acid with the molecular formula of $C_{10}H_{10}O_4$. In 1886, Hlasiwetz Barth isolated 3-methoxy-4-hydroxycinnamic acid (P. Laure, 2007) from the genus *Ferula foetida* for the structure determination (Go, M. and Cantero, 2003). FA together with dihydroferulic acid is a component of lignocelluloses which conferring cell wall rigidity by cross linking lignin and polysaccharides (Idrees et al., 2013). Biosynthesis of FA and other cinnamic acids is from the amino acids phenylalanine or tyrosine. It is commonly found in seeds of plant such as rice, wheat and oats. FA in plants usually exists as the *trans*-isomer, although *cis- trans*-isomerization across the double bond has been reported (Ferreira et al., 2007).

The abundance of these aromatic compounds in plant materials and in agricultural by-products combined with the vast array of known microbial transformations of these compounds make them attractive for biotransformation research (Gerard, 1997). FA was first synthesized in 1925 by the Knoevenagel condensation reaction. It was by condensation of malonic acid with vanillin in the presence of quinolone or by Perkin condensation of vanillin with acetic anhydride. Synthetic FA has been characterized by C-NMR and X-ray crystallography. FA occurs as colourless orthorhombic needles when crystallized from hot water. It has a molecular weight of 194 and a melting point of 174°C. FA is soluble in alcohol, ethyl acetate and hot water. However, it is moderately soluble in ether and sparingly soluble in benzene and petroleum ether (Gerard, 1997).

Isolation of FA from plant materials has been achieved by hydrolysis of FA esters with 10% H_2SO_4 followed by extraction with ethyl acetate. Separation and analysis of FA and other plant phenolic compounds has been accomplished with thin-layer chromatography (TLC), high performance liquid chromatography (HPLC) and standard column chromatography methods (Gerard, 1997). In nature, FA is found predominately esterified to a wide variety of compounds such as saccharides, glycoproteins, lignin, hemicellulose, quinic, carboxylic acids, fatty acids, betacyanins and sterols (Zhao et al., 2013).

The occurrence of FA in dietary food products and traditional medicinal preparations had prompted investigations into the pharmacology and toxicology of FA. For example, it has protective agents against UV-radiation-induced skin damage, treatment for cardiovascular diseases and cerebral thrombosis (Xie et al., 2010). Moreover, it has also been associated with other physiological effects (Mancuso and Santangelo, 2014), including inhibition of uterine contractions, anti-inflammatory, hypocholestrolemic and inhibition of viral replication. Studies of the feruloyl sterols found in rice bran oil (Gerard, 1997) showed that these ferulic acid esters caused numerous CNS effects. It includes increasing of spontaneous motor activity, decreased time to death and body temperature. In addition, FA exhibited biochemical role in the inhibition of seed germination, inhibition of indole-acetic acid and enzyme, inhibition of decarboxylation activity and other protective effect on microorganisms and pets (Zenno et al., 2003).

There are vast numbers of studies documented on the bio-medical properties of FA such as antioxidant activity (Shahidi et. al., 1999), UV-absorbing capacity and its effect of lignin as precursor in plants metabolic pathway. It is also listed as 'antioxidant' in the 'food additives' list. It has been reported to maintain the colour tone of Greenpeace, prevent discoloration of Green Tea and oxidation of banana turning black colour. Thus, it reduces bacterial contamination.

The synthesis of FA was established when FA was used as a precursor in the manufacturing of vanillin and malonic acid. Vanillin is the world's most highly prized natural flavour. It is one of the most important aromatic flavour compounds used in foods, beverages, perfumes and pharmaceuticals (Sarangi and Sahoo, 2009). Currently, 12,000 tons (Gerard, 1997) of synthetic vanillin is produced annually for the flavouring industry. The conversion of FA to vanillin has been reported to occur by *Fusarium solani* and *Paecilomyces variotii* via oxidation of 4-vinylguaiacol. It is also has been isolated from *Bacillus subtilis* when incubated with FA (Mancuso and Santangelo, 2014).

Form the previous research, free FA, feruloyl glucose and feruloylputrescine are found in citrus juices such as orange juice. These compounds are contributed to the flavour of the juice. Thus, considering the increasing interest in 'natural' products, the production of flavours via biotechnological processes offers a viable alternative to natural and chemical sources (Sarangi and Sahoo, 2009).

Currently, two methods were developed to break the cross-link and release FA from plant cell wall materials. First, an enzymatic method using FAEs and the other is alkaline hydrolysis which release FA from polysaccharides which was often used to determine the content of FA in bran (Oosterveld et al. 2000). Ferulic acid esterase (FAE) or feruloyl esterase is a subclass of carboxylic acid esterase. It catalyses the hydrolysis of the ester bond between hydroxycinnamic acids and sugars on the plant cell. FAEs can be classified into four subclasses, type A-D, based on the activities toward a set of synthetic substract (Crepin et al. 2004).

The activity of ferulic acid esterase was first discovered in culture filtrates of *Streptomyces olivochromogenes* and *Schizophyllum commune* (Settachaimongkon et al., 2014). Studies show that the type of purified ferulic acid esterase from *Streptomyces olivochromogenes* and ferulic acid esterase (FAE-I) from *Aspergillus niger* alone could not release ferulic acid from wheat bran. However, purified ferulic acid esterase (FAE-II and FAE-III) from *Aspergillus niger* has been shown in isolation to release ferulic acid from wheat bran and barley spent grains. Each ferulic acid esterase also has its own specificity with regard to the release of specific cinnamic acids such as ferulic acid, ρ-coumaric acid, sinapic acid or caffeid acid.

2.5 Soil microorganism and co-culture

Microorganisms have a long track record as important sources of novel bioactive natural products, particularly in the field of drug discovery (R. Roberts, 1998). Microbes have been shown to biosynthesize a wide array of molecules. Recent advances in genome sequencing have revealed that such organisms have the potential to yield even more structurally diverse secondary metabolites.

In the last ten years, several methods have been developed to aid in the activation of these cryptic biosynthetic pathways. One of these approaches is microorganism co-culture. It involved the cultivation of two or more microorganisms in the same confined environment. Co-culture was defined as anaerobic or aerobic incubation of different specified microbial strains under aseptic conditions (J. Bader et al.,2009). Microorganism co-culture is inspired by the natural microbe communities that are omnipresent in nature. Within these communities, microbes interact through signalling or defence molecules. Microorganism co-culture can be achieved in either solid or liquid media and has recently been used increasingly extensively to study natural interactions and discover new bioactive metabolites.

Because of the complexity of microbial extracts, advanced analytical methods are key for the successful detection and identification of co-culture induced metabolites (Bertrand et al., 2014). According to J. Bader et al (2009), the advantageous utilization of co-cultures instead of single cultivations includes the production of bulk chemicals, enzymes, food additives, antimicrobial substances and microbial fuel cells. Co-cultivation of different microorganisms may also help to identify and develop new biotechnological substances. The relevance of co-culture fermentations and the potential of improving existing processes as well as the new production of new chemical compounds in industrial biotechnology can be pointed out. Some examples for the coexistence of different micro-organisms are the forest soils, compost piles, the aerobic and the anaerobic zones of water, spontaneous fermentations of sugar-containing saps and the human skin.

Co-cultures of different microorganisms may be also advantageous for the production of enzymes. One example is the production of laccases (García et al., 2011). These enzymes are able to hydrolyse the polymer lignin and may allow the utilization of this complex biopolymer for the production of fine chemicals. Further applications of laccases may be the decolorization of textile dyes or the production of biosensors. Transition elements such as manganese or phenolic compounds results in cost-intensive waste water treatment. Another industrially important enzyme is tannase (Ferreira et al., 2007). It is used in food, feed, pharmaceutical and textile industry. These biological approaches may be an emnvironmentally friendly and cost-saving alternative for the production of these enzymes.

The usage of soil microorganism was performed in this study. Soil contains variety of microorganisms including bacteria that can be found in any natural ecosystem. Microorganisms play an important role on nutritional chains that are an important part of the biological balance in the life in our planet. Without bacteria, soil would not be fertile and organic matter such as straw or leaves would accumulate within a short time (Kummerer, 2004). The use of co-cultures of a single strain of a microorganism is a method that has the advantages of being reproducible and amenable to further process development (Gerard, 1997). Furthermore, some fermentation process needs more than one enzyme to release FA efficiently. Therefore the co-production of enzyme by microorganisms is very important to increase the production of FA (Zenno and Iwasawa, 2003).

2.6 Microbial co-culture fermentation

Co-culture is an anaerobic or aerobic incubation of different specified microbial strains under aseptic conditions (Kummerer, 2004). Chemical substances produced each year such as fuels, fine chemicals and pharmaceuticals worth several billion Euros by biotechnological processes using renewable resources (Lemos et al., 2014). Because of the sterile cultivation enables an easy way of controlling microbial milieu, growth and product formation, most of the products in industrial biotechnology today are formed using processes involving a single microbial strain (J. Bader et al., 2010). On the other hand, there are many instances where the utilization of co-cultures appears to be advantageous over a single microorganism. It is because, the potential for synergistic utilization of the metabolic pathways of all involved strains in a co-culture situation. Most biotransformation in nature takes place by the combination of metabolic pathways from different microorganisms (Bertrand et al., 2014). Moreover, co-cultivation may result in increased yields, a reduction of process costs because of cheaper substrates (Kleerebezem and Van Loosdrecht, 2007) and control of product quality.

In some cases, production of substances normally not formed by pure cultures can be observed through the induction of appropriate genes in co-cultivation processes. Co-culture fermentation may have a great impact on the development of biofuels, bioenergy and bio-based products. Examples of the utilization of co-cultures in food industry are the production of cheese, yoghurt, sourdough, African fermented dairy products and Belgian beer such as Lambic. In co-cultures, degradation and metabolization of substrates occur by the combined metabolic activity of the known microbial strains under aseptic conditions (J. Bader et al., 2010). Modification of raw materials during food production by co-cultures results in improved texture, taste and flavor and in microbial stabilization. This protection may be caused by a decreased pH-value or by the formation of growth-inhibiting substances such as lactic acid, acetic acid and ethanol.

Apart from that, energy consumption and the use of environmentally hazardous substances can often be reduced by biotechnological production processes (Bertrand et al., 2014). Further advantages may be the production of pure enantiomers, reduced steps required in synthesis of products, and less stringent security needs resulting in reduced production costs (Gerard, 1997). The risk of accidents decreases as a result of lower process temperatures and normally low pressures in biotechnological processes. Moderate process conditions result in lower required charge in the field of process security and approval procedures. These biological approaches may be an environmentally friendly and cost-saving alternative for the production of these enzymes.

The controlled cultivation of co-cultures enables the synergistic utilization of the metabolic pathways of the participating microorganisms under industrial, reproducible and controlled condition (Papagianni et. al., 2001). The optimal values of process parameters are pH, temperature, oxygen demand and the acceptable ranges of substrate and product concentrations have to be known and considered to achieve the controlled fermentation, as in pure culture cultivation (Pinar and Melek, 2014). In co-culture fermentation processes, the complexity of possible interactions which are positive or negative has to be taken into account. All aspects, the process parameters, the produced and secreted substances and possibly the occurring biotransformation, may provide an opportunity to control growth and product formation during co-culture fermentation processes. Parameters have to be found enabling the utilization of the desired part of the metabolic pathway of every single strain in co-culture to achieve the development of a controlled co-culture fermentation process and to form the favored product (J. Bader et. al., 2010).

2.7 Banana Stem Waste (BSW)

Banana is the common name for herbaceous plants of the genus *Musa* (Alwi et al., 2013) and for the fruit they produce. Banana (*Musa spp.)* is an important world food crop. It is grown and consumed in more than 100 countries throughout the tropics and subtropics. In developing countries, they are four most important food crops after rice, wheat and maize. Some study shows that there are higher content of several phenolic compounds such as dopamine and cyanidin-related compounds, lignin, and FA (Dom et al., 2008) were observed. In Malaysia, the production of commercial varieties of banana has increased by 24–27% (Alwi et al., 2013) over the decades. It gives an amount of 27,453 hectares in 2009 with Johor, Pahang, and Sarawak as the largest banana-producing states.

Bananas come in a variety of sizes and colours when they are ripe. The colours are yellow, purple, and red. In popular culture and commerce, "banana" usually refers to soft and sweet dessert. According to Alwi et al (2013), many varieties of bananas are perennial. The banana plant is the largest herbaceous flowering plant. Plants are normally not very tall. Their main or upright stem is called pseudostem that grows 6 to 7.6 meters tall. They grow from a corm. Each pseudostem can produce a single bunch of banana. After fruiting, the pseudostem dies, but offshoots may develop from the base of the plant. Leaves are spirally arranged and may grow 2.7 meters long and 60 cm wide. They are easily torn by the wind, resulting in the familiar frond look.

Each pseudostem normally produces a single inflorescence or known as the banana heart. The inflorescence contains many bracts or called as petals in between rows of flowers. The female flowers which can develop into fruit appear in rows further up the stem from the rows of male flowers. The ovary is inferior, meaning that the tiny petals and other flower parts appear at the tip of the ovary. Banana fruit develop from the banana heart, in a large hanging cluster, made up of tiers with up to 20 fruit to a tier. The hanging cluster is known as a bunch, comprising 3–20 tiers, or commercially as a "banana stem", and can weigh from 30–50 kilograms. In common usage, bunch applies to part of a tier containing 3-10 adjacent fruits.

Major factor in the economic production of FA is the cost of raw material. By-products of agriculture industries are one of the alternatives substrate and renewable resources for FA fermentation. Depending on the availability of the substrate in the country, variety of glucose was used to produce FA such as lignocellulosic biomasses (Lithchfield, 1998). Lots of studies have been done by using various agriculture resources such as wheat straw (Karagoz and Ozkan, 2014), wheat bran (Xie et al., 2010), paddy straw (Hasyierah et al., 2011) and corn. Banana stem waste (BSW) is one of the new renewable sources that have been used as substrate. According to the Table 2.1, Alwi et al., (2013) stated that glucose content in the banana pseudo stem is 74.0% higher compared to the others. The statement also supported by Sinha et al., (2012), where banana stem-central core contain 1.20% of carbohydrates per 100 gram. Therefore, in this study, the using of BSW as the substrate was established. As banana stem has no use after harvesting the fruit, it is good to make them as the carbon source for the ferulic acid fermentation process rather than throw them away.

Banana stem waste is a lignocelluloses waste, which consists of lignin, cellulose and hemicellulose. It consists of 15.42% lignin, 53.45% cellulose and 28.56% hemicelluloses (Alwi et al., 2013). In general, according to Romero et al., (2011) banana stem has potential for providing products such as manure and feet, but this practice only processes a small fraction of the total waste production. Through the exploitation of this waste material, high value compound is very significant and promising high profits with low cost (Marcia et al., 2007).

In biological pre-treatment, microorganisms, mostly fungi, are used to digest lignin and hemicellulose in waste materials. White-rot fungi such as *Pleurotus ostreatus* and *Pycnoporus cinnabarinus* 115 are preferred for biological pretreatment because of its high efficiency in degrading or modifying the lignin content in lignocellulosic biomass. Besides, some modifications have been made to fungal cultivation to improve the digestion of lignin and avoid degradation of cellulose. However, microorganism was used in this study to degrade the lignin and hemicellulose (Bajpai and Stahl, 2010).

Table 2.1: Chemical composition of different morphologic regions of banana plant

	Pseudostem	**Petioles/mid rib**	**Leaf blade**	**Floral Stalk**	**Leaf Sheaths**	**Rachis**
Glucose	74.0 [a]	68.1 [a]	60.0 [a]	79.8 [a]	74.2 [a]	31.8 [a]
Xylose	13.1 [a]	23.6 [a]	17.5 [a]	9.3 [a]	13.8 [a]	14.0 [a]
Galactose	2.5 [a]	1.1 [a]	3.8 [a]	2.9 [a]	2.2 [a]	1.7 [a]
Arabinose	9.1 [a]	4.9 [a]	15.5 [a]	5.1 [a]	7.5 [a]	4.1 [a]
Mannose	1.3 [a]	1.5 [a]	2.3 [a]	2.2 [a]	1.5 [a]	2.9 [a]
Rhamnose	-	0.8 [a]	0.9 [a]	0.7 [a]	0.8 [a]	0.7 [a]
Lignin	12.0 [a]	18.0 [a]	24.3 [a]	10.7 [a]	13.3 [a]	10.5 [a]
Cellulose	34 - 40 [a]	31.0 [a]	20.4 [a]	15.7 [a]	37.3 [a]	31.0 [a]
Holocellulose	60 - 65 [a]	62.7 [a]	32.1 [a]	20.3 [a]	49.7 [a]	37.9 [a]
Ash	14.0[a]	11.6 [a]	19.4 [a]	26.1 [a]	19.0 [a]	26.8 [a]
Potassium	33.4*	9.4*	11.6 *	23.1 [a]	21.4 *	28.0*
Calcium	7.5*	32.3*	8.0*	0.6*	5.5 *	0.6*
Magnesium	4.3*	2.9*	1.1*	0.5*	1.9*	0.3*
Silicon	2.7*	7.0*	24.9*	7.8*	2.7*	1.2*
Phosphorous	2.2*	0.7*	0.7*	0.7*	0.9*	1.7*
Pentosans	-	16.2 [a]	12.1 [a]	8.0 [a]	12.3 [a]	8.3 [a]
Starch	-	0.4 [a]	1.1 [a]	26.3 [a]	8.4 [a]	1.4 [a]
Proteins	-	1.6 [a]	8.3 [a]	3.2 [a]	1.9 [a]	2.0 [a]

2.8 Composition of natural cellulosic feedstock

The use of second generation feedstock in order to meet growing demands for future ferulic acid production is ultimately unsustainable. Furthermore, there are several limitations to starch and sugar based production. As shown in Figure 2.1, there are three main components of lignocelluloses which are are cellulose, hemicellulose, and lignin (Idrees et al., 2013). Cellulose and hemicellulose can be hydrolysed with chemicals and/or enzymes to monomeric sugars, which can subsequently be converted biologically to ferulic acid. Cellulose is the component of prime interest and can be chemically or enzymatically hydrolyzed to glucose which is the major substrate in ferulic acid fermentation.

Hemicellulose has a less compact structure than cellulose and can be significantly degraded. According to Devanand et al (2005), if hemicellulose is successfully removed from the complex and degraded to oligo or mono xylose, these smaller molecules can be further converted to other by-products. Within the complicated meshwork, lignin has a considerable impact on other existing links. The most important effect is the enhancement of the strength of hydrogen-bonds between polysaccharides, which in turn increases the stability and rigidity of the cellulose-hemicellulose structure. This type of protection successfully reduces the chance of penetration of wall-degrading enzymes, and serves as an effective barrier to pests and diseases to protect the plant body. However, this protection is not preferred in bioprocessing since it limits enzyme accessibility to the internal polysaccharides.

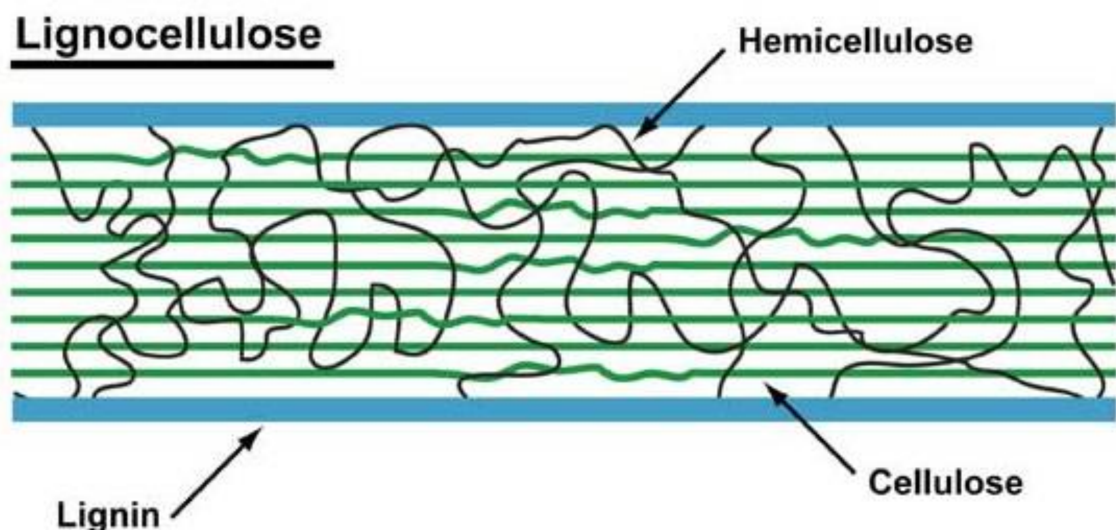

Figure 2.1: Three main components of lignocelluloses

2.8.1 Cellulose

As the most abundant organic substance on earth, cellulose is one of the most studied chemical compounds on earth. It is found in the cell wall of all plants. Cellulose is reported as the structural component of plants. It consists of linear β-1,4-linked D-glucopyranose (Saravanan and Aradhya, 2011) residues with a degree of polymerization up to at least 15000 without branches. Hydrogen bonds between cellulose molecules enable the neighbouring parallel or anti-parallel linear chains to become condensed. It will form an extremely long and thin structure hence building up the main microfibrillar phase. The crystalline structure of cellulose chain gives rise to its considerable tensile strength with minimal flexibility and water insolubility. Therefore, it is fairly resistant to biological attack, which is a major limitation to cell wall hydrolysis.

2.8.2 Hemicellulose

Hemicellulose is the second abundant polysaccharide (Saravanan and Aradhya, 2011) in plant cell wall. It constitute about 20-35% of the plant materials. Unlike the comparatively uniform composition of cellulose, hemicellulose varies tremendously among different cell types and species. Also, the degree of branching and the characteristic of the minor sugars within hemicellulose differ from plant to plant. However, there is usually one kind of hemicellulose that is predominant in a certain plant.

The major hemicellulose in cereals and hardwood is xylan. Xylan generally contains a backbone of β-1, 4- linked xylose residues. This structure is occasionally substituted by α-linked 4-O-methylglucironic acid on C2, α-linked arabinose or acetyl esters on C2 or C3 of some xylose residues. Hemicellulose non-covalently links onto cellulose with hydrogen-bonds and its C-5 sugar ring. It shows an asymmetric configuration which assists celluloses to form the liquid crystalloid.

However, xylan itself does not have a crystalline structure because it lacks the ability to form hydrogen bonds between neighbouring polysaccharide chains. Within some plant cell walls, cellulose and xylan have nearly the same amount; but since xylan is able to form hydrogen bonds only on one side, just one half of xylan could be found to directly interact with cellulose, which assists in constructing the structural backbone for the plant cell wall. This kind of intersection is sometimes identified as the sheathing of cellulose by hemicellulose, which is also considered an impediment for cellulose digestion.

2.8.3 Lignin

Certain differentiated cell types contain lignin. It is the most abundant aromatic polymer in nature that is gradually laid down when cell elongation occurs. It normally constitutes about 10-25% (Saravanan and Aradhya, 2011) of the plant material. Three different alcohols are precursors for lignin synthesis which are ρ-Hydroxyphenyl alcohol, guaiacyl alcohol and syringyl alcohol (Romero-Anayaa et al., 2011). They link with each other by a wide variety of bonds in order to form a huge network in the final phenolic compound. In addition, the lignin network continues to expand as long as the precursors are available. Hence, it will filling up the spaces that have not been occupied by other components and greatly replacing water. Within the complicated meshwork, lignin has a considerable impact on other existing links. The most important effect is the enhancement of the strength of hydrogen-bonds between polysaccharides, which in turn increases the stability and rigidity of the cellulose hemicellulose structure. This type of protection successfully reduces the chance for penetration of wall-degrading enzymes, and serves as an effective barrier to pests and diseases to protect the plant body. However, this protection is not preferred in bioprocessing since it limits enzyme accessibility to the internal polysaccharides.

2.9 Bacteria Growth Profile

Life is essentially connected with growth. The phenomenon of growth can be studied scientifically from two different points of view (F. Thomas, 1999). First view is on the level of the cell or the organism. This is where the finely tuned cellular processes undergo the increasing in length and volume, division and replication. These processes can be studied without looking at the population as a whole. On the other hand, population will be another point of view. This is where the increase of individuals number or the population size. In fact, the mathematical description and modelling are of interest. This can be studied and modelled without knowledge of the cellular mechanisms. A basic approach in microbiology is the study of growth from viewpoint of population level. This is experimentally simple because growth of bacteria can be very fast and requires only small culture volumes.

Modelling and simulation of microbial cell growth is important both theoretically and practically. Although the Monod model has been the most widely used for the prediction of cell growth, it only fits the exponential growth phase (Lin et al., 2000) of the growth, without inhibition. The characteristic of growth in liquid culture for bacteria can be display by perform their growth profile. It is divided into four phase (Widdel, 2010).

First phase is the lag phase (Widdel, 2010) where the bacteria are adapting to the conditions in the medium by synthesizing the necessary enzymes for the utilization of the available substrates. There are slight increase in cell mass and volume but no increase in the cell number. There are possibility to happen multiple lag phases. It will happen when medium contains more than one carbon source.

Then, it will come to the exponential phase (Widdel, 2010). In this phase, there is a rapid exponential increase in population, which doubles regularly until a maximum number of cells are reached and become constant. The synthesis of all cell constituents increases at as constant rate so that the cell population doubles and continue to double at regular intervals. The environment of the cells is continuously changing owing to the consumption of substrates and accumulation of products.

Towards the end of the exponential phase, the growth rate decreases until it reaches zero. It is called stationary phase (Widdel, 2010). In this phase, the number of cells undergoing division is equal to the number of cells death. The number of cell remains constant and there is no net growth. Growth may occur but it is balanced by death of the cells. The cells have stop growing because of the depletion volume of substrate.

Finally, it will come to the death phase (Widdel, 2010). It is because of the continuing depletion of nutrients and build-up of metabolic wastes, the microorganisms die at a rapid and uniform rate. During this phase, the death rate will exceed the growth rate. A straight line is obtained indicating that the cells are drying at exponential rate. Therefore, most of the researcher will take the bacteria at exponential phase to undergo fermentation process.

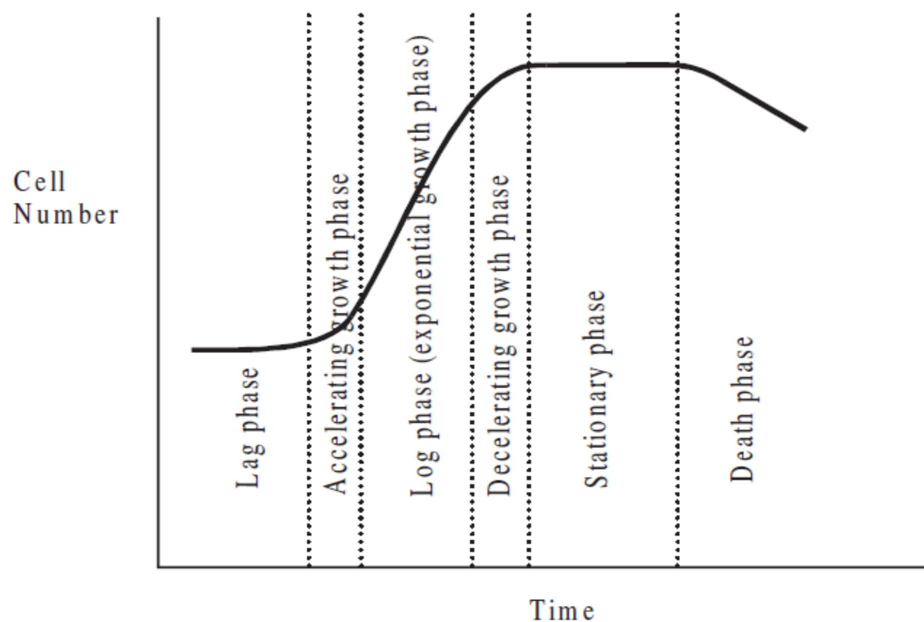

Figure 2.2: Growth curve of bacteria

Figure 2.2 above shows the growth curve of the bacteria. It indicates four phases which are lag phase at the beginning, log phase or exponential phase, stationary phase and lastly death phase.

2.10 Kinetic Study for Production of Ferulic Acid by Using Co-culture

Kinetic modelling is considered to be useful to predict the effect of operating parameters on biomass growth, substrate consumption, and product formation and also in the optimization of fermentation process. The relation between the specific growth rate, μ of a population and the substrate concentration, S is a valuable tool in biotechnology (Strigul et al., 2009). This relationship is represented by a set of empirically derived rate laws referred to as theoretical models (Liu, 2006). Several microbial growth and biodegradation kinetic models have been developed, proposed and used in bioremediation schemes. It includes Monod's, Andrews, Bungay's weighted model, General Substrate Inhibition Models (GSIM) and sum kinetic models. These models are nothing but mathematical expressions generated to describe the behaviour of a given system (Okpokwasili and Nweke, 2005).

Most process of studying bacterial growth kinetics has been largely followed using the classical Monod growth kinetic model. Monod model is a classical microbiological model often used in environmental sciences. It was suggested by Nobel Laureate J. Monod in 1942. It has been used frequently in microbiology field for more than 60 years. The model describes microbial growth kinetics in batch culture experiments using three parameters. They are maximal specific growth rate, saturation constant and yield coefficient. Though, this model has been established to be more appropriate in describing the growth process for pure culture utilizing homogenous substrates than for heterogeneous culture utilizing heterogeneous substrate (Kovárová-kovar and Egli, 1998).

Significant amount of studies on the kinetics of microbial growth and biodegradation involving mixed culture and complex substrates are still been described using the Monod growth kinetic model (Strigul et al., 2009). Development of quantitative bacteria energetics can be assumed to have commenced with the work of Monod. Monod defines the macroscopic yield of biomass on substrate as the ratio of the biomass produced to substrate consumed. He had produced his well-known kinetic expression describing the dependence of growth rate on the concentration of the growth limiting substrate (Okpokwasili and Nweke, 2005).

2.11 Analysis methods for ferulic acid production

Most commonly method used to identify metabolites of FA is High Performance Liquid Chromatography (HPLC) (Gerard, 1997) on an Agilent 1200 system using a diod array detector (DAD) at 280nm wavelength and Agilent Zorbaq SB-AQ C_{18} analytical column at flow rate of 1ml/min. Prior to analyze, samples were separated by centrifugation at 15000 rpm for 15 minutes and the supernatants were filtered. FA was identified and quantified by comparison of retention times and peak areas with authentic samples (Ferulic acid 99%, Sigma Aldrich, Malaysia). Calibration standard was prepared from pure ferulic acid dilution in range 0.1 to 0.5 g/L concentration. Based on Srinivasan et al (2007), acetonitrile was used as the mobile phase consisting of ACN: H_2O (880: 720, vol/vol). Mobile phase was used to resolve the phenolic compounds. It was used to study the production of FA by using co-culture and BSW. For the analysis of growth profile and kinetic study, indirect measurement was used in this analysis. Indirect growth measurement is by measuring the absorbance at 600nm, 250 rpm and 37°C, (Widdel, 2010) the growth profile of the production was performed. The data for absorbance was measured by using spectrophotometer. Then, the graph of absorbance versus time was plotted. Finally, the kinetic study was carried out by using Monod equation.

2.12 Summary

This literature review part presents the overview of co-culture, banana stem waste, growth profile and kinetic modelling. The using of soil microorganism was performed in this study where co-culture was isolated from the soil. Some fermentation process needs more than one enzyme (Lemos et al., 2014) to release FA efficiently. Therefore the co-production of enzyme by microorganisms is very important to increase the production of FA. Banana stem waste was act as the substrate. The four-phase pattern of growth profile and why it needs to be performed was explained above. In addition, the clarification of using Monod equation in performing kinetic study was explained too. Hence, the previous work on FA production and the analysis for the production had been stated above.

3 MATERIALS AND METHODS

3.1 Overview

This study presents a fermentation process in producing FA by using co-culture. The raw material for the process was banana stem waste. This section presents the experimental setup in completing the laboratory works. It includes in performing growth profile of the microbe, fermentation process and analysis of FA by using HPLC. Lastly, kinetic study for FA production was carried out. In this microbial process, bacteria were isolated from soil and BSW as a substrate. Preliminary studies were conducted in determining the bacteria that was chosen to further with the research of FA production from co-culture. Selection of co-culture was based on the performance of the pure culture in the release of FA from BSW.

3.2 Introduction

In this chapter, the usage of chemicals and raw materials were stated specifically. In addition, the experimental setup for the research study was explained further below. Figure **3**.**1**.1 below shows the flow of procedure in the ferulic acid production by using co-culture. There were several steps done in this study. Firstly, substrate was prepared by using banana stem waste. Banana stem waste was collected at Jalan Gambang, Pahang. Then, it was cut into small pieces with measurement 1cm by 1cm and blended. The remaining pieces were stored in $4^{o}C$ refrigerator. Then, inoculum preparation and fermentation process for pure culture started. After that, analysis was done in order to get the best pure culture producers. After the determination, growth profile for each pure culture and co-culture were performed. Then, fermentation process by using co-culture started. After that, it was analysed in order to determine the best co-culture producer. Then, experimental setups for the kinetic study were prepared. The samples were collected for every 3 hours and further calculation was done by using Runge Kutta- Fourth method.

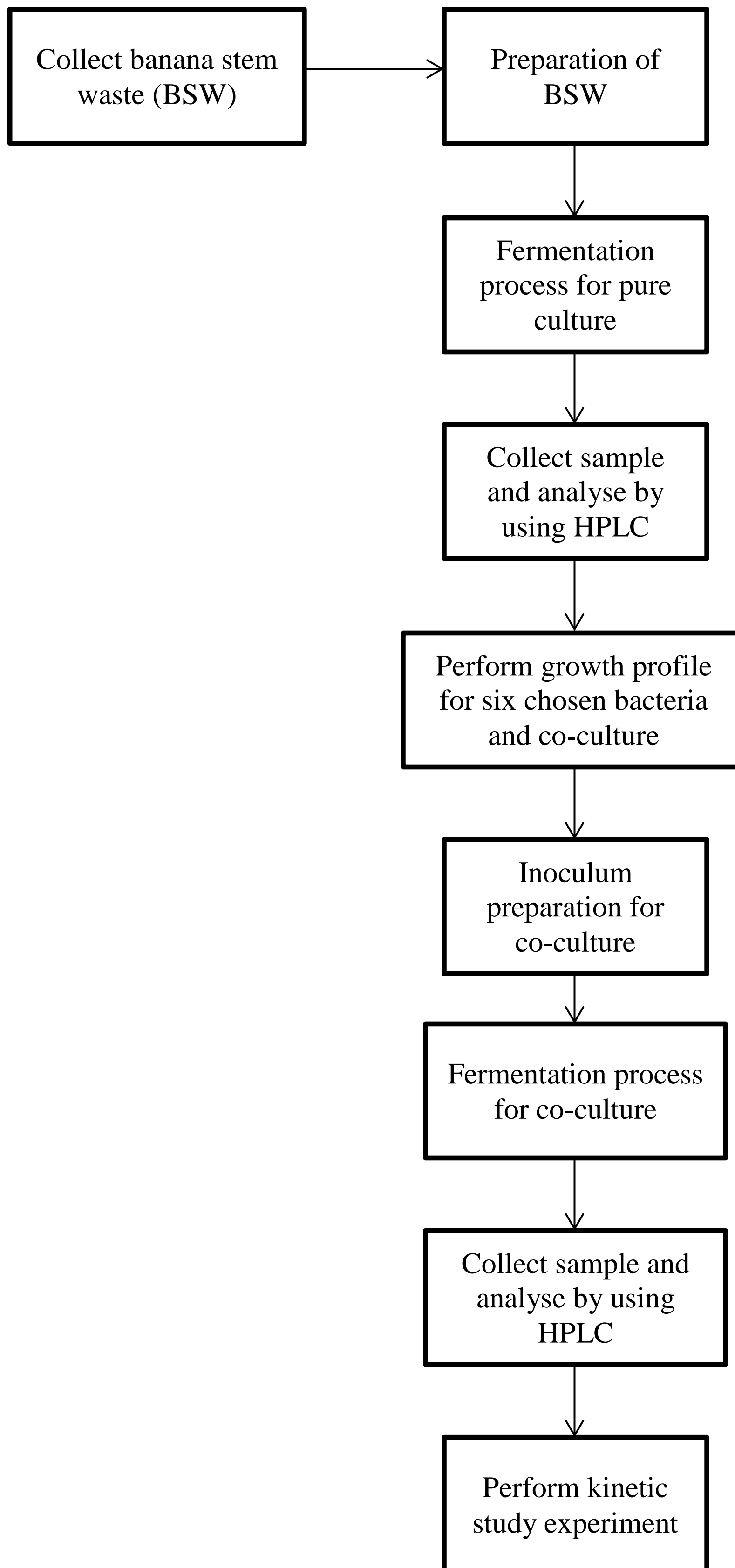

Figure 3.1: Flow-chart of the process involve in production of ferulic acid from agricultural waste

3.3 Chemicals and raw materials

3.3.1 Chemical

There were two types of chemicals used in this study. They were acetonitrile and pure ferulic acid solution. In analysing the production of FA by using HPLC, mobile phase used was acetonitrile with the ratio concentration of 55% ACN: 45% H_2O (Hasyierah et al., 2011). In addition, pure FA was used as the standard to perform a standard curve of FA. The standard curve was used to calculate the production yield of FA (Yu et.al., 2005). Moreover, distilled water was used in order to get the dry weight cell measurement. Dry weight cell measurement was used to measure the biomass concentration (Widdel, 2010). Nevertheless, raw material used in this study was banana stem waste (BSW) and co-culture.

3.3.2 Co-culture

Co-culture was prepared by mixing six best pure cultures which had been selected from 21 types of pure cultures. Preliminary studies were conducted by using 21 types of pure cultures in determining the bacteria that were chosen the best in this study. Selections of six best pure cultures were based on their performance in releasing ferulic acid from banana stem waste. Only bacteria that indicate the best ferulic acid content were prepared as inoculum for co-culture (Nelson et. al., 1999). They were bacteria *Bacillus thuringiensis* NRBC 101325, *Bacillus cereus* JCM 2152, *Bacillus cereus* ATCC 14579, *Bacillus pumilus* SAFR-032, *Lysinibacillus sphaericus* and *Brevibacillus formosus*.

3.3.3 Banana stem waste

Banana stem waste used in this experiment was obtained from Kuantan, Pahang at Jalan Gambang. The stems were cut into cube with the measurement of 1cm by 1cm (Cordeiro, 2004) and mixed with distilled water. Then they were blended and autoclaved for 15 minutes at 121°C before injected with inoculum (Marcia et. al., 2007). The purpose of autoclaved was to kill all the bacteria in the blended BSW and kept them sterilized. The banana stem wastes were blended as a batch. Extra banana stem wastes were stored in -4°C refrigerator in order to maintain their moistened (Mukherje et al., 2007). To avoid any contamination, the experiment using this banana stem waste was carried out on the same day.

Figure 3.2: Banana stem waste at Jalan Gambang, Kuantan, Pahang

3.4 Experimental setup

3.4.1 Growth profile of microorganism

Before fermentation process began, growth profile for the microorganisms had to be carried out. The purpose of it was to determine the time where stationary phase growth of bacteria occurred and fermentation process could be carried out well. Therefore, growth profile for chosen pure culture and co-culture were done. In completing this method, indirect method (Widdel, 2007) was used. The inoculums were first prepared in this process. They were prepared by using nutrient broth. In preparation of nutrient broth, 8g of nutrient broth powder were weighed. Then, 1L of pure water was added into 1L of Schott bottle. They were dissolved completely and transferred 10mL each into the universal bottle. After that, they were sterilized at 121°C for 15 minutes by using autoclave. For inoculation, a sterilized metal loop of each pure culture from the nutrient agar plate was immersed into the nutrient broth (Bertrand et al., 2014).

For indirect measurement, bacteria population in the culture was estimated by measuring its turbidity with a spectrophotometer (Bertrand et al., 2014). Firstly, a universal bottle consists of 10mL growth medium nutrient broth with inoculated media was prepared. The inoculated media consists of six types of pure culture which were bacteria A, B, C, D, E and F. The inoculums were then incubated for 24 hours at 30°C. On the next day, 10mL of the inoculums were transferred into Erlenmeyer flask containing 100mL of nutrient broth. Then, it was incubated in the incubator shaker for 24 hours at 30°C.

1 mL of sample was taken every 2 hours of interval in order to check the turbidity by using spectrophotometer. The spectrophotometer was blank with one of the media blank. The media blank was contained with sample without inoculated media. Then, the extinction value was adjusted to 0.000. It was verified from time to time that the zero point had not drifted (Widdel, 2007). The absorbance data was recorded accordingly. After that, graph of time against optical density was plotted in order to determine the stationary phase of the bacterial growth. Then, the procedures were repeated for another five pure cultures. The significant of this study was to observe the time of pure culture grew increasingly with less number of deaths.

At the stationary phase of pure cultures (J. Bader et al., 2009), the inoculums were taken out from incubator in order to mix them and performed inoculum for co-culture. Therefore, sufficient amount of bacteria can be obtained in preparing the inoculum. In preparation of co-culture inoculum, 10mL of pure culture inoculum from universal bottle was transferred into 100mL of nutrient broth in the Erlenmeyer shake flask. After 24 hours incubated, it was taken out and mixed into 1000mL of nutrient broth. This was where the inoculum for co-culture prepared. Then, it was divided into several Erlenmeyer shake flask to perform growth profile experiment. After that, growth profile for two sets of co-culture which were sets ABEF and BCDEF was performed again in order to determine the time of their stationary phase before injected into the substrate.

3.4.2 Fermentation process

Fermentation process started with twenty one types of pure cultures in order to determine the best producer from them. After the determination of six best pure cultures, fermentation process for co-culture began. Before that, each pure culture was going through identification process by using molecular method. This identification requires PCR and cycle sequencing to isolate genomic DNA from bacteria. This protocol involved breaking the cells open with a series of freeze/thaw cycles. PCR reaction was set up to amplify a region of the 16S rRNA gene. The cleaned PCR product was used as the template for a sequencing reaction which runs in a thermocycler (PCR machine). The electropheragrams was viewed from the sequencing reaction and the sequence was used in a BLAST search limited to a bacterial data base. Unknown bacteria were identified by examining the top-scoring sequences from the BLAST search results. After they were identified, the pure culture was labelled again as shown in the Table 3.1. Then, inoculums for co-culture were prepared.

For the inoculum preparation of co-culture, growth profile results for six pure cultures analysed before were used. The inoculum for co-culture was prepared at the time of stationary phase for pure cultures occurred. Firstly, full loop of each pure culture was immersed in the 10mL of nutrient broth. At the stationary hour of incubated period, they were taken out and transferred into six Erlenmeyer flask which contained 100 mL of nutrient broth each. Then, they were incubated in the incubator shaker for 24 hours at 30°C and 150 rpm. On the next day, 10mL of inoculum from each flask were transferred and mixed into 1000mL of flask containing nutrient broth. Then, they were incubated again until reached the stationary hour of co-culture. The time of stationary phase for co-cultures were analysed from their growth profile.

Figure 3.3: Inoculum preparation by using nutrient broth

At that hour, two sets of Erlenmeyer shake flasks containing blended BSW were performed by injecting 10mL of inoculum (Donaghy, 1998) into it. There were two samples containing co-culture BCDEF and ABEF. The samples were incubated in the incubator shaker for 24 hours at 30°C (Darah and Ibrahim, 1997) and 150 rpm (McMurrough et al., 1996). Therefore, fermentation processed began. In this case BSW acted as the substrate. On the next day, samples were taken out and ready for analysed. Production of FA from each sample were analysed by using High Performance Liquid Chromatography (HPLC). In preparing the sample, they were injected and filtered into vial. They were filtered by using nylon syringe filter in order to remove unnecessary things. Based on the result, the best co-culture producer was determined which from set BCDEF.

Figure 3.4: Fermentation process in Erlenmeyer shake flask with mixture of BSW and co-culture.

Table 3.1: Type of pure culture with label

Label	**Type of pure culture**
A	*Bacillus thuringiensis* NRBC 101325
B	*Brevibacillus formosus*
C	*Bacillus pumilus* SAFR-032
D	*Bacillus cereus* JCM 2152
E	*Lysinibacillus fusiformis*
F	*Bacillus cereus* ATCC 14579

Table 3.1 shows type of pure cultures with their labels. There were 21 types of pure cultures samples run in the fermentation process. After the analysis was done, six types of pure cultures had been short listed as the best producers. Then, they were labelled again as bacteria A, B, C, D, E and F. The total of six best producers were used for the co-culture fermentation process. Therefore, in Table 3.2, it shows two sets of co-culture which had been prepared.

Table 3.2: Two sets of co-culture

Number of sets	**Co-culture**
1	BCDEF
2	ABEF

There were two sets of co-culture run for the fermentation process. Set one consist of pure culture BCDEF and set two consist of pure culture ABEF.

3.4.3 Ferulic acid determination

FA content from the sample was analysed by using HPLC on an Agilent 1200 system using a diod array detector (DAD) at 280nm wavelength and Agilent Zorbaq SB-AQ C_{18} analytical column at flow rate of 1ml/min. Prior to analyse, samples were centrifuged at 15000 rpm for 15 minutes and the supernatant were filtered. After that, they were injected in the vial by using syringe and nylon syringe filter. FA were identified and quantified by comparison of retention times and peak areas with authentic samples (Ferulic acid 99%, Sigma Aldrich, Malaysia). Calibration standard was prepared from pure FA dilution in range 0.1 to 0.5 g/L concentration.

3.4.4 Kinetic study of microorganism

The hyperbolic equation proposed by Monod was modified by Lawrence and McCarty (1970). It is used to describe the effects of substrate concentration on the rate at which a given microbial concentration removes the target substrate (-dS/dt) $\frac{dS}{dt} = -\frac{qmax\ SX}{Ks+S}$ (Equation **1**). Alternatively, modified Monod equation can be written in terms of microbial growth by incorporating the net yield coefficient (Y) $\frac{dX}{dt} = \frac{Y\ qmax\ SX}{Ks+S}$ (Equation **2**) (Okpokwasili & Nweke, 2005).

$$\frac{dS}{dt} = -\frac{qmax\ SX}{Ks+S} \qquad \text{(Equation 1)}$$

$$\frac{dX}{dt} = \frac{Y\ qmax\ SX}{Ks+S} \qquad \text{(Equation 2)}$$

Based on the equation 1, dS/dt is the rate of change of substrate concentration (g/L.h). In equation 2, dX/dt is the rate of change of cell concentration (g/L.h), q_{max} is maximum consumption rate constant, S is substrate concentration (g/L), Ks is the Monod constant and X is cell concentration (g/L), Y is the net yield coefficient (g/g).

The substrate concentrations and biomass concentrations data were obtained by manipulating the time by 3 hours (Menten et. al., 2011). The samples were prepared same as the fermentation process samples. However, the data was collected for every 3 hours interval with total of 24 hours. Firstly, the sample with 3 hours incubation was taken out and checked for the optical density. The remaining sample was then centrifuged for 10 minutes. Then, the biomass and substrate was separated and dried. After they were dried, they were weighed to get the dry weight data. The concentration of biomass and substrate were obtained from the dry weight data (Kovárová-kovar and Egli, 1998). The steps were repeated for time points of 6, 9, 12, 15, 18, 21 and 24 hours. The standard curve data for biomass concentration was plotted earlier. For the preparation of biomass standard curve, very concentrated biomass concentration was prepared. Then, it was diluted to 5 times. From the optical density data collected every 3 hours, it was compared with the standard curve in order to determine the concentration

of biomass. After the data were obtained, the Runge Kutta-Fourth method was used to determine the constant value of Y, Ks and q_{max}. (Strigul et al., 2009).

3.5 Summary

The usages of chemicals in this research study were not as much as expected. In fact, the raw materials were not difficult to obtain. BSW was obtained from Kuantan, Pahang. The microorganism was obtained from soil and was further isolated. There were four steps in the experimental setup. Firstly, growth profiles for six pure cultures and two sets of co-cultures were prepared. After that, inoculum for co-culture set BCDEF and ABEF were prepared. Then, the fermentation process started when the samples were incubated in the incubator shaker for 24 hours, at 37°C and 150rpm.On the next day, FA production was determined by using HPLC. From the analysis, determination of best co-culture producer was done. The best producer for co-culture was from set of bacteria BCDEF. Lastly, kinetic study for FA production by using BCDEF set of co-culture was carried out by applying modified Monod equation.

4 RESULT AND DISCUSSION

4.1 Overview

This chapter discuss the outcome of this study which is related to the objectives and scopes. The topic covers in this chapter is growth profile for pure culture and co-culture, co-culture fermentation result and kinetic study on the product. High Performance Liquid Chromatography (HPLC) is used to measure the concentration of FA.

4.2 Introduction

In this chapter, result and discussion for the experimental study were presented. There were results for fermentation process by using pure culture and co-culture and growth profile for six pure culture and two sets of co-culture. Apart from that, kinetic study result for co-culture also discussed here.

4.3 Fermentation process by using pure culture

Fermentation process had begun with the fermentation of pure culture. It is because; the best producer from pure culture had first to be determined in order to continue with co-culture fermentation process. The result for FA production by 21 type of pure culture is shown in Figure 4.1 below.

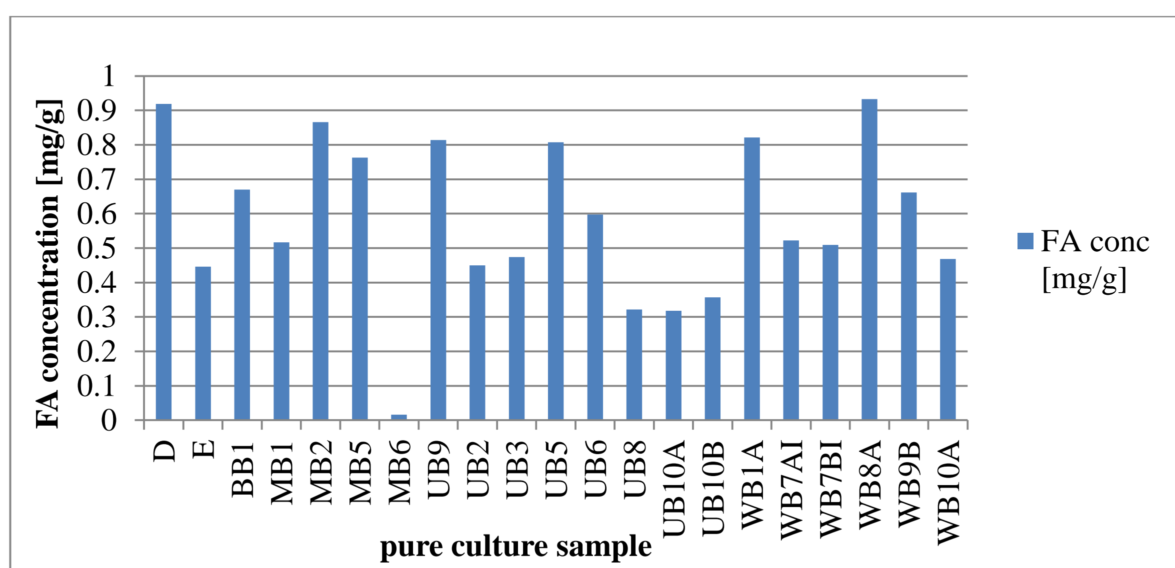

Figure 4.1: Production of ferulic acid by using pure culture

According to Yu et al (2005), ferulic acid can be obtained by the fermentation process at the range of 0 to 3 mg/g. After the analysis had been done, six pure cultures were short listed to proceed with the fermentation of co-culture. They are bacteria *Bacillus thuringiensis* NRBC 101325 (WB8A), *Bacillus pumilus* SAFR 032 (WB1A), *Bacillus cereus* ATCC 14579 (UB2), *Lysinibacillus spahericus* (MB5), *Bacillus cereus* JCM 2152 (MB2) and *Brevibacillus formosus* (UB9). They are chosen based on the performance in releasing FA. The highest producer was from bacteria *Bacillus thuringiensis* strain NRBC 101325. It was able to produce 0.9338 mg/g concentration of ferulic acid.

The second highest producer was from bacteria *Brevibacillus formosus* where it was able to produce ferulic acid concentration in the amount of 0.8139 mg/g. Moreover, bacteria *Bacillus pumilus* SAFR-032 and *Bacillus cereus* strain ATCC 14579 released 0.8216 mg/g and 0.4492 mg/g concentration of ferulic acid respectively. Bacteria *Bacillus cereus* strain ATCC 14579 considered as the lowest producer as it released only small amount of ferulic acid, however, it was still chosen as to investigate whether it able to release a lot of FA if it was mixed with the other bacteria. Lastly bacteria *Lysinibacillus sphaericus* and *Bacillus cereus* strain JCM 2152 were chosen as they were able to release 0.7631 mg/g and 0.8657 mg/g of FA respectively.

In previous case study by using paddy straw (Hasyierah et al., 2011), FA was able to release at 2.817 mg/g. Furthermore, research by Zhao S. et al (2013) which was fermentation of cornbran could release 3.2 mg/g. Cornbran contains a high amount of ferulic acid. However, the separation and purification of ferulic acid from it still encounter technical problems. Ferulic acid is found varying from 2.5 mg/g in wheat bran. Although wheat bran is rich in FA, it is expensive due to the nutrition and γ-oryzanol it contained (Garcia et al., 2009). Throughout this study, it shows that BSW was able to release ferulic acid within the range stated above. After determining six best producers, the experiment was continued by performing their growth profile. The significant of it was to identify at which hour will their number of cell increased in order to prepare the inoculum for co-culture.

4.4 *Growth profile of chosen pure culture*

Growth profiles for six types of pure culture were performed in order to determine the stationary phase of microorganisms. The inoculum preparation for co-culture had to be done at the stationary phase of microorganisms as they were able to produce bacteria in sufficient amount during that phase. The patterns of their growth were explained below.

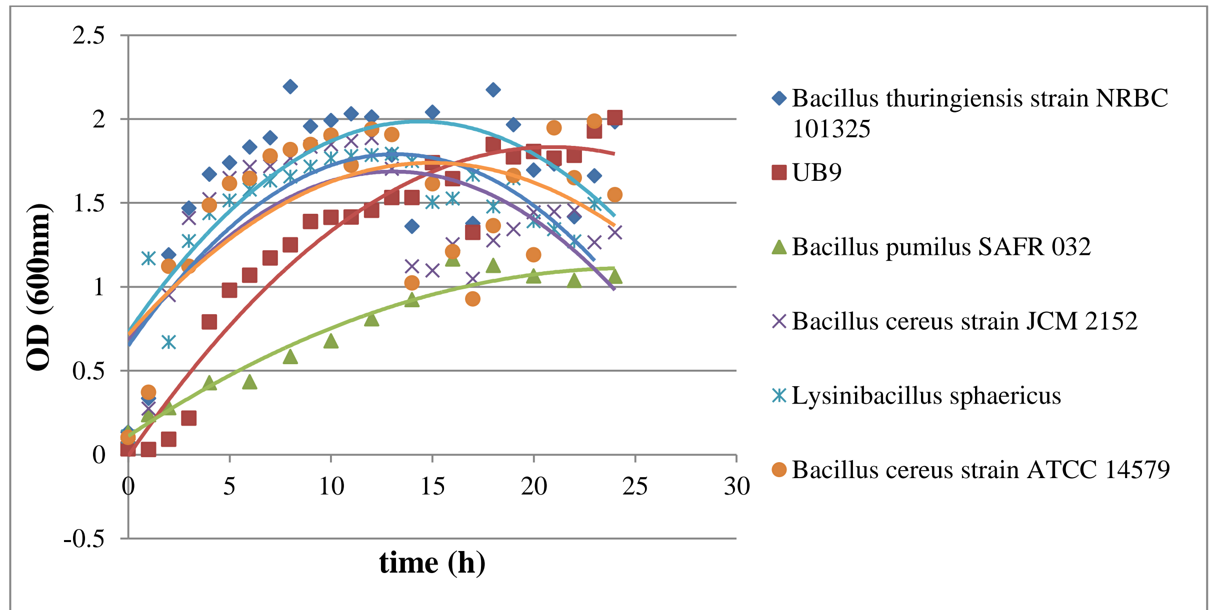

Figure 4.2: Growth pattern for six types of pure culture

Based on Figure 4.2, every pure cultures managed to adapt themselves very well as the lag phase occurred in a short time. It was proven as they started to grow rapidly at the hour of 4. This exponential phase prolonged until the hour of 10. After that, their growth started to reach stationary phase. Stationary phase is where the number of cells divided is equal to the number of cell died. It shows that the nutrient given was almost finished at this point as they were not able to increase their number of cells. Every pure culture showed their stationary phase at the hour of 12. Therefore, the inoculum preparation for co-culture could be done at the 12th hour of their growth. The significant of this method was because, the number of cells during this phase was sufficient enough to prepare the inoculum. However, there was some difference in preparing inoculum for pure culture *Bacillus pumilus* SAFR 032 (WB1A). WB1A grew very rapidly from the hour of 6th to 16th. It shows that WB1A able to live for long hours compared to the others. Then, at the 18th hour, the growth slowed down and reached stationary phase. Therefore, stationary phase for WB1A was considered at the hour of 18th.

As a conclusion, inoculum preparation for co-culture with addition of pure culture *Bacillus thuringiensis* NRBC 101325 (WB8A), *Brevibacillus formosus* (UB9), *Bacillus cereus* JCM 2152 (MB2), *Lysinibacillus sphaericus* (MB5) and *Bacillus cereus* ATCC 14579 (UB2) was done at the 12th hour of their growth. While pure culture WB1A was done at the hour of 18.

4.5 Growth profile for co-culture

Growth profile for sets ABEF and BCDEF were performed in order to determine the stationary phase of the co-culture. The significant of it was to identify the time of the stationary phase before injected into the substrate and preceded with fermentation process. Two figures below show the growth pattern for two sets of co-culture.

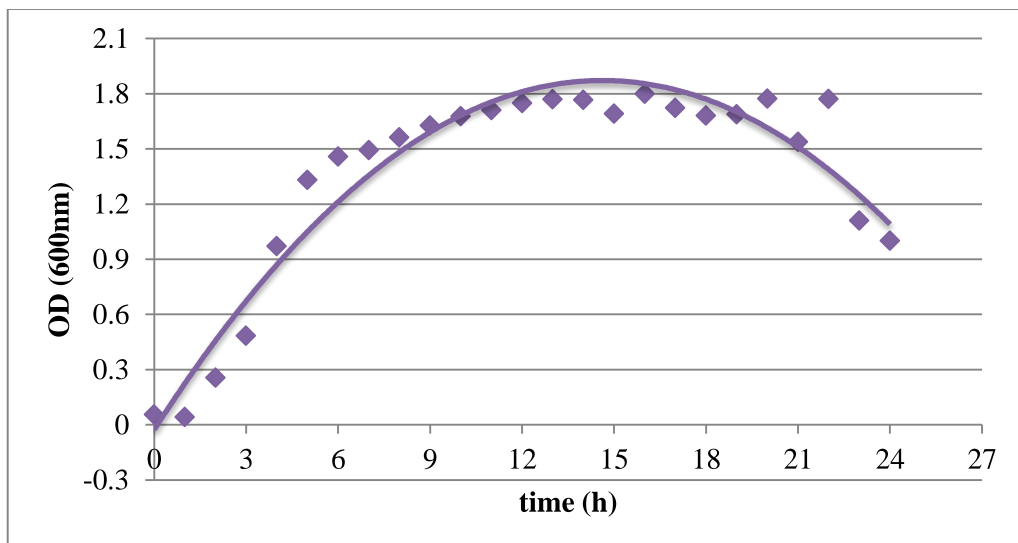

Figure 4.3: Growth profile for co-culture set ABEF

Based on Figure 4.3 above, at the hour of 3 to 9, the bacteria grew rapidly. It was where the exponential phase of the bacteria occurred. According to the duration of time, it shows that the bacteria did not need too much time for their adaptation phase. Then, at the hour of 12th, the co-culture ABEF had reached stationary phase. After that, they started to die at the hour of 21. Therefore, the inoculum could be injected at the hour of 12th as they had reached stationary phase at this hour.

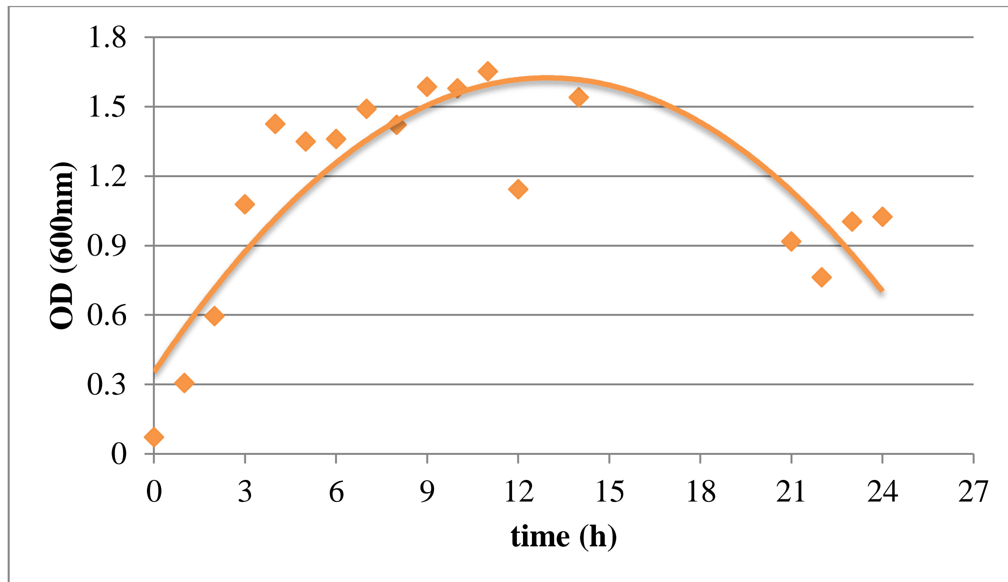

Figure 4.4: Growth profile for co-culture set BCDEF

Nevertheless, Figure 4.4 shows the growth profile for co-culture BCDEF. According to the growth pattern, the stationary phase happened at the hour of 9 until 15. Therefore, the inoculum could be injected at the hour of 12th. The bacteria also did not take too much time for their adaptation as at the hour 3 until 6, they start to reach exponential phase. Then, at the hour of 15, they started to reach death phase. The numbers of cells died was greater than the number of cells being produced in this phase. Hence, both of the co-cultures were injected into the substrate at the hour of 12th of incubation period as it was considered as their stationary phase. During that phase, the numbers of cells produced were equivalent with the number of cells died. Therefore, the numbers of cells in that phase were sufficient enough for ferulic acid production.

4.6 Fermentation process by using co-culture

After inoculum had been prepared, fermentation process was started. 10ml of inoculum was transferred to the Erlenmeyer shake flask which contained blended BSW. Then, it was incubated in the incubator shaker for 24 hours at 30°C and 150 rpm. On the next day, both samples were taken out and ready for the analysis. HPLC was used for the analysis process. Before they were presented in the HPLC, they were centrifuged and the supernatant were filtered. Results for FA production by two sets of co-cultures were shown below.

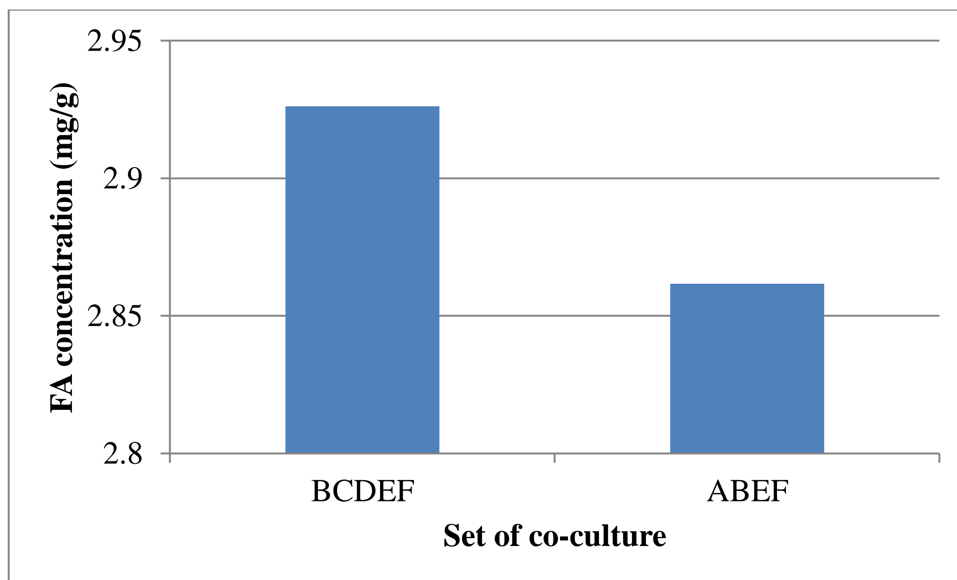

Figure 4.5: Production of FA by using co-culture

Based on Figure 4.5, there were two sets of co-culture prepared in the fermentation process. The purpose of the process was to produce ferulic acid and test for the best producer. In preparation of samples, BSW was used as the substrate and the inoculums were from co-culture ABEF and BCDEF. Both inoculums were injected into the substrate at the 12th hour of incubation period. The samples were prepared in the Erlenmeyer shake flask. After 24 hours incubated in the incubator shaker, both of them were taken out for the analysis. Based on the HPLC analysis, set co-culture BCDEF represent as the best producer. It was able to produce 2.9261 mg/g concentration of ferulic acid. Meanwhile, co-culture ABEF produced ferulic acid concentration at 2.8617 mg/g. According to the results, it shows that the more microorganisms in the sample, the more FA can be produced. Therefore, co-culture with set BCDEF was used to proceed with the kinetic study by using modified Monod equation.

4.7 Kinetic study of fermentation process

After fermentation process had completed, kinetic study on co-culture BCDEF was carried out. Kinetic study was carried out to represent the complex processes by simple mathematical or kinetic models. A successful evaluation of the kinetic constant offers better understanding into the bioreactor operation as well as the microorganism's degradation capacities and aids in the scaling up process. In this research, the hyperbolic equation proposed by Monod which modified by Lawrence and McCarty was used to describe microbial growth and biodegradation process. Since both the modified equations applied were in the form of differential equations; therefore Runge-Kutta 4th order method was compiled to solve Equation 1 and 2 simultaneously to obtain the kinetic constants: Ks, Y and q_{max}. There are several alternative numerical calculation can be applied to solve Monod equation. For example, Euler method, Runge-Kutta Fourth method and Heun's method. However, in this fermentation study, Runge-Kutta Fourth method is the best numerical calculation to be chosen.

$$\frac{dS}{dt} = -\frac{qmax\ SX}{Ks+S} \qquad \text{(Equation 3)}$$

$$\frac{dX}{dt} = \frac{Y\ qmax\ SX}{Ks+S} \qquad \text{(Equation 4)}$$

Equation 1 was used to study on the effect of substrate concentration versus time and equation 2 was used to study on the effect of biomass concentration versus time. Theoretically, q_{max} in both equations was the maximum consumption rate constant. It changed when more enzymes added into the reaction. Moreover, Ks, the Monod constant in both equation was a measurement on affinity of the enzyme on its substrate. Through the equation, the kinetic constant, q_{max} was determined from the elemental equation. Previous study carried out by Lin et al (2000) indicated that the qmax was 0.23 mmol/cell.hour by using *Lactobacillus delbrueckii*. However, in this research, the qmax was reported as 0.1669 mmol/cell.hour.

In this research, the Ks was reported as 0.9 g/L. The value for Ks was low compared to the Ks reported by other researcher got 1.88 g/L as shown in table below. Low Ks indicated that the enzyme feruloyl esterase had a high affinity on BSW. Therefore, less substrate needed to reach the q_{max}. The rate of reaction and concentration of substrate was strongly depended on the enzyme affinity on its substrate. High Ks indicated the enzyme had a low affinity on its substrate, and required a greater concentration of substrate to achieve q_{max} and vice versa. It can conclude that the enzyme feruloyl esterase had high affinity on the BSW and hence lower substrate was acquired to achieve Ks in the current study. According to Okpokwasili and Nweke (2005), the basic hypothesis of biodegradation kinetics was that substrates were consumed via catalysed reactions carried out only by the organisms with the requisite enzymes. Therefore, rate of substrate degradation are generally proportional to the catalyst concentration (concentration of organisms able to degrade substrate) and dependent on substrate concentration characteristic of saturation kinetics. This clarified the importance of these two kinetic parameters in the enzyme kinetics.

Nevertheless, Y in the equation 2 is the net yield coefficient degrades by the biomass. In a basic Monod's model, when the fermentation processes touch upon the utilization of a substrate, Y value need to be determined in the mixed microbial population. In this research, Y was reported as 0.8076 g/g. The value for Y was higher compared to the other researcher. Reardon et al (2002) obtained 0.68 g/g for Y value by using *Lactobacillus delbrueckii*. It indicated that the yield coefficient in this research study was better by using co-culture.

Previous research carried out by Zhao S et al (2013) who reported that when corn bran was used as the substrate for the production of ferulic acid, the Ks = 0.69 g/ L and qmax = 0.29 mmol/cell.h. However, when the sugar beet pulp was used as the substrate for the same production, the qmax and Ks revealed were 0.32 mmol/cell.h and 0.733 g/ L respectively. It shows that one unit of enzyme activity was defined as the amount of enzyme that deliberates 1 g of ferulic acid per litre. Different substrates used resulted in varies enzyme activity due to the dissimilar amount of enzyme that was able to release when degradation of substrate taken place. In the current study, BSW was nutritious and easy to access by soil co-cultures, the microbes can grow well and large amount of enzyme feruloyl esterase can deliberated promptly ends with more ferulic acid formation per minutes.

Yet, there were several factors that might affect the values of Ks and qmax in a microbial fermentation processes for the same production such as different substrates and incubation temperature. From the earliest study on reaction rates, it has been portrayed that the temperature must always be controlled if useful results were to be acquired from the kinetic experiment. According to Fazary, Ismadji and Ju (2010), derivations in reaction temperature as small as 1 or 2 °C might lead to the changes of 10 to 20% in the rate of reaction. The reaction rate increased with temperature until it reached the optimum temperature, then abruptly declined with further increased of temperature due to the enzyme denaturation taken place.

In an addition, temperature of a system (Liu Y, 2006) also measured the kinetic energy of the molecules in the system. Lower temperature of the system contributed to lower kinetic energy and vice versa. When temperature increased, the kinetic energy of the molecules that were able to converted into the potential energy increased due to the increased in molecules collision. In order to convert the substrate into product, enzymes must collide with and bind to the substrate at the active site which resulted in more molecules per unit time that reached the minimum activation energy to generate the enzymatic reaction. In simple word, different incubation temperature can affect the enzyme activity through the effective collisions and generated variation to the value of qmax.

The variation of enzyme activity at different temperature might yield to the diversity of result for kinetic parameters, Ks and qmax as shown in Table 4.1 below. Hence, it was crucial to ensure the enzyme functions at its optimum temperature to generate the highest enzyme activity in a fermentation process.

Table 4.1: The comparison on Ks, Y and q_{max} for the production of ferulic acid with different substrate

qmax (mmol/cell.hour)	**Ks (g/ L)**	**Y (g/g)**	**Substrate used**	**Sources**
0.543	1.96	1.22	Corn	Zhao S. et al. (2013)
11	1.88	0.65	Wheat bran	Fazary, Ismadji and Ju (2010)
0.86	13.8	1.28	Wheat straw	Hasyierah et al. (2011)
0.1669	9.0	0.8076	Banana stem waste	This research

Figure 4.6 and 4.7 below showed the graph of the experimental and predicted results for the biomass and substrate concentration as well as the R squared displayed. Commonly, R squared was a statistical measure of how well a regression line approximates the real data points. In this study on biomass and substrate concentration, the R squared revealed to be 0.8538 and 0.8833 respectively. From the earliest study mentioned that R^2 value above than 0.6 was considered acceptable for biological processes (Cornish, 1995). Since, R squared in this research was above 0.80. It can be justified that model provided good predictions for average and the experimental and predicted results were well fitted.

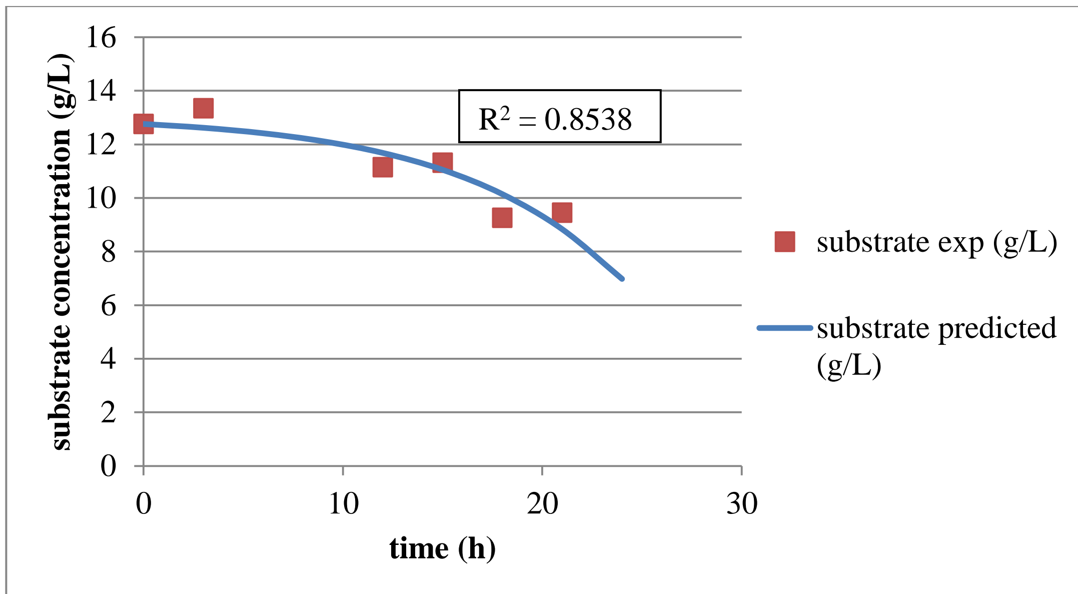

Figure 4.6: Data obtained for substrate concentration against time

Based on Figure 4.6, substrate concentration was decreased with time. According to Kovarova-kovar and Egli (1998) , for a long-term cultivation in a carbon-controlled culture, eventhough the culture was already in the steady state with respect to the biomass concentration, the residual substrate concentration will decreased by time. There was systematic study of this phenomenon performed by *E.coli* grown at different growth rates in a glucose-limited substrate. Respect to the graph above, it was shown that, the adaptation process of the bacteria occurred at the early stage of phase. After that, the bacteria proceed to grow by consuming all carbon sources in the substrate. Therefore, substrate concentration was depleted and growth of biomass increasing. The biomass concentration pattern was shown in the Figure 4.7 below.

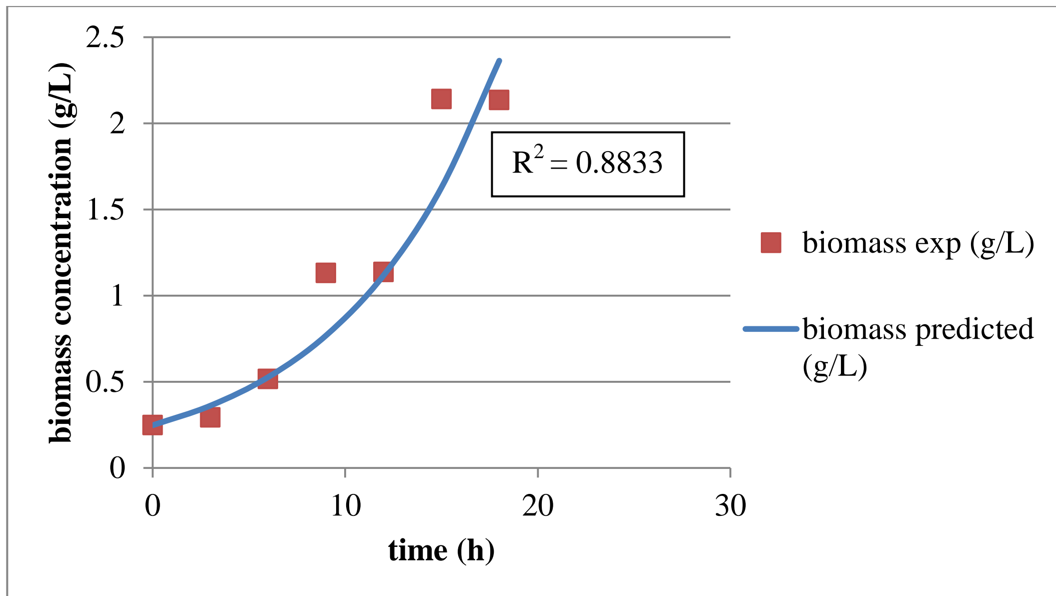

Figure 4.7: Data obtained for biomass concentration against time

Based on the graph above, biomass concentration was increasing by time. It was because the cell or biomass consumed the carbon source in the BSW as their nutrient. They used the nutrient to reproduce themselves by doubling up their number of cells. Therefore, sufficient numbers of cells were able to produce in order to degrade BSW and produce ferulic acid. From the pattern above, biomass concentration was low from the hour of 0 to 3. It shows that, they were in the adaptation mode. After that, the concentration increased rapidly from the hour of 3 to 24. There was no stationary phase occurred as a solution of the Monod model starts in the exponential growth phase. Hence, they were able to degrade BSW efficiently and produced ferulic acid in this hour.

4.8 Summary

This chapter presents result and discussion for the experimental study. The experimental studies begin with the fermentation process for pure culture. It was to determine the best ferulic acid producer for pure culture before proceed with fermentation of co-culture. From 21 types of pure culture, only six of them were chosen. They were pure culture *Brevibacillus formosus*, *Bacillus thuringiensis* strain NRBC 101325, *Lysinibacillus sphaericus*, *Bacillus cereus* strain JCM 2152, *Bacillus cereus* strain ATCC 14579 and *Bacillus pumilus* SAFR 032. Based on the six types of pure culture, two sets of co-culture were prepared. Then, growth profile for each pure culture and co-culture were carried out. The significant of their growth profile were to determine the stationary phase of bacteria. This was where the quantity of bacteria was sufficient enough to prepared for inoculum and to be injected in the substrate for the production of ferulic acid. After fermentation process ended, analysis by using HPLC was carried out. From the analysis result, co-culture BCDEF was determined as the highest producer for ferulic acid. Finally, kinetic study for co-culture BCDEF was undergone by using modified Monod equation. Based on the equation, Runge-Kutta Fourth method was used to calculate the constant value of Ks, q_{max} and Y.

5 CONCLUSION

5.1 Conclusion

Ferulic acid possess many potential in medical applications such as a scavenger for free radicals or as a protective agent against UV-radiation-induced skin damage. Sodium ferulate or known as sodium salt of ferulic acid had been used as the medicine in clinical treatment for cardiovascular disease and cerebral thrombosis (Wang et al., 2004). Recent researchers have shown that sodium ferulate could act as the non-selective relaxant to vascular smooth muscle and could be used as the cardio-protective substance (Liao et. al., 2009). Ferulic acid esterase is the key enzyme that hydrolyses the esters of ferulic acid. Therefore, this research study used co-culture as the microorganism to degrade BSW in order to release ferulic acid esterase enzyme, thus produce ferulic acid.

In this study, it found that co-culture could grow well in the BSW, and that the secretion of cellulose and ferulic acid esterase allowed the release of ferulic acid. BSW was reported to contain many sugars, protein and other minerals which provide enough nutrition to culture bacteria for ferulic acid production. There were two main objectives in this study. Firstly, to perform growth profile for co-culture and produce ferulic acid from banana stem waste thus test for the best producer. Secondly, to perform kinetic study of ferulic acid production by using co-culture.

Several methods were carried out to meet the objectives. Based on the result, co-culture BCDEF was selected as the highest ferulic acid producer than co-culture ABEF. It indicates that co-culture can released highest yield of ferulic acid. Before fermentation process started, growth profile for each pure co-culture and co-culture were carried out. Traditionally, a typical growth curve is divided into four phases which are lag phase, exponential phase, stationary phase and death phase. The purpose for it to be carried out in this study was to determine the time where the stationary phase for the cell to occur before injected the inoculum into the substrate.

The ferulic acid amount was related with the activity of cellulose and ferulic acid esterase. As the number of enzyme reaction increase, the highest yield of ferulic acid can be produced. Cellulose has the ability to decompose cell walls and releasing free ferulic acid contained in BSW. Ferulic acid esterase could cleave the ester linkages which most found in BSW, thus releasing ferulic acid. And the results indicate that co-culture BCDEF could release cellulose and ferulic acid esterase and convert to the highest yield of ferulic acid. Therefore, first objective was completed.

The Monod model is a classical microbiological model often used in environmental sciences, for example to evaluate biodegradation processes. The model describes microbial growth kinetics in batch culture experiments using three parameters which are the maximal specific growth rate, the saturation constant and yield coefficient. The Monod model uses a very convenient approximation of the batch growth process. It recognizes only two growth phase which are exponential phase and stationary phase. It is assumed that lag phase and death phase do not exist. This assumption is satisfied in many practical cases.

Modified Monod equation was used in this study which consists of three parameters. They were Ks, qmax and Y. There are several alternative algorithms that can be applied for calculation in determining the valus for parameters. For example, Euler method, Runge-Kutta Fourth method and Heun's method. However, in this fermentation study, Runge-Kutta Fourth method is the best numerical calculation to be chosen. Based on the result, values calculated for Ks, qmax and Y were 0.9g/L, 0.1669 mmol/cell.h and 0.8076g/g respectively. In addition, values for R-squared obtained were 0.8538 for substrate and 0.8833 for biomass.

5.2 Recommendation

In conjunction with the result and conclusion obtained, the following recommendations were identified to further improve the future research in this field of study:

1. The screening stage will be done in order to determine the best factors which can increase the production of ferulic acid.

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APPENDICES

1)

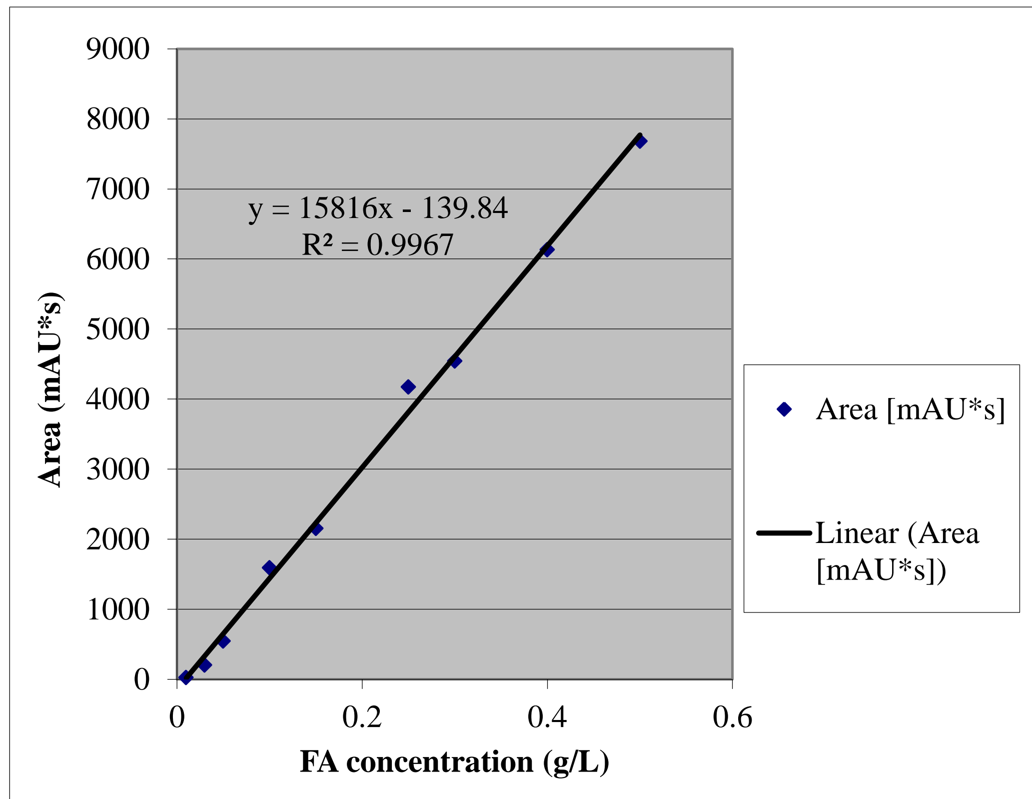

Figure 1: Standard curve for ferulic acid concentration

2)

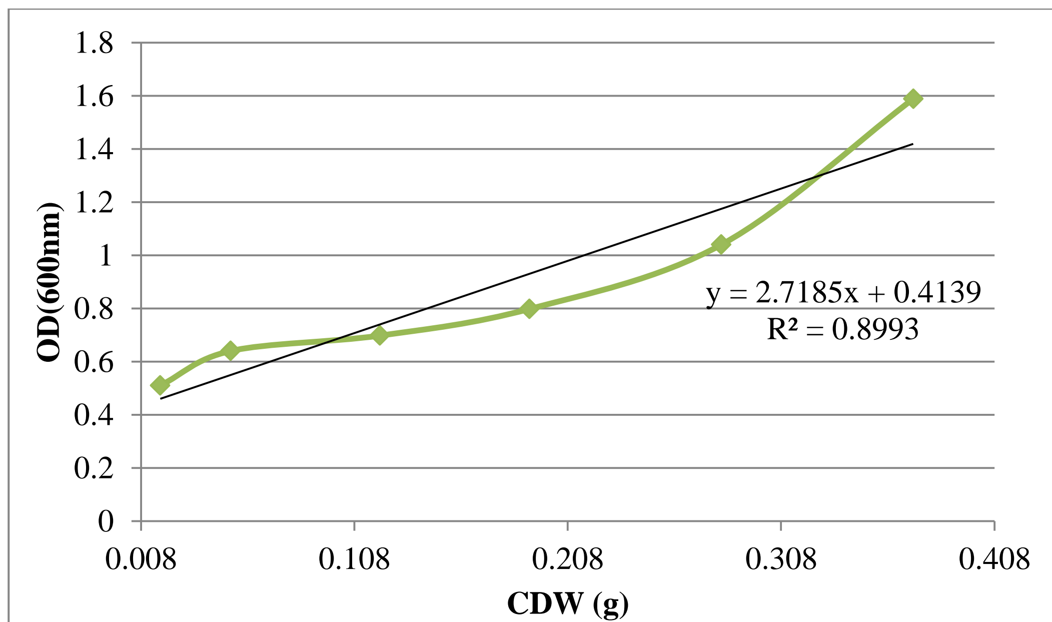

Figure 1: Standard curve for biomass concentration