FERULIC ACID PRODUCTION FROM BANANA STEM WASTE: OPTIMIZATION

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ABSTRACT

Ferulic acid is a compound well known for its anti-inflammatory and antioxidant properties, which is widely used in the food and cosmetic industry. The main objective of this study was to optimize the ferulic acid production from banana stem waste (BSW) by soil-mixed culture. Optimization of ferulic acid production was assisted by Central Composite Design (CCD). Previous screening studies have employed five processing parameters, *viz*. temperature, agitation speed, substrate-to-inoculum ratio, water-to-banana stem waste ratio and incubation time. It has been identified that the water-to-banana stem waste ratio and time factor are the most vital parameters. Consequently, the effect of time on ferulic acid production was studied in batch culture at duration ranging from 12 to 36 h and water-to-banana stem waste ratios of 0.5:1 to 1.5:1. The determination of ferulic acid was performed by High Performance Liquid Chromatography (HPLC). In addition, Design Expert software v 8.0.6 was used to design the experiment and analyse the obtained experimental data. The optimum condition obtained from this study is 1.1: 1 ratio of water-to-banana stem waste and incubation time of 27 hours. The yield of ferulic acid from this experiment was 1.17 mg FA/ g dry BSW which was lower than findings from other researchers such as Webber (2004) (21 mg phenolics/ g HDRB), Aybastier et. al.(2013) (82.12 mg phenolics/ g dried plant) and Salleh et. al. (2011) (8.17 mg FA/g paddy straw). Result from this work may be useful to optimize the ferulic acid in large scale production from BSW.

ABSTRAK

Asid ferulic adalah sebatian yang terkenal dengan ciri-ciri anti-radang dan antioksidan, yang digunakan secara meluas dalam industri makanan dan kosmetik. Objektif utama kajian ini adalah untuk mengoptimumkan pengeluaran asid ferulic dari sisa batang pisang (BSW) dengan kultur tanah-campuran. Pengoptimuman pengeluaran asid ferulic dibantu oleh Rekabentuk Komposit Pusat (CCD). Kajian penyaringan sebelum ini menggunakan lima parameter pemprosesan, iaitu suhu, kelajuan pergolakan, nisbah substrat-ke-inokulum, nisbah air-ke-sisa batang pisang dan masa inkubasi. Ia telah mengenalpastikan bahawa nisbah air-ke-sisa batang pisang dan faktor masa adalah parameter yang paling penting. Oleh yang demikian, kesan masa atas pengeluaran asid ferulic dikaji dalam kultur batch pada jangka masa yang terdiri dari 12 hingga 36 jam dan nisbah air-ke-sisa batang pisang daripada 0.5:1 ke 1.5:1. Penentuan asid ferulic dilakukan oleh Kromatografi Cecair Prestasi Tinggi (HPLC). Di samping itu, Rekabentuk Pakar perisian v 8.0.6 digunakan untuk mereka bentuk eksperimen dan menganalisis data ujikaji diperolehi. Keadaan optimum yang diperolehi dari kajian ini ialah 1.1: 1 nisbah air-ke-sisa batang pisang dan inkubasi masa 27 jam. Hasil asid ferulic dari eksperimen ini adalah 1.17 mg FA / g BSW kering iaitu lebih rendah daripada penemuan daripada penyelidik lain seperti Webber (2004) (21 phenolic mg / g HDRB), Aybastier et. al. (2013) (82,12 phenolic mg / g tumbuhan kering) dan Salleh et. al. (2011) (8.17 mg FA / g jerami padi). Keputusan dari kerja ini mungkin berguna untuk mengoptimumkan asid ferulic dalam pengeluaran berskala besar dari BSW.

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LIST OF ABBREVIATIONS

N	Numbers of experiment
k	Parameter numbers
c_p	Numbers of center point

LIST OF ABBREVIATIONS

ANOVA	Analysis of variance
BSW	Banana stem waste
CCD	Central composite design
DAD	Diode array detector
FA	Ferulic acid
FCD	Face-centered cubic design
FFD	Full factorial design
GDP	Gross domestic product
HDRB	Heat-stabilized defatted rice bran
HPLC	High performance liquid chromatography
MS	Mean of square
RSM	Response surface methodology
SS	Sum of square

1 INTRODUCTION

1.1 Motivation and statement of problem

Lignocellulosic biomass is an abundant, renewable and inexpensive energy sources that are present in the forestry, agricultural and agro-industrial wastes. Significantly, the major constituents of lignocelluloses are cellulose, hemicelluloses and lignin. Ferulic acid, a compound presents at a relatively high concentration in the cell walls of several plants is a component of lignin (Mussatto and Teixeira, 2010). Bioconversion of renewable lignocellulosic biomass to value added products are desirable in the context of green catalysis (Menon and Rao, 2012). Various biomass can be employed for ferulic acid production *viz.* maize bran, sugar cane bagasse, rice bran, wheat bran, wheat straw, pineapple peels (Tilay et al, 2008) and paddy straw (Salleh et al., 2011).

In fact, ferulic acid forms cross-linkages between lignin and cell wall polysaccharides. Hence, the release of ferulic acid from plant requires enzymatic digestion of the structures. (Fry, 1982). Apart from that, feruloyl esterase (also known as ferulic acid esterases) is an enzyme that can release ferulic acid from plant cell wall through hydrolysis process (Benoit et al., 2006). Ferulic acid is renowned for its anti-inflammatory and antioxidant agent (Graf, 1992). Therefore, it has huge potential pertaining to the medical and industry field. On top of this, it also exhibits a wide range of therapeutic effects against various diseases such as cancer, diabetes, cardiovascular and neurodegenerative (Srinivasan et al., 2007).

As environmental awareness growing among the literature society, utilization of fruits and vegetables waste in industries is one of the important and challengeable jobs around the world. Lignocellulose wastes such as banana stem waste can be utilized to produce a number of valuable products (Mussatto & Teixeira, 2010). Processing of banana plants result the generation of leaves and stem waste. Accumulation of banana stem residues in agro-industrial yards cause serious environmental problem. Moreover, disposal of the banana stem waste is expensive due to high cost of transportation and a limited availability of landfills. Therefore, utilization of banana stem waste to produce ferulic acid can reduce the environmental impacts and tackle the waste disposal problem.

In particular, ferulic acid was produced from petroleum previously. However, petroleum is predicted to be used up after about 40 years (Toriyama, Heong, & Hardy, 2005). This would consequently elevate the cost of ferulic acid and limiting the uses of ferulic acid as chemical material. Therefore, utilization of low cost substrate such as banana stem is more preferable. Besides, production of ferulic acid via alkaline hydrolysis is considered as an unnatural and non-environmentally friendly manner. This method also complicating the downstream purification process attributed to the need for product neutralization, additive and impurity removals. To overcome those issues, biologically production of ferulic acid has been developed. Producing ferulic acid via hydrolysis of lignocelluloses by microorganism is considered as a natural way and has the advantage which it is highly specific than chemical hydrolysis.

In addition, there has been no report on the optimization of ferulic acid production from banana stem waste by biological process of soil-mixed culture. Soil-mixed culture has its advantages compared to pure culture. It can be obtained easily and can produce higher production yield (Hasyierah et al., 2008). The optimum condition in production of ferulic acid can be identified through the optimization process. In industries, media compositions and conditions of process are among the factors which determine the economics of a process. Statistical designs play the role in many processes to reduce the production cost with higher production yield. Consequently, this study will contribute to industries to produce ferulic acid in high quality and quantity. Therefore, it is necessary to optimize the production of ferulic acid from banana stem waste.

1.2 Objectives

The main objective of this study was to optimize the production of ferulic acid from banana stem waste using soil-mixed culture.

1.3 Scope of this research

Bananas stem wastes together with soil-mixed culture were collected from banana plantation in Pahang, Malaysia. Thereafter, the soil mixed culture was acclimatized in banana stem waste for 7 days before the experiment started. The optimum conditions for the production of ferulic acid from banana stem waste were determined using RSM. CCD was applied to investigate the effects of two independent variables, namely water-to-banana stem waste ratio (v/v) and incubation time (h). The ranges of water-to-banana stem waste and incubation time were 0.5 to 1.5 (v/v) and 12 to 36 h respectively. CCD consists of eight experimental points and five replications at the center point. Design Expert software v 8.0.6 was used to design the experiments and analyse the experimental data. HPLC was used to detect the concentration of ferulic acid. Validation experiments were carried out for three different conditions in order to validate the model.

1.4 Organisation of this thesis

The structure of the reminder of the thesis is outlined as follow:

Chapter 2 provides a description of the lignocellulosic biomass, applications of ferulic acid, technique to produce ferulic acid and characteristics of mixed- culture. A general description on the parameters that influence the biological process of soil-mixed culture to release ferulic acid such as temperature, time, lignocellulosic material, agitation effect, water availability and inoculum-to-substrate ratio. This chapter also provides a brief discussion of the optimization tool, RSM.

Chapter 3 provides the list of raw materials and chemicals used in this study. Besides, this chapter also describes the procedures to prepare inoculum and substrate, optimization experiments, quantitative analysis of ferulic acid using HPLC and lastly the validation experiments.

Chapter 4 focuses on the findings from this study such as the results obtained from the experiments, statistical analysis, the main effect and interaction effect contributions to the optimization process and comparison the findings between this study with other researchers'.

Chapter 5 draws together a summary of the thesis and outlines the future work which might be derived from the model developed in this work.

2 LITERATURE REVIEW

2.1 Overview

2.2 Lignocellulosic Biomass

Lignocellulosic biomass is an abundant, renewable and inexpensive energy sources that are present in the forestry, agricultural and agro-industrial wastes. Such wastes include a variety of materials such as sawdust, poplar trees, sugarcane bagasse, waste paper, brewer's spent grains, switch grass, and straws, stems, stalks, leaves, husks, shells and peels from cereals like rice wheat, corn sorghum and barley (Mussatto and Teixeira, 2010). Significantly, the major constituents of lignocelluloses are cellulose, hemicelluloses and lignin form an extremely complex structure that is very difficult to break down.

Bioconversion of renewable lignocellulosic biomass to value added products are desirable in the context of green catalysis (Menon and Rao, 2012). Lignocellulosic biomass accumulates in a huge amount every year causing environmental impacts. Likewise, discarder of these materials constitutes a loss of potentially valuable sources.

Methods that have been proved to be efficient for the lignocellulose hydrolysis can be classified into chemical and enzymatic methods. Chemical method use alkali, acid or solvent to hydrolysis the lignocellulose. However, enzymatic hydrolysis has attracted increasing attention as an alternative to chemical hydrolysis because the process is highly specific. It can be performed under milder reaction conditions such as the pH around 5 and temperature less than 50^{o}C. This contributes to lower energy consumption and lower environmental impact (Mussatto and Teixeira, 2010). Figure 2.1 illustrates a representative diagrammatic framework of lignocellulosic biomass. A sugar-lignin platform potential for the generation of value added bioproducts is shown in Table 2.1.

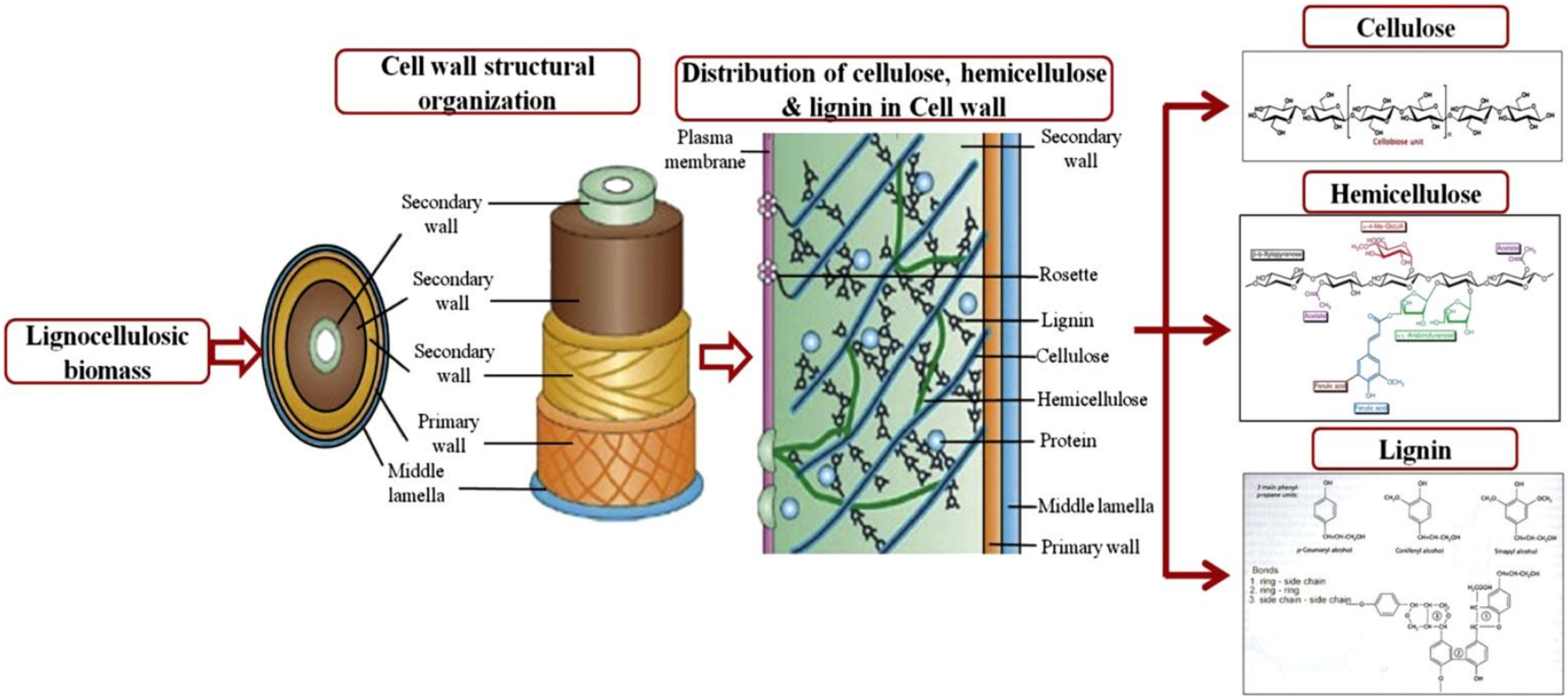

Figure 2-1: Diagrammatic illustration of the framework of lignocelluloses. (Adapted from Menon and Rao, 2012).

Table 2-1: Value added biochemicals potentially derived from cellulose, hemicelluloses and lignin. (Adapted from: Menon and Rao, 2012)

Cellulose	Polymers, Ethanol, 3-hydroxy-propanoic acid, Glutamic acid, Glucuronic acid	
	Levulinic acid	Succinic acid, THF, MTHF, 1,4 butanediol, NMP, Lactones
	Lactic acid	Acrylic acid, Acetaldehyde 2,3-pentanedione, Pyruvic acid
	Itaconic acid	3-methyl THF, 3-methyl pyrrolidone 2, methyl-1,4-butane diamide, Itaconic diamide
	Succinic acid	2-pyrrolidones, 1, 4- butanediol, Tetrahydrofurane
Hemicellulose	Xylitol, Ethanol, Butanol, hydrogen 2, 3- butanediol, Lactic acid, Furfural, Chitosan, Xylo-oligosaccharides	
	Ferulic acid	Vanillin, Vanillic acid, Protacatechuic acid
Lignin	Syngas	Methanol/Dimethy ether, Ethanol, Mixed liquid fuels
	Hydrocarbon	Cyclohexane, higher alkylates
	Phenols	Cresols, Eugenols, Coniferols, Syringols
	Oxidised products	Vanillin, vanillic acid, DMSO, aldehydes, Quinones, aromatic and aliphatic acids
	Marcomolecules	Carbon fibres, Activated carbon, polymer alloys, polyelectrolites, substituted lignins, thermosets, composites, wood preservatives, Neutraceuticals/drugs, adhesives and resins

2.3 *Ferulic acid*

Ferulic acid which is known as 4-hydroxy-3-methoxy-cinnamic acid has a molecular formula of $C_{10}H_{10}O_4$. It is a derivative of cinnamic acid. It is a component of lignocelluloses, therefore contributes to the cell wall rigidity by cross linking lignin and polysaccharides (Oryza Oil & Fat Chemical Co. Ltd, 2009). In 1935, Dutt has established that the synthesis of ferulic acid is due to its precursor characteristics in the producing of vanillin and malonic acid.

Ferulic acid is renowned on the bio-medical properties for instance UV- absorbing capacity and as precursor in plant metabolic pathway. According to Graf (1992), ferulic acid exhibits antioxidant effect. This was further proven by Maoka et al. (2008) through demonstration of food discoloration prevention, i.e. inhibition of green tea discolouration and banana oxidation. In addition, ferulic acid can be found in skin whitening formulation as melanin formation suppressing agent. Consequently, ferulic acid has been widely used in cosmetic applications as sun block or whitening agent. Apart from that, ferulic acid repressed growth of carcinogenesis cell, lowered blood sugar level by enhancing insulin secretion, lowered blood pressure and provides neuroprotection (Tournas et al., 2006). Phenolic acids like ferulic acid are typically identified and qualified by reverse-phase HPLC (Jirovsky et al., 2003). Figure 2.2 shows the structure of ferulic acid.

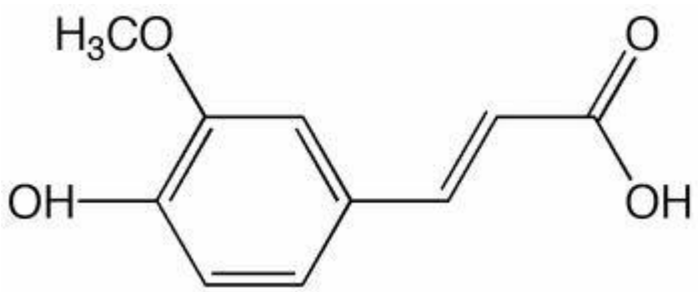

Figure 2-2: Structure of ferulic acid.

2.4 *Technique to produce ferulic acid*

Ferulic acid can be produced either by chemical synthesis, by extraction from plant cell walls, from low molecular weight ferulic acid conjugate or by microbial fermentation.

Chemical synthesis involves the condensation reaction of vanillin with malonic acid catalyzed by piperidine (Adam & Bockstahler, 1952). This method can produce high production of ferulic acid but it takes longer time, approximately 3 weeks to complete the reaction. It also leads to a mixture of trans- and cis-isomers. Therefore, it is inappropriate for commercial manufacture of ferulic acid.

There are three pathways to produce ferulic acid from natural resources i.e. from low-molecular-weight ferulic acid conjugates, from plant cell walls and by microbial fermentation. Taniguchi et al. (1994) has invented a method to manufacture ferulic acid from waste oil which obtained as by-products in the manufacture of rice salad oil. Alkali is used to hydrolyse oryzanol contained in a waste material. The hydrolysis process is carried out at 90 to 100 ^{o}C and under atmospheric pressure. The reaction time for this process is approximately 8 to 10 hours. Besides, the pH of the reaction mixture should no less than 10. The purity of the final product achieved 99.9 %.

In recent time, there has been an emerging interest in the use of naturally occurring antioxidants derived from dietary components. Enzymatic method using feruloyl esterases (FAEs) produced by microorganisms to cleave the ester bonds between plant cell wall polysaccharide and phenolic acid (Mathew & Abraham, 2004). Releasing of ferulic acid by alkaline hydrolysis from agricultural crop residues requires shorter time. The biomass is treated with diluted NaOH solution at 50-70 ^{o}C (Barberousse et al., 2009).

2.5 Agriculture Wastes

Agriculture sector represents Malaysia's third engine of growth in term of GDP just behind manufacturing industries and service sectors; hence abundance of agriculture waste. 8.44 million hectares or 25 % of land area is devoted to agriculture. Nonetheless, generation of wastes and pollutants without proper management invariably degrades the environment and affects human health. In Malaysia, oil palm, paddy, rubber, coconut, cocoa and pineapple are some of the wastes that could be potentially transformed into sought-after products. Of particular interest, banana is one of the most important fruits cultivates as indicated by the increased tonnage in the harvested fruit, from 25000 MT in the year 2005 to 28000 MT (2009). It covers 27500 ha which constitutes 11% of the total fruit area in Malaysia.

Furthermore, banana is listed as one of the six fruits for development under Entry Point Project of the National Key Economic Area for fruit production. It is exported to Singapore, Brunei, Saudi Arabia and Hong Kong. Significantly, this represents a massive source of agriculture waste in the form of lignocellulosic biomass envisaged for phenolic compound productions such as ferulic acid and caffeic acid. Johor, Pahang and Sarawak are the three major banana producing states in Malaysia (Husain & William, 2011).

A variety of agricultural by-products are potential sources of ferulic acid. According to Min et al. (2006), sweet potato is an ample source of ferulic acid. Tuber of sweet potato contains 0.54% per g dry weight. The non-tuber portion of sweet potato plant is usually thrown away after the harvest. However, it contains more than 3 % per g by dry weight of ferulic acid. In addition, Tilay (2008) presented that maize bran, rice bran, wheat straw, sugar cane baggasse, wheat bran, pineapple peels, and pomegranate peels were capable to produce esterified ferulic acid. Besides, an attempt to extract ferulic acid from paddy straw was reported by Salleh et al. (2011) and 8.17 mg/g of ferulic acid was produced.

2.6 Mixed Culture

Mixed culture is the inoculum comprised of two or more organisms. Soil-mixed culture contains protozoa, bacteria, fungi and algae growing in various numbers and kinds, subject to the nutrients available, temperature, and pH of the soil. Soil-mixed culture is interacted to each other, some as parasites, some produce essential substances and some has no effect for others. Mixed culture fermentation is far superior to the conventional pure culture fermentation *viz*. higher yield, higher microorganism growth as well as ability to perform the multistep transformation. These characteristics cannot be attained for a single microorganism.

In addition, mixed culture can provide nutrients for optimal performance whilst permits better utilization of cheap and impure substrate. Therefore, it can be easily maintained by unskilled people with a minimum training and offers more protection against contamination. Nonetheless, scientific study of mixed culture poses big challenges to scientists concerned since it involves more than one microorganism, hence contamination of mixed culture fermentation is more difficult to detect and control (United Stated National Research Council, 1992).

A variety of microorganism is capable of release ferulic acid or phenolic compound from agricultural waste. Huang et al. (2011) reported ferulic acid can be produced from lignocellulolytic agricultural biomass by *Thermofida fusca* thermostable esterase produced in *Yarrowia lipolytica*. The thermophilic actinomycete *T. fusca* was isolated from compost soils. Then, it was cloned into a *Yarrowia lipolytica* host strain. The *Y. Lipolytica* transformant was cultivated in a fermenter. The enzyme produced was then purified to hydrolysis the agricultural biomass in order to release ferulic acid.

Besides, Bonnin et al. (2002) engaged *Aspergillus niger* to release ferulic acid from sugar beet pulp. *A.niger* was grown on sugar beet pulp to generate cell wall polysaccharide-degrading enzyme such as feruloyl esterases. Significantly, *A.niger* is as competent as the commercial mixture to discharge ferulic acid from sugar beet pulp as 95 % of ferulic acid ester is solubilized. Xie et al. (2010) reported that *Hericium erinaceus* was used to release ferulic acid from wheat bran by fermentation. *H. erinaceus* produced 95.51 mg/L of ferulic acid in wheat bran broth after 4 days of culture. The activity of ferulic acid esterase was up to 2.04 $\pm$ 0.23 mU/ml.

2.7 Factors affecting ferulic acid production

Although there are many factors affecting the ferulic acid production, only five parameters were selected based on the most straightforward factors for the screening process. Therefore, incubation time, agitation, temperature, substrate-to-inoculum ratio and water-to-banana stem waste ratio were chosen in the previous study. Results obtained have indicated that water-to-banana stem waste ratio (v/v) and incubation time (h) were the two most significant parameters that influenced the production of ferulic acid from banana stem waste.

2.7.1 Effects of Temperature

Temperature is always considered as one of the most important parameters that would affect the yield of ferulic acid regardless of extraction techniques. Tilay et al. (2008) have selected temperature as one of the parameters in their optimization studies in the processes of alkaline extraction of esterase ferulic acid. The optimal temperature determined through the RSM was 21.6 ^{o}C. Besides, Salleh et al. (2011) carried out the production of ferulic acid through alkaline hydrolysis on paddy straw. In that research, they also found that temperature gave significant effects to the process. The optimum temperature for that particular process is 125^{o}C. However, there are several considerations in selecting the range of the operating temperature. The temperature should be maintained at ambient temperature if the process involve of microorganisms. This is because most of the activities of soil-mixed cultures are sensitive in temperature (Garro, de Valdez, & de Giori, 2004). Besides, according to Liao et al. (2005), biological hydrolysis on lignocelluloses can be performed under temperature less than 50 ^{o}C with lower energy consumption. Xie et al. (2010) have cultivated their culture at 25 ^{o}C to release ferulic acid from wheat bran.

2.7.2 Effect of Time

Time is the vital role in the production of ferulic acid as agreed by Salleh et al. (2011), Tilay et al. (2008) and Li et al. (2006). Time was one of the significant factors in their optimization experiments on extraction of ferulic acid. Li et al. (2006) found that only 25 min was needed to complete the reaction when the operating temperature was 110^{o}C. This suggested that the extraction time would be shorten if the operating temperature is elevated. This proposition was supported by the experiments conducted by Salleh et al. (2011), Tilay et al. (2008) and Li et al. (2006). The extraction time for Salleh et al. (2011)'s experiment was 2.5 h when operated at 125^{o}C whereas the extraction time of Tilay et al. (2008)'s experiment was 24 h when operated at 21.6 ^{o}C. According to Huang et al. (2011), the time required to enzymatic hydrolysis the agricultural biomass was 24 h at 45 ^{o}C. In addition, long reaction time is required to hydrolyse the bound ferulic acid via enzymatic hydrolysis. Since the temperature of this study is maintained at the ambient temperature, therefore, it is convincible to select time range from 12 to 36 h.

2.7.3 Effect of Lignocellulosic Material

Different lignocellulosic materials have different amount of ferulic acid present due to the different compositions in plant cell wall. Hasyierah et al. (2008) have reported that the amount of ferulic acid in corn kernels, refine wheat, wheat flour and paddy straw were 1 g/kg, 0.05 g/kg, 0.5 g/kg and 0.05 g /kg respectively. Ascensao and Dubery (2003) have proposed that lignin fraction in plant cell wall correlate directly with the amount of ferulic acid. According to Mckendry (2002) the lignin in banana stem waste was 14 %. Due to that fact, banana stem waste has a very good potential in producing high yield of ferulic acid. Table 2-2 demonstrates the composition of representative lignocellulosic feedstock.

Table 2-2: Composition of representative lignocellulosic feedstock.

Feedstock	Carbohydrate composition (% dry wt)			References
	Cellulose	Hemicellulose	Lignin	
Banana waste	13	15	14	Mckendry (2002)
Rice straw	29.2-34.7	23-25.9	17-19	Brylev et al. (2001)
Wheat straw	35-39	22-30	12-16	Prassad et al. (2007)
Wheat bran	10.5-14.8	35.5-39.2	8.3-12.5	Miron (2001)
Sugarcane bagasse	25-45	28-32	15-25	Alves et al. (2010)

2.7.4 Effect of Agitation

In aerobic fermentation agitation is important in mixing the broth for homogeneity in the fermentation and enhancing the oxygen absorption. Oxygen transfer is an important variable and is a function of agitation. Agitation helps in increasing the diffusion rate coefficient and oxygen transfer (Bartholomew et al., 1950). However, vigorous agitation will affect the dissolved oxygen in broth and lengthen the operation time. Thus, mild agitation should be provided for the homogeneity (Huang et al., 2006). In addition, Xie et al (2010) had set the rotation time of 100 rpm in fermentation process for release of ferulic acid from wheat bran. Jamal et al. (2011) used 150 rpm agitation speed in the process of phenolic acids production by *Aspergillus niger* fermentation. Therefore, 100 to 150 rpm agitation speed are considered as a moderate speed which will enhance the growth of microorganism.

2.7.5 Effect of Water Availability

Water is known as universal solvent and very important in biological system. It has a solvent function on level of organisms and cells to dissolve nutrients and scavenges wastes or metabolites. It also has a structural function in terms of stabilize the structural of molecule and cells (Gervais et al., 1996). Besides, insufficient of water in the fermentation process can cause poor diffusion of solutes and gas. This can hinder cell metabolism due to the lack of substrates in or near the cell (Gervais et al., 2003). Therefore, optimum water content in fermentation process is very important. Xie et al. (2010) have mixed the filtered wheat bran with distilled water at a ratio of 1: 20 (w/v). In this experiment, the effect of water availability to production of ferulic acid was investigated by setting the water-to-banana stem waste ratio (v/v) in the range 0.5:1 to 1.5:1. This range was much lower than the ratio in Xie et al.'s experiment due to shorter fermentation time.

2.7.6 Effect of Inoculum-to-substrate Ratio

Inoculum plays a significant role in ferulic acid production. A lower concentration of inoculums may not adequate to initiate the growth. In contrast, a high concentration of inoculum could cause competitive inhibition. A boost in inoculums size enhanced the consumption of the substrate, by this means improving the product formation. Conversely, with further amplify in inoculums size cause the rapid depletion of the nutrients which resulting in a decline in metabolic activity (Sabu et al., 2005). Significantly, the inoculum-to-substrate ratio is important in production of ferulic acid. In the experiment of Xie et al. (2010), the inoculom-to-substrate ratio was fixed to 1:10 (v:v). The ratio set by Xie et al. is higher because the fermentation is carried out in batch mode and the time of cultivation is longer (7d). Hence, the nutrients prepared should be more enough for the utilization of microorganism. The experiment time range for this research is from 12 h to 36 h; hence, the inoculum-to-substrate ratio can set in lower value. Eventually, the ratio of inoculum-to-substrate was set to unity by referring previous research.

2.8 Optimization of ferulic acid

Optimization is very important in elevating the performance of a system, a process or quality of product in order to gain the maximum benefit from it. Araújo and Brereton (1996) have agreed that in the analytical field, the optimization term alludes to applying a procedure that produces the best response.

Conventionally, optimization has been carried out by monitoring the effect of one factor at one time on the experimental response. Therefore, one factor is varied while the other factors are kept constant. However, this method has several disadvantages such as inability to distinguish the interactive effects among the parameters whilst substantial experimental works need to be carried out in order to complete the study. Ultimately, this leads to inefficiency. Alternatively, RSM, a multivariate technique was developed to improve the traditional optimization technique.

2.8.1 Response Surface Methodology (RSM)

According to Bezerra et al. (2008), RSM is a collection of mathematical and statistical technique based on the fit of a polynomial equation to the experiment data. It can be well applied when a response of interest is influenced by several variables. It is necessary to select an experimental design before applying RSM methodology. There are several types of experimental designs such as three-level factorial. Box-Behnken, central composite and Doehlert designs. Out of this pool, CCD is the most utilized for the development of analytical procedures compared to the three-level factorial design owing to the low efficiency of the latter especially for the case of large number of variables. It is noteworthy however that Box-Behnken and Doehlert designs have increased in its application in recent years.

RSM is a potent and efficient mathematical approach extensively applied in the optimization of fermentation process, e.g. media components on pyruvic acid production (Zhang and Gao, 2007), bioactive phenolic compounds production (Idris, 2011), production of vanillin from ferulic acid (Faveri et al., 2007) and biomass production optimization (Lhomme and Roux, 1991). It can provide information regarding the interaction between variables. In addition, RSM can provide information required for design and process optimization. It can also give multiple responses at the same time. Figure 2.3 illustrates the stages in application of RSM as optimization technique.

Significantly, Lundstedt et al. (1998) stated that many variables may give impact on the response of the process studied and it is impracticable to determine and control the minor contributions from each variable. Under the circumstances, it is essential to screen those variables with major effects. Screening designs can be carried out to identify the significance of several variables on the process. Generally, full factorial or two-level factorial design is used for the screening process due to its simplicity and efficiency.

The mathematical model established after fitting the function to the data may not satisfactorily express the experimental domain studied. Thus, the analysis of variance (ANOVA) is a reliable method to evaluate the quality of the model fitted. ANOVA compares the variation due to the treatment (change in the combination of variable levels) with the variation due to random errors inherent to the measurements of the generated responses. In ANOVA, the data set variation was evaluated by studying its dispersion such as the sum of square (SS) and media of the square (MS). There are many variation sources can be used to evaluate SS and MS namely total, regression, residuals, lack of fit and pure error (Vieira and Hoffman, 1989).

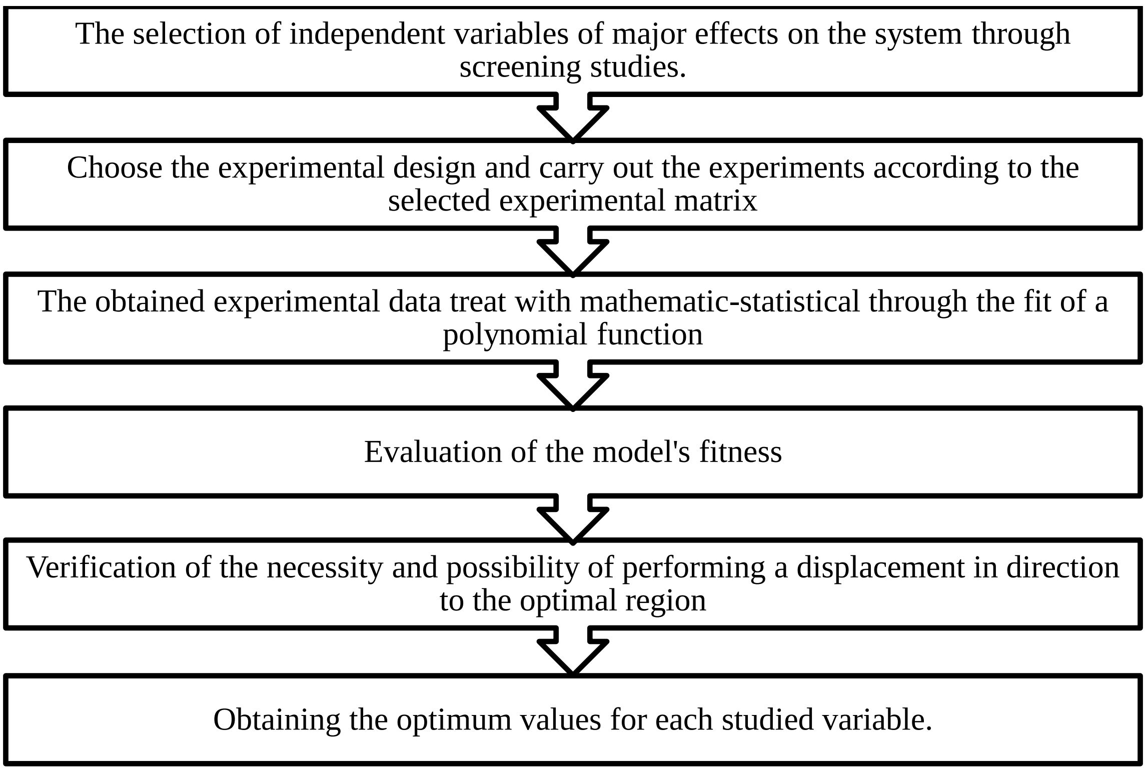

Figure 2-3: Stages in the application of RSM as an optimization technique.