BIOSYNTHESIS AND OPTIMIZATION OF METHYL 3-(3,5-DI-TERT-BUTYL-4-HYDROXYPHENYL) PROPIONATE PRODUCTION FROM OIL PALM FROND JUICE BY *Ceratocystis fimbriata*

NANG NOR AZIMAH LONG NADZRI

MASTER OF SCIENCE

UNIVERSITI MALAYSIA PAHANG

UNIVERSITI MALAYSIA PAHANG

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Thesis submitted in fulfillment of the requirements
for the award of the degree of
Master of Science

Faculty of Chemical & Natural Resources Engineering

UNIVERSITI MALAYSIA PAHANG

AUGUST 2018

This page is entirely dedicated to……

…… my lovely mother and father (Zakiah Bt Awang and Long Nadzri Bin Long Hussin), husband (Mohammad Firdaus Bin Abu Bakar), family and friends who have always been at my side and given me the encouragement and support that carries me through my study. Thanks for their never-ending love, support and care to me……

(May ALLAH S.W.T. always be with all of you)

ACKNOWLEDGEMENTS

In The Name of Allah, The Most Merciful, The Most Compassionate,
Peace and Blessings be Upon His Beloved Prophet.

I am making uncounted praises to my Allah the Almighty who has guided me to remember Him at this time. I praised Him, for it is Him who has made this master study possible. Nothing is possible unless He made it possible.

Firstly, I would like to express my sincere gratitude to my advisor Dr Mior Ahmad Khushairi Mohd Zahari for the continuous support of my master study and related research, for his patience, motivation, doa'and immense knowledge. His guidance helped me in all the time of research and writing of this thesis. Besides my advisor, I would like to thank the rest of my thesis committee: Prof. Madya Dr Saiful Nizam Tajuddin for his insightful comments and encouragement, but also for the hard question which incented me to widen my research from various perspectives.

My sincere thanks also go to Bioaromatic's Lab, Universiti Malaysia Pahang and Chemical Engineering's Lab, Faculty of Chemical and Natural Resources Engineering, Universiti Malaysia Pahang because give me opportunity for access and using the laboratory and research facilities. Without the facilities support it would not be possible to conduct this research. I would also like to express my gratitude to Universiti Malaysia Pahang, which granted me the financial assistance under UMP Grant Scheme project no. RDU 150315 and PGRS160324.

I would like to thank the graduate students of Faculty of Chemical and Natural Resources Engineering, Universiti Malaysia Pahang for the stimulating discussions, for the sleepless nights we were working together before deadlines, and for all the fun we have had in the last two years (November 2015 until December 2017). Special thanks to all my friends for their friendship.

Last but not the least, to my husband and parents, I would like to say that without your sacrifice, encouragement and understandings I may not be able to complete this study. To my brothers and sisters, thank you for supporting me spiritually throughout the writing this thesis and my life in general especially for finance.

ABSTRAK

Di Malaysia, ladang kelapa sawit dan industri minyak sawit merupakan penyumbang utama kepada pembentukan sisa pertanian. Dari kajian terdahulu, penyelidikan telah dijalankan untuk mengkaji potensi penggunaan sisa pertanian dengan cekap. Dalam kajian ini, jus kelapa sawit (OPF) digunakan untuk menggantikan fungsi glukosa semasa proses penapaian. Jus OPF dilaporkan mengandungi gula boleh diperbaharui seperti glukosa, sukrosa dan fruktosa. Jus OPF dijangka boleh menangani isu-isu alam sekitar untuk menghasilkan sebatian organik mudah meruap (VOC) terutamanya metil 3- (3,5-di-tert-butil-4-hidroksifenil) propionat kerana OPF banyak terbuang sebagai biojisim dan mudah diperoleh di seluruh Malaysia. Kajian penggunaan dan pembangunan penghasilan metil 3- (3,5-di-tert-butil-4-hidroksifenil) propionat dari kulat semakin meningkat kerana ia boleh dihasilkan secara semula jadi tanpa sintesis kimia. *Ceratocystis fimbriata* adalah kulat yang mempunyai potensi untuk mensintesis ester, ia tumbuh dengan cepat dan menghasilkan pelbagai aroma (pic, nanas, pisang, sitrus dan ros) bergantung kepada keadaan persekitaran dan kultur yang digunakan. Tujuan utama kajian ini adalah untuk menyaring dan mengoptimumkan penghasilan metil 3- (3,5-di-tert-butil-4-hidroksifenil) propionat dengan menggunakan satu siri reka bentuk eksperimen dengan teknik pengekstrakan fasa pepejal ruang (HS-SPME) dengan menggunakan gas kromotografi-spektroskopi jisim (GC-MS) untuk memisahkan kawasan puncak relatif sebatian selepas proses penapaian. Pengoptimuman penghasilan metil 3- (3,5-di-tert-butil-4-hidroksifenil) propionat dipengaruhi oleh beberapa faktor semasa proses penapaian. Siri reka bentuk eksperimen digunakan untuk menyaring dan mengoptimumkan pengeluaran sebatian itu. Dalam kajian penyaringan, kaedah rekabentuk faketorial penuh 2^4 telah digunakan untuk mencari faktor-faktor penting yang mempengaruhi pengeluaran metil 3- (3,5-di-tert-butil-4-hidroksifenil) propionat, yang merupakan suhu inkubasi (25 °C - 35 °C), medium pH awal (pH4 - pH 8), kelajuan agitasi (100 rpm - 150 rpm) dan kepekatan glukosa (20 g/L - 30 g/L) dalam jus OPF. Respon dalam penapisan dipadankan dengan persamaan regresi linear berganda dan memperoleh korelasi (R^2 = 0.8960) antara data eksperimen dan data model. Reka bentuk komposit pusat (CCD) digunakan sebagai reka bentuk eksperimen dan model regresi polinomial dengan istilah kuadrat digunakan untuk menganalisis data eksperimen menggunakan analisis varians (ANOVA). Analisis ANOVA menunjukkan bahawa model sangat signifikan ($p < 0.0001$) untuk menghasilkan metil 3- (3,5-di-tert-butil-4-hidroksifenil) propionat. Respon tersebut dipadankan dengan persamaan polinomial urutan kedua dengan korelasi tinggi (R^2 = 0.9598) di antara nilai ujikaji dan nilai yang diramalkan. Keputusan proses pengoptimuman menunjukkan bahawa penghasilan propionat maksimum metil 3- (3,5-di-tert-butil-4-hidroksifenil) propionat diperolehi dalam keadaan medium pH awal (8), kelajuan agitasi (100 rpm) dan inkubasi suhu (25 ° C). Di bawah keadaan optimum ini, pengeluaran metil 3- (3,5-di-tert-butil-4-hidroksifenil) propionat tertinggi didapati apabila masa penahanan adalah pada 32.80 minit dan kawasan puncak relatif 0.29% kawasan kromatogram dengan menggunakan GC-SPME. Kesimpulan kajian ini telah memberikan garis panduan yang signifikan dan kefahaman awal bagi penghasilan sebatian metil 3- (3,5-di-tert-butil-4-hidroksifenil) propionat dengan menggunakan jus OPF sebagai substrat tunggal, boleh diperolehi dan mampan oleh *C. fimbriata* pada skala yang lebih besar pada masa hadapan.

ABSTRACT

In Malaysia, oil palm plantations and the palm oil industries were the main contributors to the generation of agricultural waste. From previous studies, researchers have identified the potential of utilizing the agricultural waste efficiently. In this research, oil palm frond (OPF) juice was used to replace the function of glucose during fermentation. OPF juice is reported to contain renewable sugars such as glucose, sucrose and fructose. OPF juice is expected to address the environmental issues to produce volatile organic compounds (VOCs) especially for production of methyl 3-(3,5-di-tert-butyl-4-hydroxyphenyl) propionate as OPF is abundantly available as a biomass and easily available throughout Malaysia. The utilization and development of methyl 3-(3,5-di-tert-butyl-4-hydroxyphenyl) propionate production fungus have been of increasing interest as they are naturally produced without chemical synthesis. *Ceratocystis fimbriata* is a fungus which has the potential for synthesizing esters, it grows quickly and produces a variety of aromas (peach, pineapple, banana, citrus and rose) depending on the strain and culture conditions. The aim of this study was to screen and optimize methyl 3-(3,5-di-tert-butyl-4-hydroxyphenyl) propionate using a series of experimental design by a head space-solid phase micro extraction (HS-SPME) technique combined with gas chromatography-mass spectroscopy (GC–MS) was used to separate the relative peak area of the compound during the fermentation. Optimization of methyl 3-(3,5-di-tert-butyl-4-hydroxyphenyl) propionate production are affected by several factors during the period of fermentation. Series of experimental designs were applied to screen and optimize the production of the compound. In the screening study, 2^4 full factorial design were used to find significant factors affecting production of methyl 3-(3,5-di-tert-butyl-4-hydroxyphenyl) propionate, which are incubation temperature (25 °C-35 °C), initial pH medium (pH4, pH8), agitation speed (100 rpm, 150 rpm) and concentration of glucose (20 g/L, 30 g/L) in OPF juice. The responses in screening were fitted with a multiple linear regression equation and obtained a correlation ($R^2 = 0.8960$) between the experimental data and model data. Then central composite design (CCD) was applied as the experimental design and a polynomial regression model with quadratic term was used to analyze the experimental data using analysis of variance (ANOVA). ANOVA analysis showed that the model was very significant ($p < 0.0001$) for the methyl 3-(3,5-di-tert-butyl-4-hydroxyphenyl) propionate production. The responses were fitted with the second order polynomial equation with high correlation ($R^2 = 0.9598$) between the observed and predicted values. The results of optimization process showed that a maximum methyl 3-(3,5-di-tert-butyl-4-hydroxyphenyl) propionate production was obtained under the condition of initial pH medium (8), agitation speed (100 rpm) and incubation temperature (25°C). Under these optimized conditions, the highest 3-(3,5-di-tert-butyl-4-hydroxyphenyl) propionate production was obtained when the column retention time was 32.80 minutes and the relative peak area was 0.29 % of chromatogram area by using GC-SPME. As a conclusion, this study provides a significant guideline and basic of understanding for the production of methyl 3-(3,5-di-tert-butyl-4-hydroxyphenyl) propionate compound at larger scale using OPF juice as sole, renewable and sustainable substrate by *C. fimbriata* in the near future.

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LIST OF SYMBOLS

mL	milliliter
L	Liter
kg	kilogram
g	gram
°C	temperature
rpm	Rotation per minute
g/L	Gram per Liter
mL/min	Milliliter per minute
spores/mL	Spores per milliliter

LIST OF ABBREVIATIONS

GC-MS	Gas chromatography mass spectrometry
SPME	Solid phase micro extraction
HPLC	High performance liquid chromatography
ANOVA	Analysis of variance
C. fimbriata	*Ceratocystis fimbriata*
VOCs	Volatile organic compounds
CCD	Central composite design
DOE	Design of experiments
OPF	Oil palm frond
OPT	Oil palm trunk
EFB	Empty fruit bunches
PKC	Palm kernel cake
POME	Palm oil mill effluent
PPF	Palm press fibre
FFB	Fresh fruit bunches
MARDI	Malaysian Agricultural Research and Development Institute
CDW	Cell dry weight
FFD	Full factorial design
RSM	Research surface methodology
PDMS	Polydimethylsiloxane
CAR	Carboxen
DVB	Divinylbenzene
MPOB	Malaysian palm oil board
PKS	Palm kernel shells
MF	Mesocarp fibres
NaOH	Sodium hydroxide
HCL	Hydrochloric acid
MSM	Mineral salt medium

$(NH_4)_2SO_4$	Ammonium sulfate
KH_2PO_4	Monopotassium phosphate
$Ca(NO_3)_2.4H_2O$	Calcium nitrate tetrahydrate
$MgSO_4.7H_2O$	Magnesium sulfate heptahydrate
$Fe(NO_3)_3.9H_2O$	Iron(III) nitrate nonahydrate
$ZnSO_4.7H_2O$	Zinc sulfate heptahydrate
$(MnSO_4.4H_2O)$	Manganese(II) sulfate tetrahydrate
MSM	Mineral salt medium
ATCC	American type of culture collection
PDA	Potato dextrose agar

CHAPTER 1

INTRODUCTION

1.1 Background

Oil palm, *Elaeis guinensis*, is widely used as crops in Malaysia and other tropical countries. The palm oil production has undergone advanced growth and becomes one of the most important contribution to the national income. The palm oil consumption recorded significant increased from 6% in year 1976 to 28% in year 2009. The sharp increase in global consumption is contributed mainly by Indonesia and Malaysia, which are the major palm oil producing countries in the world (Abdullah and Wahid, 2010). In 1920, the total oil palm planted area in Malaysia was only 400 hectares with the first commercial planting was set up in year 1917 (Ooi et al., 2014). Since then, the oil palm plantation was expanded due to the main thrust of Malaysia government to avoid highly dependency of plantation companies merely on rubber plantations. This drives Malaysia to become world's largest producer of palm oil for decades until Indonesia overtakes its role in year 2006 (Ooi et al., 2014; Santosa, 2008).

In year 2006, Malaysia is the second largest producer of palm oil with 15.88 million tonnes or 43% of the total world supply. Indonesia is the world's largest producer of palm oil with 15.9 million tonnes of oil or 44% of the total world supply. In 2007, productive oil palm plantations in Malaysia are 4.3 million hectares, a 3.4% increase from year 2006 which stood at 4.2 million hectares (Shuit et al., 2009). The increase in oil palm plantation area in Malaysia is mainly because of growing global demand for edible oil especially palm oil. The palm oil industry has been proven to play an important role in the growth of Malaysian economy. This was proven when the industry helped to maintain the economy of this country during the economic crisis through its export-oriented activities in the late 1990s (Ujang et al., 2010).

The Malaysian palm oil industrial complex can be defined as the various direct linkages, processing chains and products created as a consequence of the cultivation of oil palm and the production of the main product which is palm oil and secondary products, palm kernel oil and cake (Ujang et al., 2010). There are several by-products of oil-palm and these include oil palm trunk (OPT), oil palm fronds (OPF), empty fruit bunches (EFB), palm kernel cake (PKC), palm oil mill effluent (POME) and palm press fibre (PPF) (Zahari et al., 2003). Lately, OPF is emphasized as it has a great potential to be utilized as a roughage source or as a component in complete feed for ruminants such as castles and goats and pulp production (Dahlan, 2000; Hassan et al., 1996; Shuit et al., 2009; Wanrosli et al., 2007). OPF also can be obtained when the palms are pruned during the harvesting of fresh fruit bunch (FFB), and therefore, it is available daily.

Previous studies have reported, since OPF has a high moisture content and contains a large amount of sugar, it is potential to be used as a carbon source during fermentation (Dahlan, 2000; Wanrosli et al., 2007). Another studies by (Zahari et al., 2012), report that pressed juice by using a simple sugarcane press from oil palm frond (OPF) contained renewable sugars such as glucose, sucrose and fructose. The palm oil industry has attracted great interest from researchers due to the abundance of valuable residues generated from the palm oil mill. Both the solid waste and waste water from the palm oil industry are rich in carbon source and can be good substrates for microorganisms. There are several reports on bioconversion of palm oil mill residues for value-added products, such as utilization of POME for biogas, organic acids and P(3HB) production (Hassan et al., 1997; Mumtaz et al., 2008; Yacob et al., 2006; Yee et al., 2003; Zakaria et al., 2010), bioconversion of oil palm empty fruit bunch (OPEFB) for cellulase and sugar production (Ariffin et al., 2006; Roslan et al., 2011), and composting of oil palm empty fruit bunch (Baharuddin et al., 2010).

This has inspired researchers to search another value-added product by using OPF. The utilization and development of volatile organic compounds (VOCs) from microorganisms have been of increasing interest as they are naturally produced without chemical synthesis. VOCs are carbon-based solids and liquids that readily enter the gas phase by vaporizing at 0.01 kPa at a temperature of approximately 20 °C (Pagans et al., 2006). Methyl 3-(3,5-di-tert-butyl-4-hydroxyphenyl) propionate was one of VOCs production from this study by using *Ceratocystis fimbriata* (*C. fimbriata*). The methyl

3-(3,5-di-tert-butyl-4-hydroxyphenyl) propionate is using as an antioxidant in stabilizer reaction for production organic materials (Li et al., 2014). *C. fimbriata* have been identified as aroma producers. The potential of solid waste residues, such as oil palm frond (OPF), for the production of methyl 3-(3,5-di-tert-butyl-4-hydroxyphenyl) propionate has yet to be studied.

1.2 Problem statements

Flavored volatile organic compounds (VOCs) such as alcohols and esters are highly demanded as they are widely used in the food, beverage, pharmaceutical and cosmetic industry as key ingredients (Cheetham, 1997; Welsh et al., 1989). In 1994, the size of the flavor market being worldwide estimated at 9.7 billion US dollar (Cheetham, 1997). In recent years, there has been an increasing in consumer preferences toward natural flavors although chemical synthesis still remains a more convenient technology (Cheetham, 1997).

However, since compounds extracted from plant sources, for example fruits; there are problems of seasonal supply, high cost and regional variation in VOCs profile and quantity (Krishna and Karanth, 2002; Lomascolo et al., 1999), other non-conventional sources such as free living microbial cells, including food-grade microorganisms (Lomascolo et al., 1999), have gathered considerable interest from an industrial viewpoint (Lomascolo et al., 1999; Vandamme, 1996; Welsh et al., 1989). As a result, in the past years most industries have carried out extensive screening programmes on naturally occurring microorganisms for the selection of industrially relevant VOCs producers (Cheetham, 1997; Steele and Stowers, 1991).

Approximately 250 VOCs have been discovered from fungi where they occur as mixtures of simple hydrocarbons, heterocycles, aldehydes, ketones, alcohols, phenols, thioalcohols, thioesters and their derivatives, including, among others, benzene derivatives, and cyclohexanes (Chiron and Michelot, 2005; Korpi et al., 2009; Ortíz-Castro et al., 2009). VOCs also have varieties of odors so it is not surprising that attentiveness in fungal VOCs began with the fungi that humans can smell. There is a large volume of published studies describing the distinct bouquets of macrofungi such as mushrooms and truffles, highly valued in the culinary arts, including mixtures of different VOCs, of which alcohols, aldehydes, terpenes, aromatics and thiols dominate (Breheret

et al., 1997; Cho et al., 2008; Fraatz and Zorn, 2011; Splivallo et al., 2007; Tirillini et al., 2000). Therefore, the production of VOCs by fungus has become an importance objective for the industry.

Methyl 3-(3,5-di-tert-butyl-4-hydroxyphenyl) propionate is a one of VOCs production that able be produce from microorganism. It is one of the most important hindered hydroxyphenylcarboxylic acid esters, because it is an effective antioxidant and also a key starting material for the preparation of many other antioxidants through transesterification reactions with other alcohols. Many organic materials, such as lubricants, fuels and polymers, are susceptible to oxidative and thermal deterioration from heat or mechanical stress, or in the presence of chemicals such as atmospheric oxygen or metallic impurities (Strlič et al., 2009). This may result in the loss of the desirable physical properties of these materials and the failure of their proper functions. By using, methyl 3-(3,5-di-tert-butyl-4-hydroxyphenyl) propionate as an antioxidant as a stabilizer into organic materials for retarding the deterioration (Seguchi et al., 2012).

Fungi from the genus *Ceratocystis* produced a large diversity of fruit-like aromas and among this genus, *C. fimbriata* seems to be interesting because of its relatively rapid growth, its good ability for spore production and the variety of aromas synthesized (Senemaud, 1988). In general, the production of VOCs is by biosynthesis. The biosynthesis always uses the common substrate as it sources of energy or carbon source, that is glucose. The glucose is a competitive carbon source for many kinds of fermentation in the manufacturing of biobased products. In fact, glucose is made from lignocellulose biomass. However, the hydrolysis of lignocellulose is very difficult to do as its natural lignin component that recalcitrant to degradation. As an alternative, the oil palm frond (OPF) juice could replace the function of glucose for the fermentation.

The effects of various factors in production of VOCs are using experimental design (DoE) such as Box-Behnken and Central Composite Design (CCD). This method is better experimental method due to its systematic ways in collecting data and data analysis. Besides that, in term of time consuming, the experimental work, could be shorten. For the screening the full factorial design was chosen because to study the main effects and interactions effects of the factors in this study. In order to determine the optimum process parameters, such as incubation temperature, agitation speed and initial pH medium for methyl 3-(3,5-di-tert-butyl-4-hydroxyphenyl) propionate production, the

experiments were designed according to a CCD in three variables following the Response Surface Methodology (RSM).

Since OPF is an abundant solid waste at oil palm plantation and is currently under-utilized, it has great potential to be used as sustainable, and renewable fermentation feedstock to produce methyl 3-(3,5-di-tert-butyl-4-hydroxyphenyl) propionate. Hence this study was done to explore and clarify the potential of OPF juice as sustainable promising sources for methyl 3-(3,5-di-tert-butyl-4-hydroxyphenyl) propionate production.

1.3 Objectives of study

The main objective of this research is to utilize glucose derived from OPF juice as a novel and renewable carbon source to produce VOCs such as methyl 3-(3,5-di-tert-butyl-4-hydroxyphenyl) propionate through fermentation using fungus strain of *C. fimbriata*.

The specific objectives of the study are:

1) To investigate the ability of *C. fimbriata* to produce VOCs by using OPF juice as a sole carbon source in the shake flask experiment.

2) To investigate the factorial analysis affecting methyl 3-(3,5-di-tert-butyl-4-hydroxyphenyl) propionate production such as effect of initial pH medium, effect of agitation speed, effect of incubation temperature and effect of glucose concentration in OPF juice using the full factorial design method.

3) To optimize methyl 3-(3,5-di-tert-butyl-4-hydroxyphenyl) propionate production using Response Surface Methodology (RSM) with Central Composite Design (CCD).

1.4 Scope of the study

In order to achieve the objective, the following scopes have been identified:

1) The potential of OPF juice as sole substrate for the VOCs production during fermentation using *C. fimbriata* was done by using cell dry weight (CDW) method whereby the characterization of sugar composition and consumption of the OPF juice by *C. fimbriata* were carried out using High Performance Liquid Chromatography (HPLC). The relative peak area of chromatogram area of VOCs production was separated and analyzed by Gas Chromatography-Mass Spectroscopy (GC-MS) with Solid Phase Micro Extraction (SPME).

2) As for the comparison study, technical grade glucose was used as a carbon source for the growth and production of VOC's using *C. fimbriata*.

3) Factors affecting the methyl 3-(3,5-di-tert-butyl-4-hydroxyphenyl) propionate production one of VOCs production such as the effect of incubation temperature (25 °C, 35 °C), initial pH medium (pH 4, pH 8), agitation speed (100 rpm, 150 rpm) and concentration of glucose (20 g/L, 30 g/L) in OPF juice were observed by 250 mL flask as the fermentation system by using full factorial design method.

4) The optimum condition for the methyl 3-(3,5-di-tert-butyl-4-hydroxyphenyl) propionate production were examined using central composite design (CCD) and analyzed the response pattern using Response Surface Methodology (RSM). The parameter studied for the optimization of methyl 3-(3,5-di-tert-butyl-4-hydroxyphenyl) propionate production were obtained from the factorial analysis which were as follows; incubation temperature at (15°C – 35 °C), initial pH medium (pH 4 – pH 12) and agitation speed (40 rpm – 160 rpm).

CHAPTER 2

LITERATURE REVIEW

2.1 Introduction

In this chapter, some information on biosynthesis of VOCs by using microorganism were discussed briefly. A review on the production of VOCs from low cost (waste-based) substrates were discussed as well. The discussions were specified on the potential of waste substrates for VOCs production and the analytical method for analysis and separate the relative peak area of chromatogram area. Besides that, brief information on factors affecting the production of VOCs production such as methyl 3-(3,5-di-tert-butyl-4-hydroxyphenyl) propionate, incubation temperature (°C), initial pH medium, agitation speed (rpm) and concentration of glucose (g/L) in OPF juice were also reviewed. At the end of this chapter, elaboration on the potential use of oil palm waste as an alternative, renewable and cheap substrate for the production of methyl 3-(3,5-di-tert-butyl-4-hydroxyphenyl) propionate and design of experiment were also discussed.

2.2 Volatile organic compounds and microorganisms

Volatile organic compounds (VOCs) are known as the organic chemicals which has a high vapor pressure at standard room temperature. Volatile organic compounds have a low boiling point which results in their high vapour pressure. With a low boiling point, it causes a huge number of molecules to evaporate and sublimates from the compounds, in solid or liquid form, and enter the surrounding air. This process is known as volatility. VOCs are carbon-based solids and liquids that readily enter the gas phase by vaporizing at 0.01 kPa at a temperature of approximately 20 °C (Pagans et al., 2006). About 250 VOCs have been discovered from fungi where they occur as mixtures of

simple hydrocarbons, heterocycles, aldehydes, ketones, alcohols, phenols, thioalcohols, thioesters and their derivatives, including, among others, benzene derivatives, and cyclohexanes (Chiron and Michelot, 2005; Korpi et al., 2009; Ortíz-Castro et al., 2009). It has conclusively been shown that, fungal VOCs are derived from both primary metabolism and secondary metabolism pathways (Korpi et al., 2009). VOCs are ideal "infochemicals", because they can diffuse through the soil and atmosphere. VOCs also have varieties of odors so it is not surprising that attentiveness in fungal VOCs began with the fungi that humans can smell. There is a large volume of published studies describing the distinct bouquets of macrofungi such as mushrooms and truffles, which are highly valued in the culinary arts, including the mixtures of different VOCs, of which alcohols, aldehydes, terpenes, aromatics and thiols dominate (Breheret et al., 1997; Cho et al., 2008; Fraatz and Zorn, 2011; Splivallo et al., 2007; Tirillini et al., 2000). More recent studies had discovered that wine-associated yeasts (*Saccharomyces spp.* and non-*Saccharomyces* yeasts) have been well known as flavoured VOC producers (Comi et al., 2001; Fernández et al., 2000; Kunkee and Amerine, 1970; Rojas et al., 2001; Patrizia et al., 1998; P Romano et al., 1997; Patrizia et al., 1997).

In addition, microorganisms play a crucial role in the generation of natural flavor compounds particularly in the field of food aromas. As pointed out recently by Bigelis (1992), filamentous fungi are useful in this field because of their ability to produce a great number of flavouring compounds and also to release aroma modifying enzymes. Many fungi and yeast have been discovered to produce *de novo* odorous compounds. *Ceratocystis sp.* is one of the filamentous fungi that are able to produce *de novo* odorous compounds, including floral flavours. The yeast *Williopsis saturnus* synthesize *de novo* fruity esters flavours such as volatile branced acetates and their yields can also be improved by feeding fuel oil as a cheap source of precursor branched alcohols to the fermentation process (Vandamme, 2003). Meanwhile, *Geotrichum klebahni* produces a broad spectrum of ethylsters of branched carboxylic acid and also generating a pleasant fruity flavour (Vandamme, 2003). *Ceratocystis sp.* and the yeast *Kluyveromyces lactis* and *Sporidiobolus salmonicolor* produce a wide range of terpenes and lactones with fruity or floral flavours (Vandamme, 2003). Moreover, the capacity of some *Ceratocystis sp.* to produce fruit-like aromas has already been reported (Lanza et al., 1976). Moreover, strains of the fungi *Ceratocystis* have been identified as aroma producers (Medeiros et al., 2006). Christen et al. (1997) also studied the production of aroma compounds by

employing different substrates such as wheat bran, cassava bagasse, and sugar cane bagasse complemented with a synthetic medium.

2.3 *Ceratocystis fimbriata*

C. fimbriata is a fungal plant pathogen that attacks a variety of temperate and tropical plants (Engelbrecht and Harrington, 2005). *C. fimbriata* also known as *Ceratocystis variospora*, is known to synthesize monoterpenes such as geraniol, citronellol, nero, linalool, and others (Schindler, 1982). *C. fimbriata* usually grows best between the temperatures off 18 °C to 30°C and is able to produce ascospores within a week. The fungus probably survives adverse conditions as mycelium within the plant host, or as aleurioconidia in the soil or in plant hosts or debris. *C. fimbriata* grows readily on most agar media at first with a fluffy appearance. Mycelium is hyaline at first, becoming brown, gray or olive-green after 2 to 4 days with sweet odor, often with banana scent (Johnson et al., 2005).

Within a few days, there are usually abundant conidiophores that produce chains of hyaline conidia, sometimes called endoconidia, characteristic of the anamorph genus *Chalara*. However, *Chalara* species are anamorphs of discomycetes, and the genus *Thielaviopsis* is now used for anamorphs of *Ceratocystis sp*. (Paulin et al., 2002). Endoconidia are cylindrical and may vary in size from 11 to 16 mm long by 4 to 5 mm wide (Hunt, 1956). Specialized conidiophores give rise to thick-walled, pigmented aleurioconidia and sometimes called chlamydospores, probably a survival spore. Aleurioconidia produced blastically, singly or in chains, orange-brown to brown are typically 9-16 mm long and 6-13 mm wide. Endoconidia may also darken and become thick walled chlamydospores, thus resembling aleurioconidia. Endoconidia, chlamydospores formed from endoconidia, and aleurioconidia may be produced on and within the substratum.

C. fimbriata and *Ceratocystis moniliformis* are particularly interesting because of their relatively rapid growth, the variety of complex aroma mixtures synthesized, the utilization of waste substrates and the potential for solid state fermentation (Christen et al., 1997). Moreover, *Ceratocystis sp.* are well-known to produce a wide range of terpenes, with fruity or floral odour (Vandamme, 2003). It also has the potential for synthesizing esters, grows quickly and produces a variety of aromas (peach, pineapple,

banana, citrus and rose), depending on the strain and culture conditions (Bluemke and Schrader, 2001; Medeiros et al., 2003; Pandey et al., 2000; Soccol et al., 2007). Previous research has shown that, there are some volatile compounds with fruity characteristics are produced by *C. fimbriata*. There are twelve compounds detected, among them: ethanol, acetaldehyde, ethyl acetate, ethyl propionate, and isoamyl acetate (Medeiros et al., 2006). In another study, Soares et al. (2000) also discovered that, the compounds such as acetaldehyde, ethanol, isopropanol, ethyl acetate, ethyl isobutyrate, isobutyl acetate, isoamyl acetate and ethyl-3-hexanoate are potentially produced by *C. fimbriata*. In 1998, Bramorski et al. (1998) published a paper in which they described the major volatile compounds found were alcohols, esters and, in a lesser amount, ketones.

2.4 Volatile organic compounds by biosynthesis

Some volatile organic compounds, such as alcohols, esters, aldehydes and ketones are potentially produced by biosynthesis.

2.4.1 Alcohol

The alcohols that produced by biosynthesis from *C. fimbriata* were ethanol, isoamyl alcohol, 2-hexanol, 1-propanol, 2-propanol, and 1-butanol (Bramorski et al., 1998). Alcohols do not play a predominant role in flavours but are known to contribute to the overall flavor quality and are precursors of fruit-like flavoring esters, which are definitely present in almost all fruits (Senemaud, 1988). Isoamyl alcohol has a fusel oil, whisky character with a pungent odor and repulsive taste with an aroma threshold value of 250 ppb to 4.1 ppm, and its usage comprise of alcoholic beverages, gelatins, puddings, baked goods, hard candy, chewing gum, non-alcoholic beverages, frozen dairy and soft candy. This alcohol occurs in vinegar, cheeses, butter, cognac, rum, whiskies, cider, sherry, grape wines, arctic bramble, olive, gin, quince, sake and buckwheat (Burdock, 2016). The bioproduction of isoamyl alcohol using *Neurospora sp.* has been widely reported before (Brigido, 2000; Brown and Hammond, 2003; Kobayashi et al., 2008; Pastore et al., 1994; Yamauchi et al., 1991). Meanwhile, bioproduction of isoamyl alcohol using *C. fimbriata* was reported by Bramorski et al. (1998).

2.4.2 Ester

The esters that produced by biosynthesis from *C. fimbriata* were ethyl acetate, ethyl propionate, isoamyl acetate, butyl acetate, and ethyl butyrate (Bramorski et al., 1998). Meanwhile, ethyl acetate, ethyl isobutyrate, isobutyl acetate, isoamyl acetate and ethyl-3-hexanoate are potentially produced by *C. fimbriata* (Soares et al., 2000). Ethyl acetate is a fruity smelling liquid with a brandy note and is the most common ester in fruits (Bauer et al., 2008). Butyl acetate has a strong, fruity odor and a burning, then sweet taste reminiscent of pineapple. It occurs in many fruits and is a constituent of apple (Bauer et al., 2008). Its bioproduction is by using *Kluyveromyces marxianus* in cassava bagasse (Medeiros et al., 2001). Isoamyl acetate is a compound that has a fruity, banana, sweet, fragrant, powerful odor with a bittersweet taste reminiscent of pear with an aroma threshold of 2–43 parts per billion (ppb).

Its usage comprises of alcoholic beverages, gelatins, puddings, baked goods, hard candy, chewing gum, non-alcoholic beverages, confectionary, frosting, soft candy, frozen dairy and sweet sauce in a range of 19–112 parts per million (ppm). It occurs naturally in some fruits like apple, apricot, banana, grape, berries, melon, papaya, peach, pear and pineapple, and products such as vinegar, wheat and rye bread, cheeses, butter, alcoholic and non-alcoholic beverages (Burdock, 2016). Biotechnological production was reported using *Saccharomyces cerevisiae* (Brown and Hammond, 2003; Kłosowski and Czupryński, 2006; Kobayashi et al., 2008), *Staphylococcus* (Talon et al., 1998), *C. fimbriata* (Soares et al., 2000) and *Ceratocystis moniliformis* (Bluemke and Schrader, 2001). In this study, methyl 3-(3,5-di-tert-butyl-4-hydroxyphenyl) propionate was discovered by using *C. fimbriata*.

2.4.3 Methyl 3-(3,5-di-tert-butyl-4-hydroxyphenyl) propionate

Methyl ester are a family of plant or animal fat-based materials that are used to produce other products such as industrial solvents, cleaners, lubricants, and fuels. Methyl esters can be produced from a variety of raw materials such as fat by-products and plant oils. A chemical process called transesterification turns this feedstock into methyl esters. The other method is by enzymatic processes carried out by microorganism during fermentation.

Methyl 3-(3,5-di-tert-butyl-4-hydroxyphenyl) propionate is one of VOCs production that able be produce from microorganism. It is one of the most important hindered hydroxyphenylcarboxylic acid esters, because it is an effective antioxidant and also a key starting material for the preparation of many other antioxidants through transesterification reactions with other alcohols (Fung et al., 2014; Gatto et al., 2011). Lubricants, polymers and fuels are examples of organic materials that are vulnerable to oxidative and thermal deterioration from mechanical stress or heat, or in the presence of chemicals such as atmospheric oxygen (Strlič et al., 2009).

This may affect in the loss of the desirable physical properties of these materials and the failure of their proper functions. For example, lubricants undergo thermal degradation when used during periods of elevated temperature, forming resins and sludge. One way to slow down the deterioration is to incorporate an antioxidant as a stabilizer into these materials (Seguchi et al., 2012). Sterically hindered hydroxyphenylcarboxylic acid esters have attracted considerable interest in this field. They have been widely used in polymer processing, and in industrial and automotive lubricating oils and greases (Bertoldo and Ciardelli, 2004). The excellent antioxidant properties of sterically hindered hydroxyphenylcarboxylic acid esters ensure process stability, long-term thermal stability of polymers and resilience of the corresponding products. They also enhance the performance of lubricant formulations by improving thermal stability, enabling oil drains and providing extended equipment life.

Continued efforts have been devoted to develop and improving the preparation processes of these hindered hydroxyphenylcarboxylic acid esters so that they become environmentally friendly and cost effective (Baranski, 2008). Methyl 3-(3,5-di-tert-butyl-4-hydroxyphenyl) propionate is one of the most excellent hindered hydroxyphenylcarboxylic acid esters. Figure 2.1 shows the chemical structure meanwhile in Table 2.1, show the physical properties of methyl 3-(3,5-di-tert-butyl-4-hydroxyphenyl) propionate.

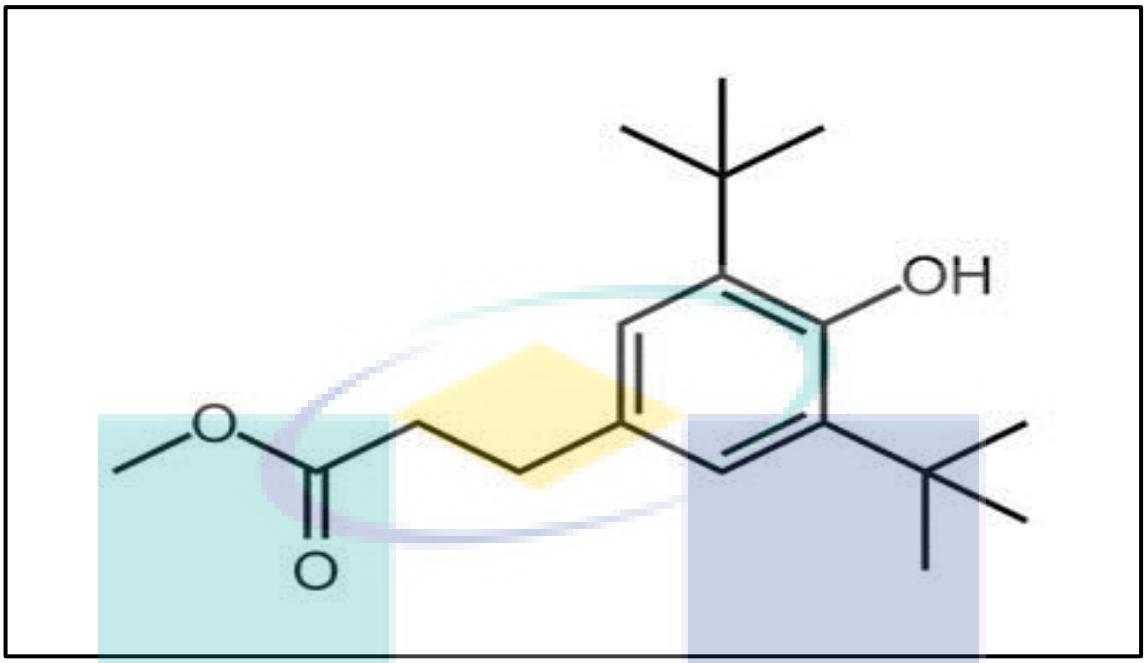


Figure 2.1 Methyl 3-(3,5-di-tert-butyl-4-hydroxyphenyl) propionate

Source: Li et al. (2014)

Table 2.1 Physical properties of methyl 3-(3,5-di-tert-butyl-4-hydroxyphenyl) propionate

Category	Properties
CAS number	006386-38-5
Empirical formula	$C_{18}H_{28}O_3$
Molecular weight	292.4131
IUPAC name	Methyl 3-(3,5-di-tert-butyl-4-hydroxyphenyl) propionate; Benzenepropanoic acid, 3,5-bis(1,1-dimethylethyl)-4-hydroxy-, methyl ester; Methyl 3-(3,5-ditert-butyl-4-hydroxyphenyl) propanoate
Melting point	259.65 °C
Boiling point	525.18 °C

2.4.4 Aldehyde

Among the aldehydes, benzaldehyde (almond aroma) and vanillin are the most important and widely used by the food industry. Benzaldehyde is used in aroma compositions due to its bitter almond odor and is applied as a starting material for many aliphatic fragrance and flavour materials. It possesses a sweet, floral and spice-like odor (Burdock, 2016). It naturally occurs in many products such as bitter almond, peach, apricot kernel, cheeses and black tea (Surburg and Panten, 2016). This compound can be

obtained in natural form, by extraction and distillation from botanical sources, or synthesized from benzyl chloride. Natural benzaldehyde, generally extracted from fruit kernels such as apricots, is used as an ingredient in cherry and other natural fruit flavours and has a market of ~20 tons per year and a price of approximately EUR 240 per kg. Benzaldehyde obtained from natural cinnamaldehyde can be purchased for EUR 100 per kg with an estimated market of more than 100 tons per year (Schrader, 2007). The screening of several white rot fungi has shown that numerous species are able to synthesize benzaldehyde through *de novo*, such as *Pleurotus sapidus*, *Polyporus sp.* and others (Lomascolo et al., 1999). Berger et al. (1987) studied the formation of a methoxy benzaldehyde in Ischnoderma benzoicum.

2.5 Analytical method for analysis volatile organic compounds

In view of the physical–chemical properties of volatile organic compounds, most common analytical methods are including separation by gas chromatography (GC) followed by on-line mass spectrometry (MS), flame ionisation detection (FID) or electron capture detection (ECD) (Dewulf et al., 2002; Eiceman et al., 2004; Mangani et al., 2003; Santos and Galceran, 2002). Recently, atomic emission spectroscopy (AES) has been recognized, if applicable, as a sensitive and highly selective detection system for GC (Campillo et al., 2004; van Stee et al., 2002). In a limited number of cases, high performance liquid chromatography (HPLC) or ion chromatography (IC) is used, particularly for the analysis of carbonyl compounds after derivatization (Claeson and Sunesson, 2005; Fujita et al., 2003; Hellen et al., 2006; Kot-Wasik et al., 2004; Shendell et al., 2004; Uchiyama et al., 2006). Moreover, the technique of solid phase micro extraction (SPME) coupled with GC-MS offers a simple and sensitive technique for volatile compound analysis.

The technique of (SPME) offers a simple and sensitive technique for volatile compound analysis. It represents a convenient alternative to more conventional methods of extraction of organic volatile and semi-volatile chemicals from different sample sources. It eliminates the usage of organic solvents, is significantly rapid, low-cost and simple, and integrates extraction, pre-concentration and sample introduction into a single step (Zhang and Pawliszyn, 1993; Zhang et al., 1994). SPME fibers are used in many areas of volatility studies and also an obvious choice for fast screening of volatile sesquiterpernoids on GC-MS (Bach et al., 2014; Merfort, 2002; Petronilho et al., 2014;

Rubiolo et al., 2014; Zhan et al., 2014). Initial concepts of SPME application was first introduced and published in 1989 by Belardi and Pawliszyn. Rapid development in this technique leads to the first SPME device in 1990, which was commercialised in 1993 by Supelco (Zain et al., 2017).

Since its introduction, SPME has developed furthermore because of its many advantages in terms of sample preparation (Kataoka and Saito, 2011). Although a large number of SPME applications have been reported for biomedical analysis (Souza et al., 2015), it also has great potential in analyzing environmental samples (Popiel and Sankowska, 2011; Saraji and Ghani, 2015; Yegemova et al., 2015; Zygmunt et al., 2007) as in the case of HCN analysis. SPME fibers often consist of a fused silica core coated with different materials that make them suitable for different analytes. Commercially available SPME fibers can be divided in two groups with regards to the mechanism involved in the interaction between the analytes and the SPME phase, absorption and adsorption (Górecki et al, 1999).

With absorption, the analytes are extracted by partition in the bulk of the SPME stationary phase; with adsorption, the analytes physically interact or chemically bind to the surface of the stationary phase (Andersen et al., 2015). Stationary phases that have absorption properties include polydimethylsiloxane (PDMS) and polyacrylate. The diffusion of small molecules in PDMS is similar to that in organic solvents; this provides a fast diffusion of the analytes into PDMS and an absorption type of extraction. Diffusion in polyacrylate is significantly lower than PDMS, but still fast enough to allow for absorption of the analytes to the stationary phase (Górecki et al., 1999). The adsorption type of fibers contains carboxen (CAR), divinylbenzene (DVB), or a mixture of these two plus PDMS. In the CAR/DVB/PDMS fiber material the primary extracting mechanism is adsorption to the porous material (Górecki et al., 1999).

Overall, in these materials, the diffusion is poor, and the analytes are retained at the surface by adsorption to the stationary phase. The pore size of the material is then very important for the capacity of the fiber and when the maximum is reached, there is constant equilibrium with the surrounding phase (Górecki et al., 1999; Pawliszyn, 2000). CAR and DVB have a similar surface area, but while the former has an even distribution of macro, meso, and micro pores, the latter is mainly a meso porous material with a moderate amount of macro pores. Macro pores are mainly present on the surface of the

material and trap analytes by hydrogen bonding or van der Waals interaction while micro- and meso pores physically trap analytes (Shirey and Mindrup, 1999). Fibers with PDMS/DVB/CAR are a combination of CAR-PDMS coated with DVB-PDMS. Thus, the larger analytes are retained in the meso and macro pores of the outer DVB layer, while the smaller analytes migrate through this layer and are retained by the micropores in the inner layer of CAR. This enables the fiber to cover a wide range of analytes in the range of 40–275 Da (Matich et al., 2008).

2.6 Production of VOCs from low cost (waste-based substrate)

The biotechnological processes application for industrial production can be classified as promising for sustainable development, although biotechnological production strategies for a range of products have not yet passed the test of economic viability. This is often caused by the cost of the raw materials. At this point, a viable solution strategy can be identified in the utilization of a wide range of waste and surplus materials that can be upgraded to the role of feedstocks for the biomediated production of desired end products. Such materials are mainly produced in agriculture and industrial branches that are closely related to agriculture (Braunegg et al., 1998; Khanna & Srivastava, 2005; Koller et al., 2010). Moreover, over the past few decades, an increasing trend towards efficient utilization of natural resources has been observed around the world. The direct disposal of agroindustrial residues as a waste on the environment represents an important loss of biomass, which could be bioconverted into different metabo-lites, with a higher commercial value (Albuquerque et al., 2006; Vendruscolo et al., 2007; Villas Bôas and Esposito, 2000).

Besides that, alternative uses of waste are now encouraged to decrease environmental pollution while in the past, organic wastes were discharged directly into the environment. These uses may increase the waste value (Damasceno et al., 2003). In addition to that, the production of VOCs is widely used because aroma production by microorganisms using waste-based substrate such as cassava wastewater, OPF juice, apple pomace, soy bean, amaranth grain and soy bean oil possibly may contribute to an economical VOCs production and also has been the focus of several studies. This is the result of secondary metabolism and labelled as natural aroma.

2.6.1 Production of VOCs from cassava wastewater

Cassava industry effluents are being researched for useful applications. Considered as a non-exhausting source, cassava industry wastewater can be the source of raw material for fermentative processes. Its high organic load and cyanogenic glycoside content originate from cassava plants. One of the alternative uses for cassava wastewater is as substrate for *Geotrichum fragrans* cultivation (Damasceno et al., 2003). This aerobic microorganism, isolated from cassava wastewater, is cyanide-resistant. *Geotrichum sp.* has been reported to be a producer of fruit aromas by generation of volatile compounds (Farbood, 1991; Latrasse et al., 1987; Pastore et al., 1994; Welsh, 1994) and such aromas have been detected in cassava processing industries.

2.6.2 Production of VOCs from apple pomace

Many researchers, looking for value-added products, have proposed the use of apple pomace for the production of enzymes (Berovič and Ostroveršnik, 1997; Favela-Torres et al., 2006; Shrikot et al., 2004; Zheng and Shetty, 2000a), organic acids (Shojaosadati and Babaeipour, 2002), protein-enriched feeds (Albuquerque et al., 2006; Bhalla and Joshi, 1994; Devrajan, et al., 2004; Vendruscolo et al., 2007), edible mushrooms (Worrall and Yang, 1992; Zheng and Shetty, 2000b), ethanol (Ngadi and Correia, 1992a, 1992b; Paganini et al., 2005), aroma compounds (Bramorski et al., 1998; Medeiros et al., 2000; Medeiros et al., 2006; Tsurumi et al., 2001), natural antioxidants (Foo and Lu, 1999; Lu and Foo, 2000), and edible fibers (Grigelmo-Miguel and Martín-Belloso, 1999; Masoodi et al., 2002; Paganini et al., 2005), among many others.

2.6.3 Production of VOCs from soybean

Soybean-based foods, including soy milk, tofu and fermented products, are widely consumed in Eastern countries and are expanding in consumption around the world. The volatile compounds in various fermented soybean products, such as Japanese miso and natto, Chinese sufu and Thai thua nao, have been studied extensively (Chung, 1999; Chung et al., 2005; Ku et al., 2000; Leejeerajumnean et al., 2001; Mori et al., 1983; Sugawara, 1991), and nearly 100 different volatile compounds representing a variety of chemical classes were identified.

Whilst there is wide variation in the volatile components of fermented soybean products, studies have shown that the most frequently present compounds include esters (ethyl 2-methyl butyrate, ethyl hexanoate), acids (acetic acid, 2/3methyl butanoic acid), pyrazines and phenolic compounds.

2.6.4 Production of VOCs from amaranth grain

The use of amaranth for diets is partly limited by presence of antinutritional substances – trypsin inhibitor, phenols, tannins, and phytohemagglutinins (Correa et al., 1986; Imeri et al., 1987). (Ciganek et al., 2007) reported that hexanal and acetic acid were found as the most abundant compounds detected in amaranth samples.

All VOCs emitted from plants can originate from biogenic and anthropogenic sources. Many plants emit substantial amounts of phytogenic volatile organic compounds (PVOCs), which include alkanes, alkenes, alcohols, aldehydes, ethers, esters and carboxylic acids. Defense, communication and protection against extreme environmental conditions have been proposed as reasons for these emissions. PVOCs are produced by a range of physiological processes in many different plant tissues and are themselves also extremely diverse, more than 30 000 compounds were predicted (Niinemets et al., 2004).

2.6.5 Production of VOCs from OPF juice

Malaysia as a tropical country experiences hot and wet weather throughout the year. This climate encourages the growth of the oil palm and consequently the development of oil palm cultivation in Malaysia. This development has made Malaysia a major global oil palm biomass producer (Yusoff, 2006). As a main exporter and producer of palm oil in the world, the total oil palm planted area in Malaysia reached 4.98 Mha as of September 2011 (MPOB, 2011b), which covers approximately 73% of the agricultural land and makes oil palm a promising raw material for renewable energy generation. The distribution of oil palm plantations in Malaysia is shown in Figure 2.2 In 2010, a total of 16.99 Mt of crude palm oil was produced (MPOB, 2011a).

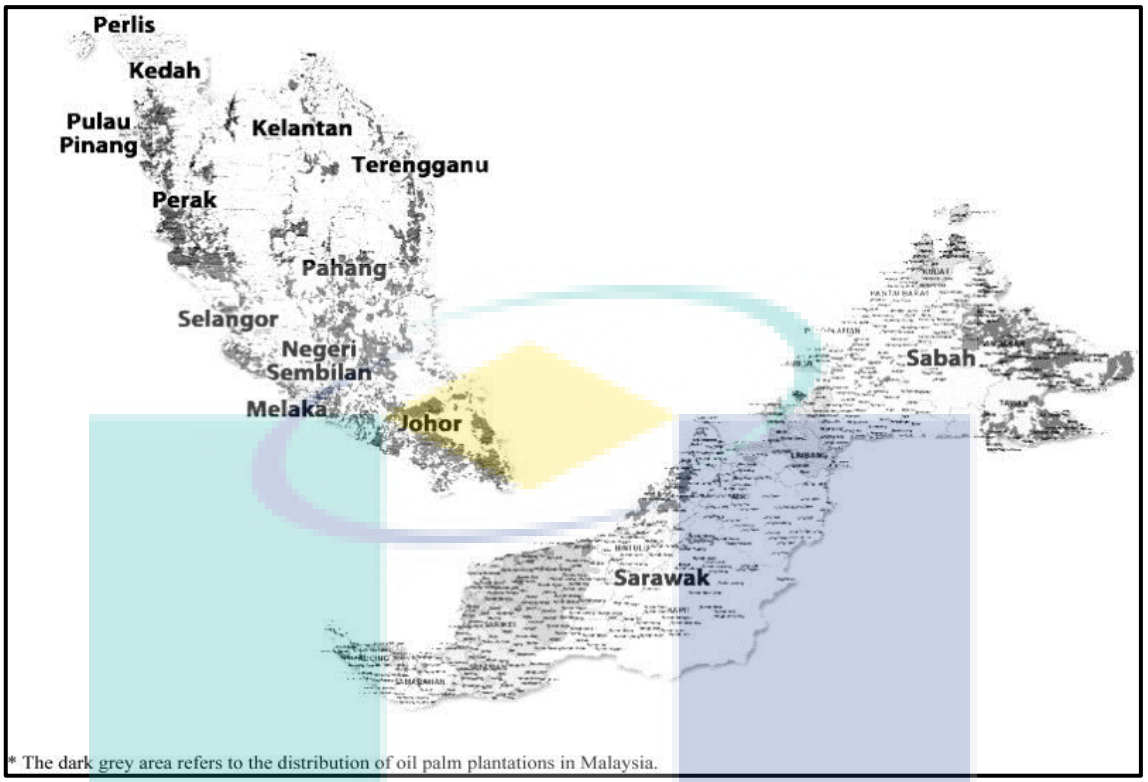


Figure 2.2 The distribution of oil palm plantations in Malaysia
Source: MPOB (2010)

Moreover, government support for downstream activities has been targeted at palm oil-based products such as oleochemicals and, more recently, at strengthening the role of the private sector in this industry as part of the Palm Oil National Key Economic Area (NKEA). At the same time, the palm oil industry generates significant amounts of biomass every year, which is mostly used as fertilizer in the plantations (Agency, 2011). There are six types of waste generated from oil palm industry and could be categorized into two groups; which are oil palm biomass and palm oil mill effluent (POME). Oil palm fronds (OPF) are available in the plantation throughout the year as they are regularly cut during harvesting of fresh fruit bunches (FFBs) and pruning of the palm trees. Additional fronds as well as oil palm trunks (OPT) will only be available in the plantations during the replanting of oil palm trees every 25 to 30 years.

Each of the trees produces approximately 10% of palm oil, while the remaining 90% is biomass residue. The different types of residues are produced by the mill and plantation activities. The palm kernel shells (PKS), mesocarp fibers (MF), and empty fruit bunches (EFB) are the main residues produced during the milling process, while the fronds and trunks are the major residues obtained from the plantation during felling. The

fronds are also obtained during harvesting and pruning (Abnisa et al., 2013). The sources and types of residues are shown in Table 2.2. The volume and type of oil palm residues are expected to rapidly increase and will become a serious problem in the future.

Table 2.2 Sources and types of oil palm residues

Sources of residue	**Type of residue**	**Weight of the total source (%)**	**Quantity per hectare (ton/ha)**
Fresh fruit bunch (from palm oil mill)	Palm kernel shell	5.5	1.10
	Empty fruit bunch	22	4.42
	Mesocarp fiber	13.5	2.71
Oil palm tree at felling (from plantation)	Trunk[a]	70	41.07
	Frond	20.5	16
	Leaf	6.53	7.69
	Other	2.97	19.44
Oil palm tree at pruning (from plantation)	Frond[b]	27.03	10.40

[a] Palm trunks felled once every 25–30 years

[b] Consists of the leaf and measured in dry weight

Source: Abnisa et al. (2013)

Nowadays, the residues of the oil palm are mainly contributing to biomass waste in Malaysia, and these wastes require extra initiative to handle. A survey of the literature indicates that most of them are negatively handled which leads to the negative impact to the environment. Most of the residues from the plantations are incinerated or dumped as organic fertilizer to decompose naturally, and only 40% of the trunks are used in plywood manufacturing (Asma et al., 2010). In palm oil mills, the PKS, EFB, and MF residues are generally sent to the boiler to be burned as fuel for steam generation (Mahlia et al., 2001). Many studies are being conducted to manage these wastes, producing different by-products such as activated carbon (Alam et al., 2007), xylose (Rahman et al., 2006), cellulase (Alam et al., 2009), polyhydroxyalkanoates (Mumtaz et al., 2010), protease (Wu et al., 2006), hydrogen (Morimoto et al., 2004).

The utilization of OPF into more beneficial product has been reported recently as a livestock feed (Bengaly et al., 2010; Dahlan, 2000; Kawamoto et al., 2001), biofuel regeneration (Lee et al., 2010; H. T. Tan et al., 2010) , absorbent for heavy metal ions in waste water (Salamatinia et al., 2010), renewable sugar (Sabiha et al., 2011; Zahari et al.,

2012), composite board (Rasat et al., 2011; Rozman et al., 1997) and it has also been recognized as a promising raw material to produce paper through chemical pulping processes (Rosli et al., 2004; Wanrosli et al., 2007). Hussin et al. (2013) reported that, the treatment of OPF with alkaline and organic alcohol solution is practically suitable for the isolation of lignins. Besides that, it discovered that the VOCs such as phenol group contain higher amount of phenolic -OH in the OPF lignin structure.

Other researchers, also looking for value added products, have proposed the use of OPF juice for the production of VOCs such as succinic acid (Tan et al., 2016), bioethanol (Lee and Halim, 2014), and biobutanol (Nasrah et al., 2017). Succinic acid is produced by using *Actinobacillus succinogenes* 130Z in fermentation of OPF juice and give a final concentration up to 21 g/L after 60 hours of anaerobic fermentation (Tan et al., 2016). Meanwhile, by using *S. cerevisiae* HC10 in OPF juice for production bioethanol is high (79.77%). Optimized fermentation conditions for bioethanol production are OPF juice (40%), inoculums size (20%), pH (4.5), and fermentation time (24 hour) (Lee and Halim, 2014). For biobutanol production, from OPF juice by *Clostridium acetobutylicum* ATCC 824 using response surface methodology (RSM) produce 0.3054 g/g after 144 hours of incubation period.

2.7 Renewable sugar from OPF

OPF juice had been identified as a good source to replace the function of glucose for the fermentation. It was reported that pressed juice from oil palm frond (OPF) contained renewable sugars such as fructose, glucose and sucrose (Zahari et al., 2012). For obtaining 50% (wt/wt) of OPF juice, the fresh OPF was pressed using a simple sugarcane pressing machine. The glucose content in the juice was 53.95 ± 2.86 g/l, which accounts for 70% of the total free sugars. OPF juice contains high amount of sugars, making it a potential fermentation feedstock for various value-added products such as polyhydroxyalkanoates (PHA), bioethanol, biobutanol, lactic acid, and succinic acid (Zahari et al., 2012). In order to be a good industrial fermentation feedstock as a renewable sugar, there are some criteria that need to be fulfilled. The substrate needs to be cost effective, be consistently and locally available, be able to produce high yield of biomass and product of interest to be easily operated, have low risk on health and safety to contain no impurities, and meet the local government legislation (Che Maail et al.,

2014). Table 2.3 shows the sugars concentration and composition of renewable sugars in OPF juice at different section of OPF petiole.

Table 2.3 Amount of sugars contained in the OPF juice from different section of fresh oil palm frond

OPF section[a]	Fructose (g/L)[b]	Glucose (g/L)[b]	Sucrose (g/L)[b]	Total sugar (g/L)
X_A	1.91	61.17	16.95	80.03
Y_A	0.78	57.48	19.89	78.15
Z_A	1.10	52.98	22.99	77.07
Average	1.26	57.21	19.94	78.42
X_B	1.26	54.66	16.47	72.39
Y_B	1.42	51.94	20.18	73.54
Z_B	1.30	49.66	21.16	72.12
Average	1.33	52.09	19.27	72.68
X_C	1.97	54.51	19.60	76.08
Y_C	2.00	52.34	22.10	76.44
Z_C	3.35	50.83	24.77	78.95
Average	2.44	52.56	22.16	77.16
Average*	1.68 (±0.75)	53.95 (±2.86)	20.46 (±1.56)	76.09 (±2.85)

*values are means of triplicates samples

X is initial of OPF, Y is middle of OPF, Z is edge of OPF

a A, B and C represent three (3) different OPF from different oil palm tree

b Determined by HPLC

Source: Zahari (2013)

2.8 Characterization of OPF and OPF juice as fermentation substrate

Recent study by Zahari (2013), shown that moisture content in the freshly chopped OPF is about 70%, which is similar to that in the outer part of oil palm trunk (OPT) (Kosugi et al., 2010). OPF also contains high dietary fiber (21%) and carbohydrate (28%), with a low crude protein of 3.2%. Lower protein content in the petiole part of OPF compared to the leaflet was also reported by other researchers, whereby most of the protein is accumulated in the leaflet (Dahlan, 2000). The cellulose, hemi-cellulose, and lignin contents of the fresh OPF are 41.7%, 16.4% and 15.5%, respectively (Zahari,

2013). According to Zahari (2013), the OPF and OPF juice contained high percentages of carbon, i.e. 49% and 39%, respectively by using CNHS (carbon, nitrogen, hydrogen, sulfur) analyzer. The high carbon content in the OPF juice indicates its suitability as a renewable carbon source to produce value-added products such as, bioethanol, lactic acid and biobutanol through fermentation. In addition to that, OPF juice also contained suitable C/N (carbon/nitrogen) ratio i.e. 50, which is essential for microorganisms' growth.

The metal concentrations in the OPF and OPF juice are shown in Table 2.4. In addition, Table 2.4 also shows metals concentrations in the OPF and OPF juice. The results showed low heavy metals concentration (<100 ppm) in the OPF juice, showing its suitability as fermentation feedstock. The nutrients and heavy metals in the OPF and OPF juice are determined using ASTM D 3682 method (Omar, Idris, Yunus et al., 2011) and the content is then determined using Inductively Coupled Plasma (ICP). Meanwhile the free amino acids and total amino acids are analyzed according to the method described in Official Journal of the European Communities 19.9.98, L257/16 are shown in Table 2.5 (Zahari, 2013).

Table 2.4 Nutrient and metallic elements in OPF and OPF juice *

Analysis	**OPF**	**OPF juice**
N (%)	0.9	0.8
C (%)	49	39
C/N (%)	56	50
Organic Carbon (%)	37	29
Composition of nutrients and metal elements		
Sulfur (%)	0.2	0.4
Phosphorus (%)	0.02	0.02
Potassium (%)	0.2	2.3
Calcium (%)	1.4	2.9
Magnesium (%)	0.2	0.5
Boron (ppm)	4	2
Manganese (ppm)	61	2
Copper (ppm)	2	2
Iron (ppm)	100	66
Zinc (ppm)	3	9

*Data is the mean of duplicate samples

Table 2.5 Amino acids content in OPF juice

Parameter	Concentration (µg/g OPF juice)
Asparagine	1.6
Threonine	0.2
Serine	111.0
Glutamic acid	22.7
Glycine	2.3
Alanine	1.6
Cystine	0.7
Valine	0.6
Methionine	0.2
Isoleucine	0.3
Leucine	0.7
Tyrosine	0.3
Phenylalanine	2.0
Histidine	0.6
Tryptophan	0.9
Lysine	0.1
Arginine	1.2
Proline	27.1
Total amino acid	174.1

Source: Zahari (2013)

Based on the sugars content, minerals and nutrients analysis, it is suggested that OPF juice has potential to be used as fermentation substrate to produce value-added products. Previous study has shown that OPF juice is suitable to be used as fermentation feedstock as there was no inhibition on microbial growth or product formation, there were no impurities, it was easy to be operated, and it had no risk on health and safety (Zahari et al., 2012). The use of OPF juice as renewable fermentation feedstock should be of wide range of applications in various industries as OPF is readily available all year round, no enzymatic or chemical pretreatment will be needed unlike lignocellulosic materials and most importantly, there are no inhibitors or salts that will affect the fermentation yield. Salts and inhibitors like weak acids, furan derivatives, and phenolic compounds which are produced during steam pretreatment and hydrolysis of lignocellulose materials may

affect the performance of product-generating microbes (Palmqvist and Hägerdal, 2000; Rumbold et al., 2010).

2.9 Factors affecting production of methyl 3-(3,5-di-tert-butyl-4-hydroxyphenyl) propionate

There were several other factors related to the biosynthesis of methyl 3-(3,5-di-tert-butyl-4-hydroxyphenyl) propionate. Factors such as temperature (°C), initial pH of medium, agitation speed (rpm) and concentration of glucose (g/L) in OPF juice will influence the growth and biosynthesis of methyl 3-(3,5-di-tert-butyl-4-hydroxyphenyl) propionate production.

2.9.1 Incubation temperature

Temperature is one of the parameter that will give effection on fermentation performance of selected yeast strains for volatile compound productivity such as ethanol, were reported (Baranyi and Roberts, 1994; Carvalheiro et al., 2005; Clark and Blanch, 1997; Dalsenter et al., 2005; Dragone et al., 2004; Evans et al., 2002; Hettenhaus, 1998; McMeekin et al., 2002; Messens et al., 2003; Salmon and Mauricio, 1994; Sanchez et al., 2004). Besides that, temperature also can exert different effects on the growth and production phases of secondary metabolism (Rizk, Abdel-Rahman, and Metwally, 2007). Higher temperatures result in more ester production. Saisons and other beers with very fruity characteristics (usually ales) are fermented between 70 °F and 75 °F (21 °C - 24 °C). Lower fermentation temperatures in the range of 64°F to 70 °F (18 °C – 21 °C) result in the production of less of these fruity or floral esters and produce spicier phenolic compounds like vanillin, 4-vinyl guaiacol, and eugenol which impart vanilla, smoky, or clove-like flavors to the beer. Lager fermentations usually occur under even lower temperatures, 50 °F to 55 °F (10 °C- 13 °C), resulting in the production of very few ester compounds (Bushman, 2015). From the previous study, it has been suggested that the suitable temperature for *C. fimbriata* in shake flasks fermentation was 30 °C (Bramorski et al., 1998; Christen et al., 1997; Christen and Raimbault, 1991; Christen et al., 1994; Medeiros et al., 2003; Soares et al., 2000).

2.9.2 Initial pH medium

The pH level of the growth medium has a marked effect on secondary metabolite production with synthesis falling rapidly either side of an optimal level. The hydrogen or hydroxyl ion concentration may have a direct effect on cell, or it may act indirectly by varying the degree of dissociation of substances in the medium. Therefore, the change of pH is also important for the enzyme activity of microorganisms, for the intermediate products, their dissociation and solubility (Rizk et al., 2007). The important of initial medium pH was described elsewhere (Lee et al., 2004; Loo and Sudesh, 2007; Shimizu et al., 1994) in which the optimal pH shall be determined at desired level to obtain optimal growth and desired by- products. (Bramorski et al., 1998; Christen et al., 1997; Christen and Raimbault, 1991; Christen et al., 1994; Medeiros et al., 2003; Soares et al., 2000) discover that pH 6 is suitable for production of fruity aroma by using *C. fimbriata*.

2.9.3 Agitation speed

Agitation speed is a very important factor in the fermentation process since it will increase the amount of dissolved oxygen in the cultivation medium (Purwanto et al., 2009). Excessive agitation would produce greater mechanical forces or hydrodynamic shear stresses and this condition is known to damage fungal mycelia and that lead to cell destruction, thus lowering the production of microorganisms pellets (Darah and Ibrahim, 1996; Porcel et al., 2005). Moreover, agitation speed of the culture broth has a variety of effects on microorganisms, including rupture of the cell wall, change in the morphology of filamentous microorganisms, variation in the efficiency and rate of growth and also variation in the rate of formation of the desired product (Porcel et al., 2005). It has been suggested that the suitable agitation speed for *C. fimbriata* in shake flasks fermentation was 150 rpm (Christen and Raimbault, 1991) and 180 rpm (Christen et al., 1994).

2.9.4 Glucose concentration

Glucose concentration is one of the factors which influences fungal morphology and product concentration. The glucose concentration in this study were using OPF juice for production of VOCs especially for methyl 3-(3,5-di-tert-butyl-4-hydroxyphenyl) propionate production through fungal fermentation. In addition, from the previous study, the metal concentration in the OPF and OPF juice also contain low heavy metals concentration (<100 ppm) in the OPF juice, showing its suitability as fermentation stock

(Zahari et al., 2012). According to Zahari et al. (2012), the pressed juice from OPF contained renewable sugars such as glucose, sucrose and fructose. Christen et al. (1994) reported that the more characteristics and intensive notes such as banana or nut are obtained with glucose or sucrose as carbon sources. Christen and Raimbault (1991) discover that 30 g/L of glucose was suitable for production of fruity aroma by using *C. fimbriata*.

2.10 Design of experiment

Design of experiments (DOE) is a systematic method to determine the relationship between factors affecting a process and the output of that process. In other words, it is used to find cause-and-effect relationships. It is also to study whether response surfaces are important for a few reasons. For instance, the response function is characterized in the area of interest to the experimenter, statistical inferences can be made on the sensitivity of the response to the factors of interest, factor levels can be determined for which the response variable is optimum (e.g., maximum or minimum), and factor levels can be determined that simultaneously optimize a few responses (Mason et al., 2003). However, it is a powerful technique used for discovering a set of process variables or factors which are most important to the process and then determine at what level these factors must be kept optimizing the process performance. Statistical design of experiments is a quick and cost-effective method to understand and optimize any manufacturing processes (Antony and Roy, 1999). The experiments in which the effects of more than one factor on response are investigated are known as full factorial experiments (Barka et al., 2014).

2.10.1 Factorial analysis

In an unoptimized methyl 3-(3,5-di-tert-butyl-4-hydroxyphenyl) propionate production, there might exist factors or variables which do not have significant or any effect on methyl 3-(3,5-di-tert-butyl-4-hydroxyphenyl) propionate process. Factorial analysis can be used to screen variables which are relevant to methyl 3-(3,5-di-tert-butyl-4-hydroxyphenyl) propionate production. A screening experiment explores which input variables (factors) that are causing most of the variability in the output (responses). A screening experiment usually involves only two levels of each factor and can also be called characterization testing or sensitivity analysis (Telford, 2007). Factorial

experimentation is a method in which the effects due to each factor and the combinations of factors are estimated. Factorial designs are geometrically constructed and vary all the factors simultaneously and orthogonally (Lazic, 2006).

In a full factorial experiment, both of the (-1) and (+1) levels of every factors are compared with each other and the effects of each of the factor levels on the response are investigated according to the levels of other factors. Doing so with the factorial planning of the experiments, it is possible to investigate simultaneously the effect of all the variables (Montgomery, 2017). In the study, full factorial design introduced a design space for further optimization using central composite design (CCD) with four significant factors, which were temperature, pH of initial medium, agitation speed and glucose concentration by performing a set of 16 experiments.

2.10.2 Optimization design

Optimizing can be referred as improving the performance of a system, a process, or a product in order to attain the maximum benefit from it. The term optimization has been widely used in chemistry by means of discovering conditions at which a procedure produces the best possible response (Diaconescu et al., 2011). Response surface methodology (RSM) is one of the most practical statistical optimization tools in biological and chemical process. RSM has been actively used for a number of phases of an optimization process in fermentation. RSM is a collection of statistical and mathematical techniques useful for designing experiments, developing models and evaluating the effects of variables in which a response of interest is influenced by several variables and the objective is to optimize this response. Fundamentally, RSM includes central composite design (CCD), Box-Behnken design, one factor design, D-optimal design, user-defined design and the historical data design.

The most popular statistical methods are CCD and Box-Behnken design. For one numeric variable, CCD has 5 levels (-a, -1, 0, +1, +a) whereas the Box- Behnken design only has 3 levels (-1, 0, +1). RSM also accommodate an experimental model that infers the correlation and interaction between a set of experimental variables and observed results, and finally provides optimized conditions (Baş and Boyacı, 2007; Bezerra et al., 2008; Nasrah et al., 2017). The current research aimed to optimize methyl 3-(3,5-di-tert-

butyl-4-hydroxyphenyl) propionate production using RSM with CCD by investigating the effect of pH of initial medium, incubation temperature, and agitation speed.

CHAPTER 3

METHODOLOGY

3.1 Overall research methodology

Figure 3.1 illustrates the overall research methodology for biosynthesis of methyl 3-(3,5-di-tert-butyl-4-hydroxyphenyl) propionate from OPF juice. For the preparation of OPF juice for biosynthesis of methyl 3-(3,5-di-tert-butyl-4-hydroxyphenyl) propionate, several amounts of fresh OPF (without leaves) were collected from the oil palm plantation at Lembaga Kemajuan Pertubuhan Peladang (LKPP), Lepar Hilir, Gambang, Pahang, Malaysia. The OPF juice was extracted by pressing the frond using a conventional sugarcane press machine by following the previous method described earlier by Zahari et al. (2012). The OPF juice was centrifuged at 10,000 rpm for 10 minutes at the temperature of 4 °C (Eppendorf, Germany) and the supernatant was filtered to remove the solid particles. The precipitate (pellet) was decanted and the supernatant (OPF juice) was used in the fermentation. The filtrate was stored in the freezer at the temperature of -20 °C (Liebherr, Malaysia) before use.

For the biosynthesis of methyl 3-(3,5-di-tert-butyl-4-hydroxyphenyl) propionate, pH of the OPF juice that was supplemented with synthetic medium was adjusted to 6 by adding sodium hydroxide (NaOH) 0.5 M and sterilized by autoclaving at the temperature of 121 °C for 20 minutes. The initial pH value of the OPF juice was approximately 4.6. Upon cooling, the OPF juice was introduced into a 250 mL Erlenmeyer flask containing 100 mL of OPF juice supplemented with synthetic medium aseptically in a laminar flow (Esco, Singapore) and inoculated with *C. fimbriata*. The OPF juice supplemented with synthetic medium and inoculum was biosynthesized for the production of methyl 3-(3,5-di-tert-butyl-4-hydroxyphenyl) propionate in 250 mL Erlenmeyer flask and was carried

out at the temperature of 27 °C on a rotary shaker (Infors, Switzerland). The methyl 3-(3,5-di-tert-butyl-4-hydroxyphenyl) propionate produced from biosynthesis experiment was analyzed and the relative peak area of chromatogram area was separated by Gas Chromatography-Mass Spectroscopy (GC-MS) with Solid Phase Micro Extraction (SPME) (Agilent Technologies, USA).

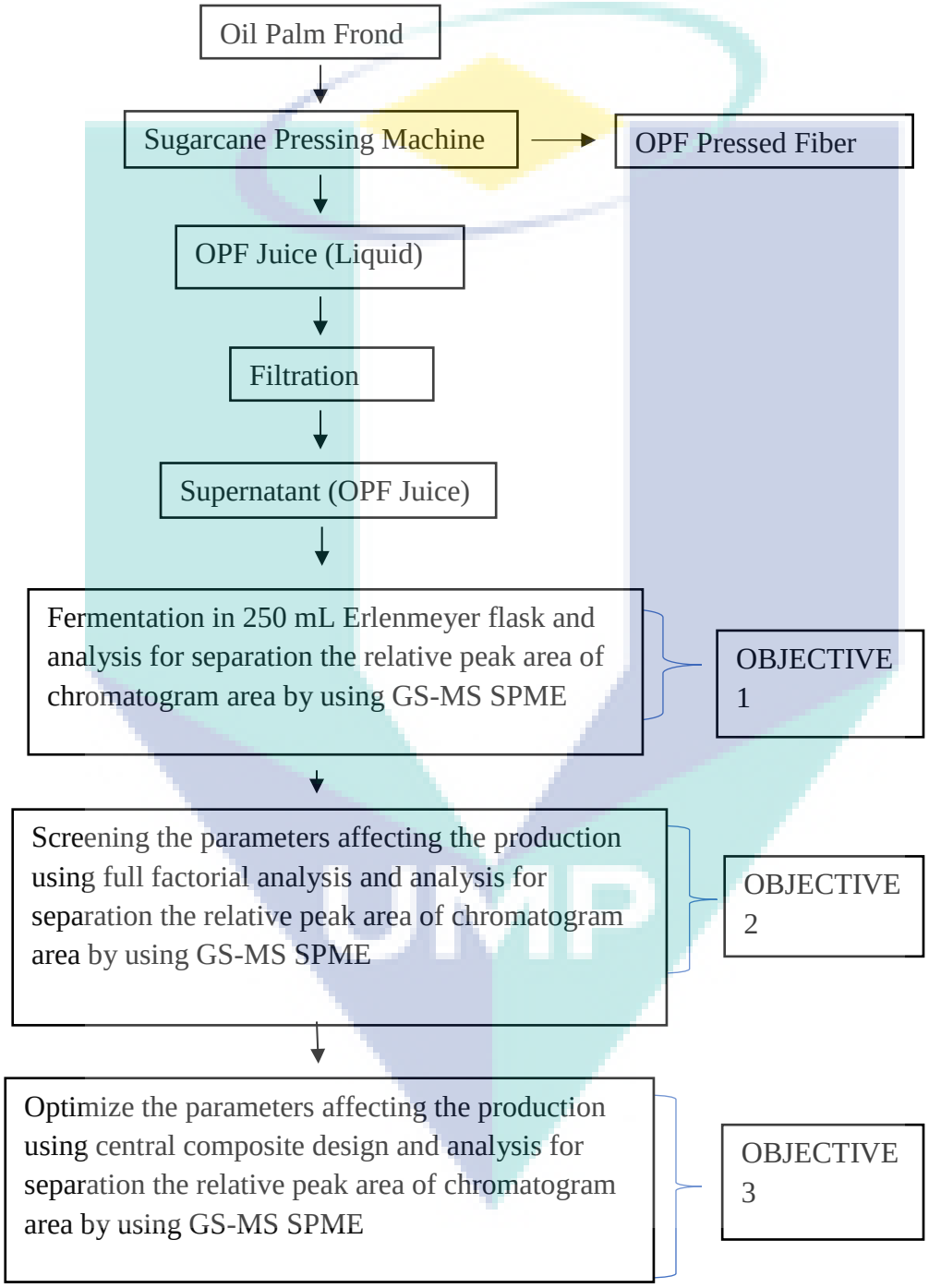


Figure 3.1 Research methodology for production methyl 3-(3,5-di-tert-butyl-4-hydroxyphenyl) propionate from OPF juice

3.2 Preparation of OPF juice

In this study, fresh OPF (petiole part without leaves) were collected from the oil palm plantation at Lembaga Kemajuan Pertubuhan Peladang (LKPP), Lepar Hilir, Gambang, Pahang, Malaysia. The OPF juice was collected by crushing the fresh OPF petioles using a conventional sugarcane press machine following a method described earlier by Zahari et al. (2012). OPF juice was centrifuged at 10,000 rpm for 10 minutes at the temperature of 4 °C (Eppendorf, Germany) and the supernatant was filtered to remove the solid particles. The precipitate (pellet) was decanted and the supernatant (OPF juice) was used in the fermentation. The supernatant was stored in the freezer at the temperature -20 °C (Liebherr, Malaysia) before use (Zahari et al., 2012). Figure 3.2 below showed the extraction of OPF juice to use for fermentation.

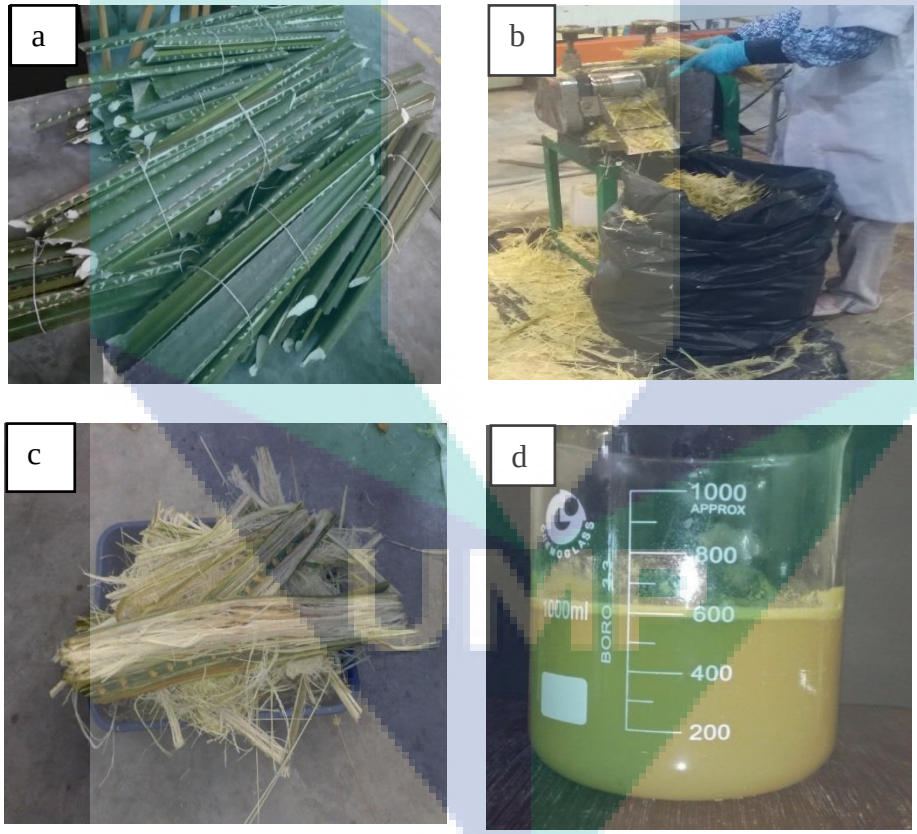
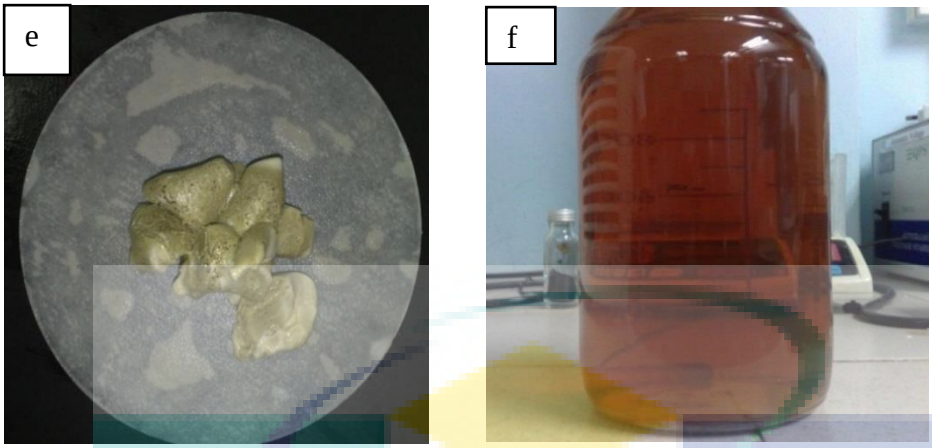


Figure 3.2 (a) Freshly OPF without leaves, (b) Sugarcane pressing machine used in this study, (c) OPF after pressing, (d) Fresh OPF juice after pressing



(e) Precipitate from OPF juice after centrifugation, (f) OPF juice after centrifugation

3.3 Sugar content

The sugar content in the OPF juice was determined by High Performance Liquid Chromatography (HPLC) (Agilent Series 1200, USA) using the Supelcosil LH-NH2 column (Sigma Aldrich) (25 cm x 4.6 mm ID, 5 µm particles) with a RI detector operated at the temperature of 30 °C. The mobile phase consisted of a ratio of acetonitrile: water (75%: 25%) at a flow rate of 1.0 mL/min. Meanwhile, standard sugars for HPLC analysis such as glucose, sucrose and fructose were obtained from Fisher Scientific (Leicestershire, UK) by comparing their retention times (Zahari et al., 2012).

3.4 Fungus strain

C. fimbriata (ATCC 12866) was obtained from freeze-dried microorganism purchased from American Type of Culture Collection (ATCC) had been used for biosynthesis of VOCs.

3.5 Growth and production medium for *C. fimbriata*

The growth medium for *C. fimbriata* was grown and transferred periodically onto Potato Dextrose Agar (PDA). To prepare 1 liter of PDA, 39.0 gram of PDA was weighed and dissolved in 1 liter of distilled water. Then, all the solutions were heated and stirred until it was completely dissolved, and the solution looked slightly clear. After that, these agars were autoclaved for 15 minutes to make sure it was in sterilized condition. The PDA agar was poured into sterilized petri dishes and left for solidification. As for culture maintenance or preservation purposes, the strain was sub-cultured once for every 5 weeks onto fresh petri dishes. For long preservation period up to 30 months, the strain was

streaked onto agar slant that contain 50% glycerol stock and kept at the temperature of 4 °C (Paul et al., 2015). The seed culture was prepared after 7 days of growth at the temperature of 30 °C. After 7 days, the spores were collected from the surface of the plate by adding distilled water containing a few drops of Tween 80 and some glass beads. The spore suspensions contained 108 spores/mL, which were prepared by dilution with sterilized distilled water and counted with the Neubauer's chamber (Medeiros et al., 2006).

For the seed culture preparation, 1 mL of spores were taken aseptically and transferred into 50 mL centrifuge tube containing 9 mL of basal growth medium. The seed cultures were then incubated at the temperature of 27 °C for 9 days on a rotary shaker at (150 rpm) in an aerobic condition. The basal growth medium for *C. fimbriata* was shown in Table 3.1. The initial pH of medium was adjusted to 6.0 with (NaOH) 0.5 M before autoclaved at 121 °C for 20 minutes. The trace element solution comprised of: Iron(III) nitrate nonahydrate (Fe(NO3)3.9H2O) 723.8 mg/L; zinc sulfate heptahydrate (ZnSO4.7H2O) 439.8 mg/L; manganese(II) sulfate tetrahydrate (MnSO4.4H2O) 203.0 mg/L (Christen and Raimbault, 1991).

Table 3.1 Basal growth medium for *C. fimbriata*

Items	**g/L**
Glucose	20.0
Urea	0.75
Ammonium sulfate ($(NH_4)_2SO_4$)	2.25
Monopotassium phosphate (KH_2PO_4)	1.0
Calcium nitrate tetrahydrate ($Ca(NO_3)_2.4H_2O$)	0.5
Magnesium sulfate heptahydrate ($MgSO_4.7H_2O$)	0.5
Chloramphenicol	0.5
Trace elements	2 mL

Source: Christen and Raimbault (1991)

3.6 Fermentation procedure

The experiments were done by using OPF juice containing 30 g/L of glucose using 250 mL Erlenmeyer flasks and were mixed with a mineral salt medium (MSM). OPF juice with initial concentration of glucose at ± 43.96 g/L was diluted using distilled water to a final concentration of 30 g/L. The dilution factor was calculated based on the formula shown in Equation 3.1. The MSM was prepared based on Christen and Raimbault (1991) and the MSM compositions are shown in Table 3.2. All compositions were mixed together, and the initial pH of medium was 4.6 then adjusted to 6.0 with NaOH 0.5 M before autoclaved. The trace element solution comprised of: Iron(III) nitrate nonahydrate (Fe(NO3)3.9H2O) 723.8 mg/L; zinc sulfate heptahydrate (ZnSO4.7H2O) 439.8 mg/L; manganese(II) sulfate tetrahydrate (MnSO4.4H2O) 203 mg/L. The flasks were covered with cotton and sterilized at the temperature of 121 °C for 20 minutes. One mycelium cell from seed cultures was taken aseptically and introduced into a 250 mL Erlenmeyer flask containing 100 mL of the OPF juice supplemented with MSM. The fermentation was carried out at the temperature of 27 °C on a rotary shaker at 150 rpm for 8 days and all experiments were conducted in triplicates.

$$M_1V_1 = M_2V_2 \qquad 3.1$$

Table 3.2 MSM for *C. fimbriata*

Items	**g/L**
Glucose in OPF juice	30.0
Urea	0.75
Ammonium sulfate ($(NH_4)_2SO_4$)	2.25
Monopotassium phosphate (KH_2PO_4)	1.0
Calcium nitrate tetrahydrate ($Ca(NO_3)_2.4H_2O$)	0.5
Magnesium sulfate heptahydrate ($MgSO_4.7H_2O$)	0.5
Chloramphenicol	0.5
Trace elements	2 mL

Source: Christen and Raimbault (1991)

3.7 Fermentation procedure for screening and optimization

For screening and optimization process, the experiments were done by using OPF juice containing 20 g/L and 30 g/L of glucose using 250 mL flasks and mixed with a MSM. To prepare 20 g/L and 30 g/L of glucose concentration in fermentation broth, a similar method was applied as explained in Section 3.6. Both medium had the following composition: urea, 0.75 g/L; (NH4)2SO4, 2.25 g/L; KH2PO4, 1 g/L; Ca(NO3)2.4H2O, 0.5 g/L; MgSO4.7H2O, 0.5 g/L; trace element solution, 2 ml/L; chloramphenicol 0.5 g/L and the pH was adjusted to pH 4 and pH 8 with NaOH 0.5 M and hydrochloric acid (HCL) 1.0 M before autoclaved. The flasks were covered with cotton and sterilized at the temperature of 121 °C for 20 minutes (Christen et al., 1997). One mycelium cell from seed cultures was taken aseptically and introduced into a 250 mL Erlenmeyer flask containing 100 mL of the MSM. The fermentation was carried out at the incubation temperature of 25 °C and 35 °C on a rotary shaker for 100 rpm and 150 rpm for 4 days all experiments were conducted in triplicates.

3.8 Cell dry weight (CDW) measurement

The mycelial were taken at every 24 hours to measure the total dry weight. The mycelial were centrifuged at 10,000 rpm for 15 minutes at the temperature of 4°C (Eppendorf, Germany) and the supernatant of sample were proceeded for analytical procedure. Meanwhile the solids were washed with distilled water and centrifuged for two consecutive times. In order to determine the cell dry weight (CDW) of fermentation sample, 50 mL of centrifuge tube were dried at the temperature of 100 °C overnight and kept in desiccators at room temperature prior weighing. The weight of the centrifuge tube was measured by electronic precision balance model GR-200 (AND, JAPAN) until consistent reading was obtained and recorded (W1). CDW was measured by centrifuged the mycelial and the supernatant was decanted, and the mycelial residue was dried at the temperature of 100 °C for 24 hours in the oven (Memmert, Germany). The dried mycelial were kept in the desiccators and weigh until consistent reading was recorded (W2) (Christen and Raimbault, 1991). The CDW can be calculated from the Equation 3.2

$$\text{CDW}\left(\frac{\text{g}}{\text{L}}\right) = (\text{W2} - \text{W1})\ \text{g} \div 100\ \text{mL} \qquad 3.2$$

3.9 Determination of residual sugar

Samples withdrawn from 250 mL Erlenmeyer flask at certain operating hour were subjected to residual sugars analysis. Samples were dispensed into 1.5 mL eppendorf tubes and were spun down for 10 minutes at 10,000 rpm. The supernatant was withdrawn using 3 mL syringe and passed through a nylon membrane filter 0.20 µm (Milipore, USA). The filtrate was analyzed by using HPLC. The sugars content in the OPF juice was determined by HPLC (Agilent Series 1200, Germany) by following the method explained in Section 3.3 (Zahari et al., 2012).

3.10 Analytical procedure

The volatile compounds were detected by gas chromatography-mass spectroscopy solid phase micro extraction (GC-MS SPME). In this study, fiber DVB-CAR-PDM (50/30 µm), which have been frequently employed for the extraction of the volatile fraction from natural products, was tested to analyse the present of volatile compounds. The coating was 1 cm long for the fiber. Before GC–MS analysis, the fiber was conditioned in the injector of the GC system, according to the instructions provided by the manufacturer. 1 mL amount of sample was placed in a 4 mL flat-bottom headspace vial sealed with screw cap with PTFE/silicone septum (Agilent). The sample was heated for 45 minutes on a hot plate at the temperature of 60 °C. The SPME device was then inserted into the sealed vial by manually penetrating the septum and the fiber was exposed to the headspace for 45 minutes during the extraction time. After sampling, the SPME fiber was immediately inserted into the GC injector and thermally desorbed. A desorption time of 1 minute at the temperature of 230 °C was used in the splitless mode. Before sampling, the fiber was reconditioned for 5 minutes in the GC injector port at the temperature of 230 °C (Pellati et al., 2013).

3.11 Screening process using full factorial analysis

For this research, factorial design for experimental data was chosen because the design allows determination of factors with the highest impact on the process. Full factorial design of 2k runs, where K is the number of variables, was selected for the screening design. The full factorial screening design involved runs at every possible combination at the defined high and low limit for each variable, refer Table 3.3. In total, sixteen (16) experiments were done based on a 24 full factorial design. This design was

chosen in this study to reduce the number of experiments without losing a lot of information on the possible influences that affect the factors of VOCs production. Methyl 3-(3,5-di-tert-butyl-4-hydroxyphenyl) propionate was one of compounds used in the VOCs production. In this research, this compound was chosen for screening and optimization process. Initially the glucose contains in OPF juice was ± 43.96 g/L and for screening according to parameters such as 20 g/L and 30 g/L of glucose, can be calculated from the Equation 3.1. The initial pH medium was 4.6, and it was adjusted according to parameters such as pH 4 and pH 8, by using NaOH 0.5 M and hydrochloric acid (HCL) 1.0 M. The methyl 3-(3,5-di-tert-butyl-4-hydroxyphenyl) propionate factors studied were pH of initial medium, temperature, agitation speed and glucose concentration in OPF juice. Table 3.3 presents the variable factors with the coded and actual values for each set of parameters for the experiment. The experimental design and analysis of data were done using Design Expert version 7.1 (State-Ease, Inc., Minneapolis, MN). With the help of Design Expert software, the experimental design for this study was established as shown in Table 3.4. All experiments were done in triplicates and the results were recorded as mean values of methyl 3-(3,5-di-tert-butyl-4-hydroxyphenyl) propionate production.

Table 3.3 Factors and levels used in the 2^4 factorial design study

Parameter name	**Code**	**Units**	**Low**	**High**
Initial pH medium	X_1	-	4	8
Incubation temperature	X_2	°C	25	35
Agitation speed	X_3	rpm	100	150
Glucose concentration	X_4	g/L	20	30

Table 3.4 Experimental design matrix for screening

Std	Factor 1 Initial pH medium	Factor 2 Incubation temperature	Factor 3 Agitation speed	Factor 4 Glucose concentration
		°C	rpm	g/L
1	4	25	100	20
2	8	25	100	20
3	4	35	100	20
4	8	35	100	20
5	4	25	150	20
6	8	25	150	20
7	4	35	150	20
8	8	35	150	20
9	4	25	100	30
10	8	25	100	30
11	4	35	100	30
12	8	35	100	30
13	4	25	150	30
14	8	25	150	30
15	4	35	150	30
16	8	35	150	30

3.12 Optimization process using central composite design

The RSM applied in the study is the CCD along with three different factors. The factors were pH, temperature and agitation speed. The experimental design used a three variable (A, B, C) with five level (-α, -1, 0, +1, +α) CCD. Having four factors in section 3.10, three out of the four factors were selected for optimization. These three factors namely, temperature, pH and agitation speed had the highest positive effect on methyl 3-(3,5-di-tert-butyl-4-hydroxyphenyl) propionate production. The three selected factors for optimization using RSM and CCD with the levels and the center point of the design was shown in Table 3.5.

Table 3.5 Experimental range and levels of the factors

Factor	**-α**	**-1**	**0**	**+1**	**+α**
A: Initial pH medium	4	6	8	10	12
B: Incubation Temperature	15 °C	20 °C	25 °C	30 °C	35 °C
C: Agitation speed	40 rpm	70 rpm	100 rpm	130 rpm	160 rpm

A total of twenty (20) experiments were performed according to the range and levels of the factors for methyl 3-(3,5-di-tert-butyl-4-hydroxyphenyl) propionate production as stated in Table 3.5. Meanwhile, in Table 3.6 showed the experimental design were established by using Design Expert version 7.1 (State-Ease, Inc., Minneapolis, MN).

Table 3.6 Experimental design matrix using RSM with CCD and response for methyl 3-(3,5-di-tert-butyl-4-hydroxyphenyl) propionate production

Std	**Agitation speed**	**Initial pH medium**	**Incubation temperature**
	rpm		**°C**
1	70	6	20
2	130	6	20
3	70	10	20
4	130	10	20
5	70	6	30
6	130	6	30
7	70	10	30
8	130	10	30
9	40	8	25
10	160	8	25
11	100	4	25
12	100	12	25
13	100	8	15
14	100	8	35
15	100	8	25

Table 3.6 Experimental design matrix using RSM with CCD and response for methyl 3-(3,5-di-tert-butyl-4-hydroxyphenyl) propionate production (continue)

Std	Agitation speed	Initial pH medium	Incubation temperature
	rpm		°C
16	100	8	25
17	100	8	25
18	100	8	25
19	100	8	25
20	100	8	25

3.13 Validation experiments

Following the design and analysis of the both screening and optimization experiment, the best and optimized condition proposed was by the using of fitted model to predict the highest possible methyl 3-(3,5-di-tert-butyl-4-hydroxyphenyl) propionate production factor that can be achieved within the range of factor studied. Experiments were conducted according to the suggested experimental conditions and results of the experiments were compared with the suggested results to verify the significance of the factorial model. An error below 10 % was desired between the predicted and experimental methyl 3-(3,5-di-tert-butyl-4-hydroxyphenyl) propionate production factor, calculated using the Equation 3.3.

$$Error(\%) = \frac{\left| \text{predicted value - experimental value} \right|}{\text{experimental value}} \times 100\% \quad 3.3$$

CHAPTER 4

RESULTS AND DISCUSSION

4.1 Preliminary study

For the preliminary study, the ability of *C. fimbriata* to produce VOCs by using OPF juice as a sole carbon source in the 250 mL Erlenmeyer flasks was discussed. The experiments were conducted by using OPF juice containing 30 g/L of glucose and complemented with mineral salt medium (MSM). The fermentation process was carried out at the temperature of 27 °C on a rotary shaker at 150 rpm for 8 days (Christen and Raimbault, 1991).

Generally, the whole branch of OPF consisted of petiole and leaflets. However, in this study, the OPF juice was extracted only from the petiole part, since the leaflet should remain in the plantation for soil conservation, erosion control and nutrient recycling. The OPF juice was extracted using a conventional sugarcane pressing machine without any pre-treatment such as acid, alkali or enzyme. By using sugarcane pressing machine, 44.29 g/L of glucose was obtained from a bunch of fresh OPF. Table 4.1 showed the sugar concentration by using HPLC by following the method explained in Section 3.3 (Zahari et al., 2012). The total sugars concentration was ±53.82 g/L. Glucose was found to be dominant sugar in OPF juice (±43.96 g/L) followed by sucrose (±9.86 g/L).

The finding was almost similar with finding of past studies by (Zahari et al., 2012), which pointed out that glucose was the major sugar component in OPF juice followed by sucrose. This might be due to the different locations of the OPF collection, environment of the OPF plantations and fertilizers used at the plantations. The OPF in this experiment was obtained from the oil palm plantation in Gambang, Pahang, meanwhile Zahari et al. (2012) obtained the OPF from a plantation in Serdang, Selangor.

The presence of sugar in OPF was expected due to the process of photosynthesis. Photosynthesis was a process by plants that converts carbon dioxide and water into glucose by using energy from the sunlight. Oil palm trees had a high leaf area index, which means that aside from providing shade to wildlife like cobras, a significant amount of photosynthesis occurred.

Based on the sugars content that showed in Table 4.1, it was suggested that OPF juice had a potential to be used as fermentation substrate to produce VOCs especially for methyl 3-(3,5-di-tert-butyl-4-hydroxyphenyl) propionate. A good fermentation substrate also should create minimal problems in other aspects of the production process particularly aeration and agitation, extraction, purification and waste treatment. Based on the previous research (Zahari et al., 2012), characterization study on the OPF and OPF juice, it showed that most of the characteristics had been met and OPF juice could be considered as a potentially good fermentation substrate for the production of VOCs and also methyl 3-(3,5-di-tert-butyl-4-hydroxyphenyl) propionate.

Table 4.1 Amount of sugar contain in OPF juice by using HPLC

OPF juice	Glucose g/L	Sucrose g/L	Total sugar g/L
Sample 1	44.27	10.29	54.56
Sample 2	43.92	9.48	53.4
Sample 3	43.68	9.82	53.5
Average	43.96	9.86	53.82

4.1.1 Cell biomass and growth profile of *C. fimbriata*

Figure 4.1 (a) showed the *C. fimbriata* after 7 days on PDA plate at incubation temperature of 30 °C. *C. fimbriata* on PDA hyaline at first with a fluffy appearance. Becoming brown, grey or olive-green after 5-7 days, odor sweet, often with banana scent and found similar results to those obtained by (Johnson, 2005). In Figure 4.1 (b) *C. fimbriata* under microscope had cylindrical endoconidia, unicellular, hyaline to light brown, smooth, mainly cylindrical with flattened end, straight and borne in chains of variable length by Engelbrecht et al. (2005) also showed similar results. Meanwhile in Figure 4.1 (c), it had wide-mouth endoconidiophore with emerging doliiform endoconidium. The finding was consistent with findings of past studies by Johnson

(2005) which borne near the phase of perithecia. Doliiform endoconidium produced from wide mouthed phialides, often remained in chains.

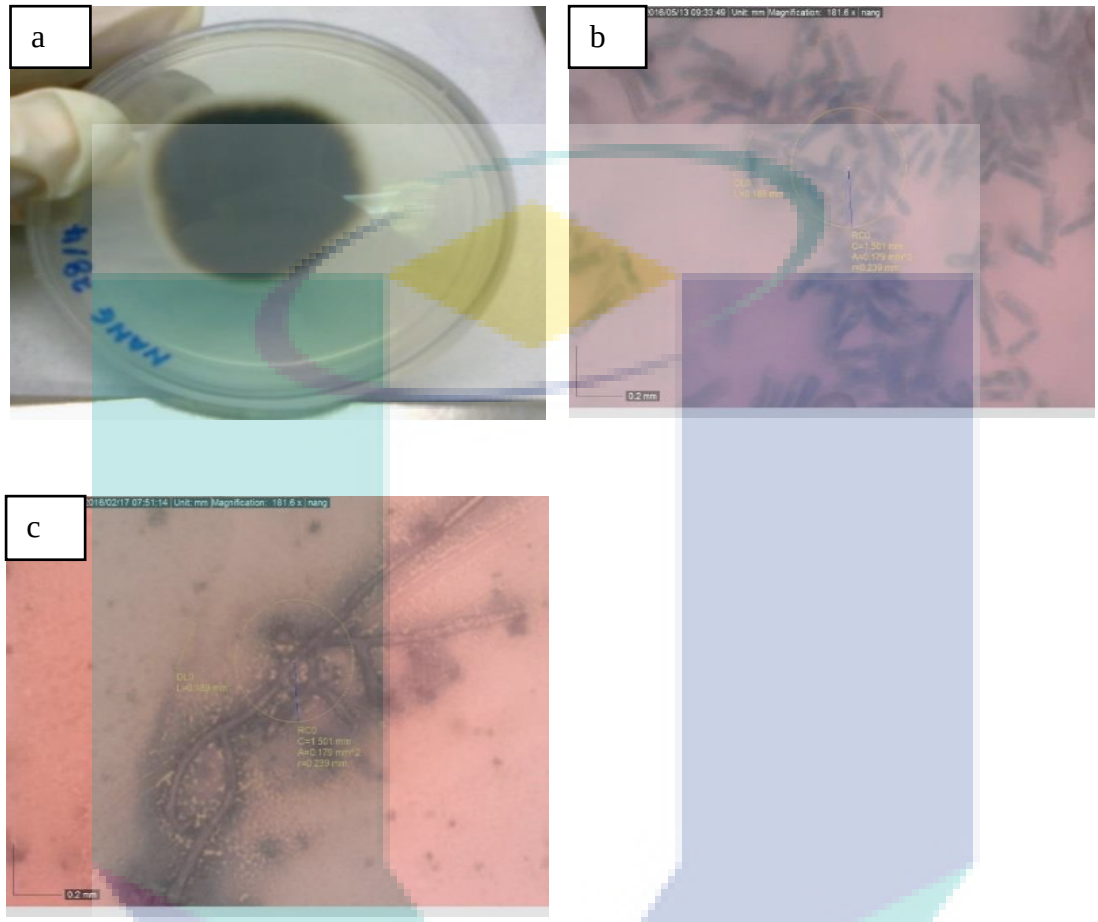


Figure 4.1 (a) *C. fimbriata* after 7 days on PDA plate at incubation temperature of 30 °C, (b) *C. fimbriata* under microscope had cylindrical endoconidia (c) *C. fimbriata* had wide-mouth endoconidiophore with emerging doliiform endoconidium

Figure 4.2 (a), *C. fimbriata* showed the seed culture in basal growth medium for the nine days on a rotary shaker at (150 rpm) in an aerobic condition and was used as the inoculum in the fermentation of OPF juice. The inoculum was then transferred to the fermentation medium which consisted of 30 g/L glucose in OPF juice supplemented with an MSM. This was consistent with the finding from Johnson, (2005) which showed that the fluffy appearance for the culture. Figure 4.2 (b), (c) (d) and (e), revealed the mycelium germinated with increasing period of fermentation from second day until the fourth day, consistently. Figure (b), showed that the flask had one cell of mycelia after the first day of fermentation. With reference to figure (c), after two days of the fermentation period, the fungus germinated to two big mycelium followed by the third day of fermentation, it had seven mycelium cells. On the fourth day until the end of the fermentation, the fungus

germinated and had grown into many myceliums of cells. The morphology of this fungus in supplemented OPF juice also found similar results to those obtained by Johnson, (2015) which had the fluffy appearance.

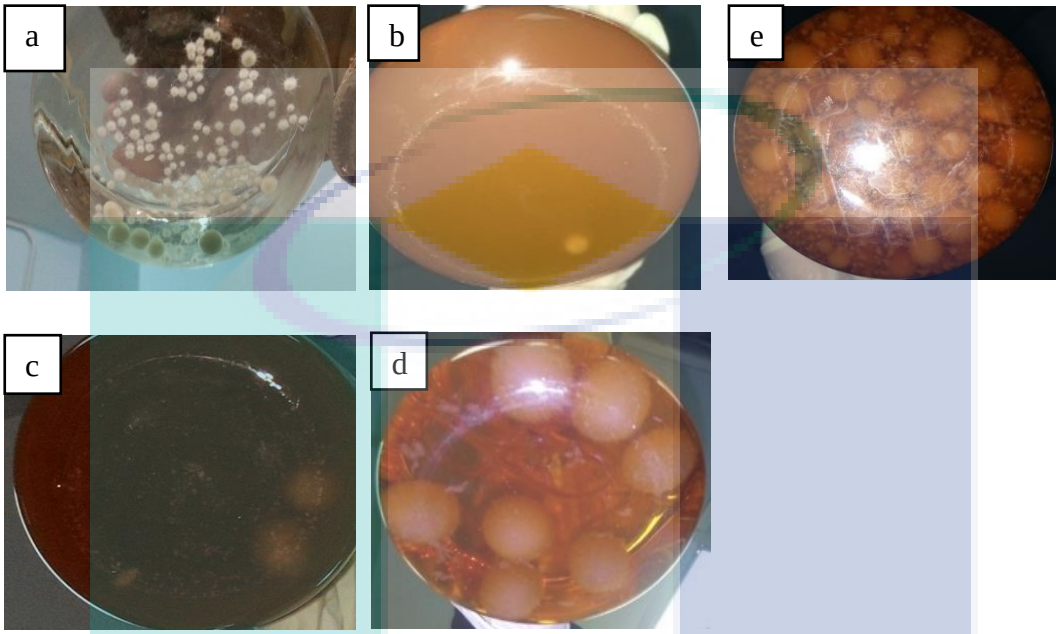


Figure 4.2 (a) Seed culture of *C. fimbriata* in basal growth medium for the nine days on a rotary shaker at (150 rpm) in an aerobic condition, (b) The mycellium cell on the first day of fermentation, (c) The second day of fermentation, (d) third day of fermentation, (e) on the fourth day of fermentation

The results of the biomass produced by *C. fimbriata* in MSM supplemented with OPF juice is shown in Figure 4.3. The biomass production was calculated from the first day until the eighth day of fermentation. As shown in Figure 4.3, it was apparent that the *C. fimbriata* biomass had increased sharply from the second day to the fourth day and increased slowly thereafter. Based on the growth profile, the lag phase for *C. fimbriata* began from the first day until the second day. Then, followed by the exponential phase where the productions of biomass increased significantly up to the fourth day.

After that, the stationary phase started to occur slowly from the fourth day until the end of the fermentation period. On the other hand, the results of the glucose consumption of *C. fimbriata* in MSM supplemented with OPF juice was shown in Figure 4.4. The glucose consumption was analysed from the first day of fermentation until the eighth day. In Figure 4.4, the glucose consumption of *C. fimbriata* decreased sharply

from second day to the fourth day of fermentation period and decreased slowly thereafter as it was consumed by the fungus.

With reference to Figure 4.3 and Figure 4.4, it was shown that the biomass production profile was consistent with the glucose consumption profile by *C. fimbriata*. Based on both figures, glucose was fully utilized by *C.fimbriata* from the first to the fourth day. After fourth day, fructose concentration was increased due to the sucrose converted to glucose and fructose. This might be due to the presence of invertase enzyme (Hernández-Carbajal et al., 2013). The invertase enzyme, hydrolyze the residual sucrose from the OPF juice to glucose and fructose during the fermentation. After the fourth day, the fructose was increased, and the sucrose was decreased.

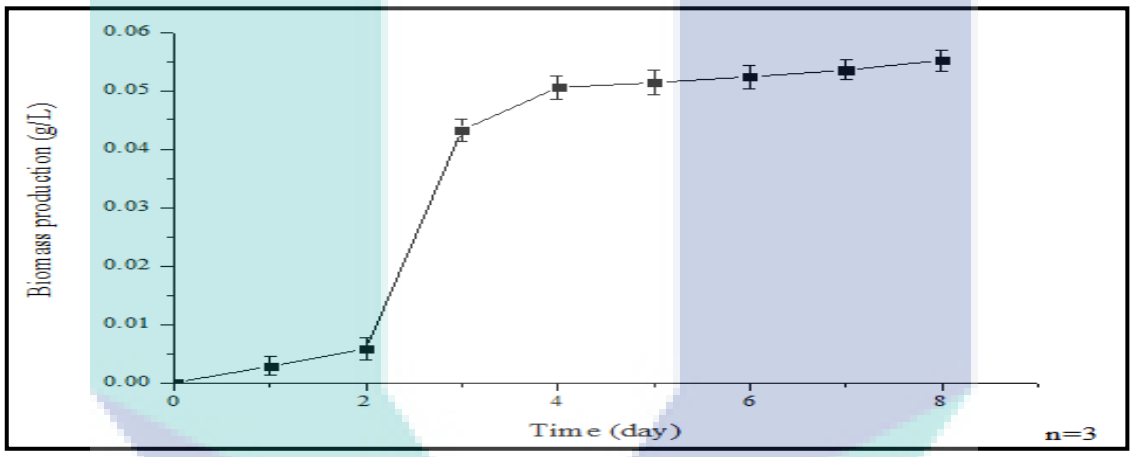


Figure 4.3 Biomass production of *C. fimbriata* in OPF juices supplemented with MSM as a sole substrate for 8 days of fermentation

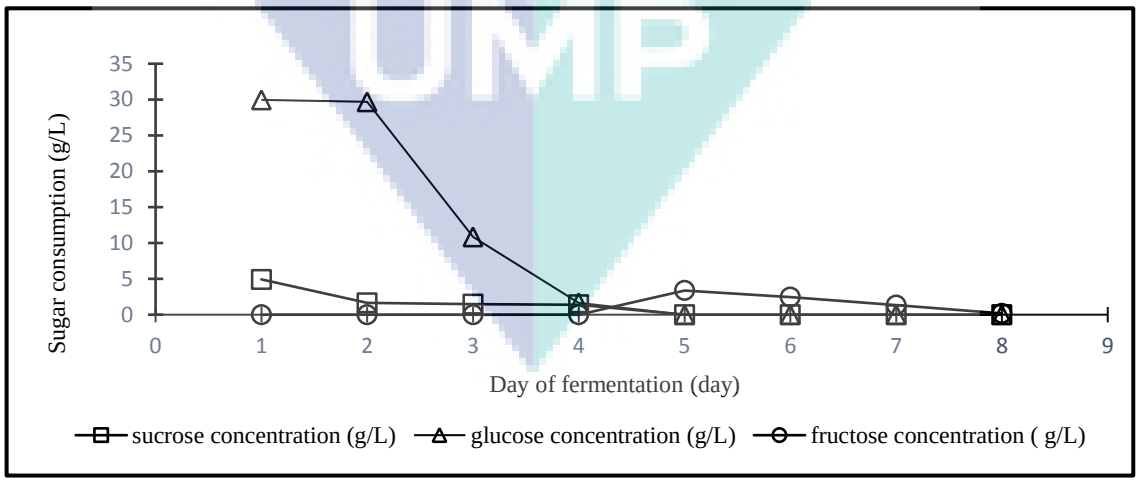


Figure 4.4 Glucose consumption profile for eight day of fermentation period by *C. fimbriata* in OPF juice supplemented with MSM.

4.1.2 VOCs production

From the fermentation of *C. fimbriata* using OPF juice (as the sole carbon source), it was found that at least 45 peaks of compounds were detected by GC-MS SPME. The compounds for eight days of fermentation was shown in Table 4.2 meanwhile in Table 4.3 was summarized from Table 4.2. The volatile compounds identified from fermentation of *C. fimbriata* were mostly alcohol, phenol, ester, aldehyde as the major compounds. While other compounds such as alkane, alkene, ketone, amide, fatty acid and others were also produced during the fermentation. Based on the GC-MS SPME analysis, eleven peaks were classified as alcohols from the second day until the eighth day. The compounds include 3,4-dihydroxybenzyl alcohol, tris (trimethylsilyl); 1-octen-3-ol; 2-ethyl-1-hexanol; phenylethyl alcohol and (Z)- 3-hexen-1-ol. As commonly known, alcohols did not play a predominant role in flavors but were known to contribute to the overall flavors quality and were precursors of fruit-like flavoring esters (Christen et al., 1997). In microorganisms, all alcohol, except ethanol was formed by the reduction of α-keto acids which were derived from amino acids metabolism (Welsh et al., 1989). Phenolic compounds were also detected consisting of 2-methoxy-4-vinylphenol; p-tert-butyl- phenol and 2,4-bis(1,1-dimethylethyl)- phenol on the first day until the eighth day of fermentation.

Seven peaks were recorded as ester between the first day until the sixth day of fermentation. The esters comprised of methyl 3-(3,5-di-tert-butyl-4-hydroxyphenyl) propionate; (dimethylsilylene) bismethanol diacetate and oxalic acid, isobutyl nonyl ester. From the data in Figure 4.5, it was apparent that on the fourth day of the fermentation period, there were two ester groups were present, which were methyl 3-(3,5-di-tert-butyl-4-hydroxyphenyl) propionate and (dimethylsilylene) bismethanol diacetate. Methyl 3-(3,5-di-tert-butyl-4-hydroxyphenyl) propionate was an ester group and it present during the fermentation period from the first day until the fifth day. Among the fifth day of fermentation, methyl 3-(3,5-di-tert-butyl-4-hydroxyphenyl) propionate at the fourth day was recorded as the highest production at the retention time 32.858 minutes and the relative peak area was 0.21 % of chromatogram area. The ester group plays a pivotal role in enhancing the different fruit fragrances such as methyl-butyrate (apple fragrance), methyl butanoate (pineapple), ethyl butyrate (orange), ethyl butanoate (pineapple), pentyl-butyrate (pear) and pentyl-butanoate (apricot) (Zawirska et al, 2009).

Four aldehydes were detected from the six peaks of compounds during the fermentation period except on the third day and the eighth day of fermentation. These aldehydes were methyl, 2-(4-ethoxyphenyl)-2- propanal; 2- benzothiazolecarbox aldehyde; 3,4-dimethyl-benzaldehyde; 3,5-dimethyl-benzaldehyde and 2,6-dimethylbenzaldehyde. Three alkanes were recorded including dimethyl (isopropyl)silyloxycyclohexane on the third day; 2,6,10-trimethyl- dodecane on the seventh day and 1,3,5-trioxane on the eighth day.

Forty-five peaks were recorded during the fermentation which corresponded to 11 alcohols, 10 phenols, 7 esters, 6 aldehydes, 3 alkanes, 2 ketones, 2 alkenes, 1 amide, 1 fatty acid and 2 others. During the fermentation process, the volatile compounds were produced from the first day until the eighth day of the fermentation period. However, more volatile compounds were produced after the fourth day of fermentation period compared to the other days. With reference to the past study which had used coffee husk as a substrate, the major compound produced by *C. fimbriata* were ethyl acetate, ethanol and acetaldehyde (Medeiros et al., 2006). Other compounds, including ethylpropionate, propylacetate, ethyl isobutyrate, butyl acetate were also produced during fermentation (Medeiros et al., 2006). Another researcher found that nine volatile compounds were detected when citric pulp was used as the substrate in fermentation. These compounds were acetaldehyde, ethanol, ethyl acetate, propyl acetate, ethyl isobutyrate, 2-hexanone, 2-hexanol, isoamyl acetate and one unidentified compound (Rossi et al., 2009).

From this study, methyl 3-(3,5-di-tert-butyl-4-hydroxyphenyl) propionate was chosen for next objective such as screening and optimization. This study indicated that the methyl 3-(3,5-di-tert-butyl-4-hydroxyphenyl) propionate was present consistently from first day until fifth day. The relative peak area of this compound from first day was 0.10 %, for the second day was 0.15 %, at third day was 0.16 %, at the fourth day was 0.21 % and the fifth day was 0.18%. The production was increased slowly from first day to fourth day and decreased at the fifth day. Besides that, this compound also was consistent with the glucose consumption profile by *C. fimbriata* that depicted in Figure 4.4. Based on the period of fermentation, there were other compounds that showed higher percentage area of compound compared with methyl 3-(3,5-di-tert-butyl-4-hydroxyphenyl) propionate such as 2,4-bis(1,1-dimethylethyl)- phenol, (dimethylsilylene) bismethanol diacetate, nonanoic acid, 3,4-dimethyl- benzaldehyde,

phenylethyl alcohol, 2-ethyl-1-hexanol, 1-Octen-3-ol, dimethyl (isopropyl) silyloxycyclohexane and 2-Methoxy-4-vinylphenol. Although the other compounds showed higher percentage area of compound, but they were not being considered as chosen compounds because they were also present in blank medium. Besides that, they were not consistently present from the first day until the fifth day of period of fermentation. From the first day until the fifth day period of fermentation, the glucose was fully consumed by the fungus, and afterward the fungus consumed another non-dominant carbon source such as fructose. This study had found that generally, methyl 3-(3,5-di-tert-butyl-4-hydroxyphenyl) propionate was compatible with glucose consumption and the compound presence during the period of fermentation.

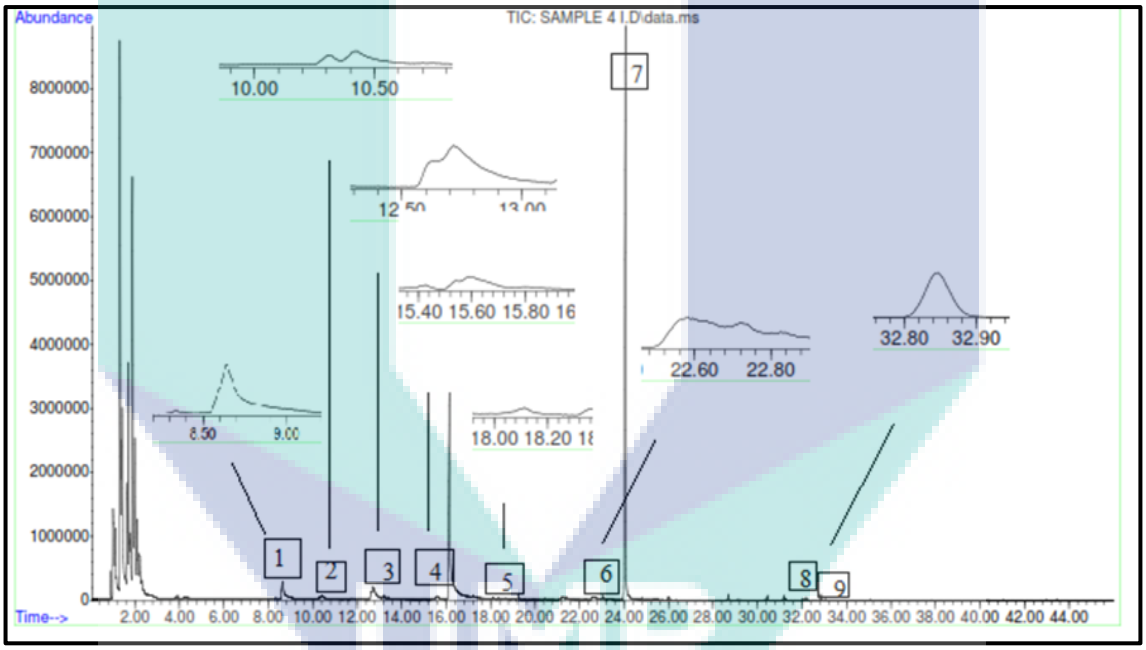


Figure 4.5 (GC-MS) with (SPME) analysis produced 9 peaks on the fourth day of fermentation. Peak (1) retention time at 8.661, peak (2) retention time at 10.421, peak (3) retention time at 12.732 were the alcohol group; peak (6) retention time at 22.643 and peak (9) retention time at 32.858 were the ester group; peak (4) retention time at 15.604 was the aldehyde group; peak (5) retention time at 18.107 was fatty acid; peak (7) retention time at 24.044 was the phenol group; and peak (8) retention time at 32.174 was the ketone group

Table 4.2 Volatile organic compounds produced by *C. fimbriata* using OPF juice as the sole carbon source

Day of fermentation	R_{time}	Area (%)	Volatile organic compounds
1	18.589	0.84	2-Methoxy-4-vinylphenol
	21.225	0.60	2-(4-ethoxyphenyl)-2-methyl-propanal
	24.033	0.31	2,4-bis(1,1-dimethylethyl)- phenol
	32.832	0.10	Methyl 3-(3,5-di-tert-butyl-4-hydroxyphenyl) propionate
2	15.582	0.58	3,4-dihydroxybenzyl alcohol, tris (trimethylsilyl)
	18.369	0.69	P-tert-butyl- phenol
	21.231	1.36	2-benzothiazolecarboxaldehyde
	23.621	14.69	2,4-bis(1,1-dimethylethyl)- phenol
	32.826	0.15	Methyl 3-(3,5-di-tert-butyl-4-hydroxyphenyl) propionate
3	8.688	2.47	1-Octen-3-ol
	12.774	1.49	Dimethyl(isopropyl)silyloxycyclohexane
	24.033	19.42	2,4-bis(1,1-dimethylethyl)- phenol
	31.184	0.09	2-(1-phenylethylidene)- hydrazinecarbothioamide
	32.826	0.16	Methyl 3-(3,5-di-tert-butyl-4-hydroxyphenyl) propionate
4	8.661	2.40	1-Octen-3-ol
	10.421	0.95	2-ethyl-1-hexanol
	12.732	2.50	Phenylethyl Alcohol
	15.604	0.47	3,4-dimethyl- benzaldehyde
	18.107	0.67	Nonanoic acid
	22.643	0.71	(Dimethylsilylene) bismethanol diacetate
	24.044	27.09	2,4-bis(1,1-dimethylethyl)- phenol
	32.174	0.08	7,9-di-tert-butyl-1-oxaspiro [4,5] deca-6,9-diene-2,8-dione
	32.858	0.21	Methyl 3-(3,5-di-tert-butyl-4-hydroxyphenyl) propionate

Table 4.2 Volatile organic compounds produced by *C. fimbriata* using OPF juice as the sole carbon source (continue)

Day of fermentation	R_{time}	Area (%)	Volatile organic compounds
5	4.339	2.67	(Z)- 3-hexen-1-ol
	12.694	10.98	Phenylethyl Alcohol
	15.588	0.88	3,4-dimethyl- benzaldehyde
	21.241	0.70	Ethyltriethoxysilane
	24.022	29.01	2,4-bis(1,1-dimethylethyl)- phenol
	32.832	0.18	Methyl 3-(3,5-di-tert-butyl-4-hydroxyphenyl) propionate
6	8.682	2.06	1-Octen-3-ol
	12.694	8.45	Phenylethyl Alcohol
	15.593	0.78	3,5-dimethyl- benzaldehyde
	19.909	0.13	Oxalic acid, isobutyl nonyl ester
	24.038	35.54	2,4-bis(1,1-dimethylethyl)- phenol
	32.158	0.15	7,9-Di-tert-butyl-1-oxaspiro (4,5) deca-6,9-diene-2,8-dione
7	8.693	2.43	1-Octen-3-ol
	11.737	1.56	cis-Linalool Oxide
	14.272	1.11	1,4-dimethoxy- benzene
	16.139	18.21	1,3,5-Trioxane
	19.968	0.36	1,2,4-Trimethoxybenzene
	24.033	37.05	2,4-bis(1,1-dimethylethyl)- phenol
8	12.716	6.74	Phenylethyl Alcohol
	15.593	0.80	2,6-Dimethylbenzaldehyde
	19.899	0.18	2,6,10-trimethyl- dodecane
	24.039	42.11	2,4-bis(1,1-dimethylethyl)- phenol

Table 4.3 Volatile organic compounds produced by *C. fimbriata* using OPF juice as the sole carbon source for 8 days of fermentation (summarized from Table 4.2)

Chemical groups	**Alcohol**	**Ester**	**Aldehyde**	**Phenol**	**Alkane**	**Alkene**	**Keto ne**	**Other**
1st day		1	1	2				
2nd day	1	1	1	2				
3rd day	1	1		1	1			1
4th day	3	2	1	1			1	1
5th day	2	1	1	1				1
6th day	2	1	1	1			1	
7th day	1			1	1	2		1
8th day	1		1	1	1			
Total no. of compound	11	7	6	10	3	2	2	4

4.1.3 Summary for preliminary study

Based on the results and discussion, it could be concluded that volatile organic compounds could be produced from the utilization of OPF juice as the carbon source by *C. fimbriata*. With the reference to the GC-MS SPME analysis, the highest number of VOCs (3 alcohols, 1 aldehyde, 1 fatty acid, 1 phenol, 1 ketone and 2 ester) were produced by *C. fimbriata* on the fourth day of the fermentation period. Methyl 3-(3,5-di-tert-butyl-4-hydroxyphenyl) propionate was chosen for future analysis because its production was consistent with glucose consumption and was present consistently from the first day until the fifth day of the fermentation period. The other compounds were disregarded because they present in blank medium. For future objective, further investigation on the parameters affecting methyl 3-(3,5-di-tert-butyl-4-hydroxyphenyl) propionate from OPF juice by *C. fimbriata* were to be looked upon to improve the yield.

4.2 Factorial analysis for production of methyl 3-(3,5-di-tert-butyl-4-hydroxyphenyl) propionate

2^4 full factorial design with total of 16 experiments were performed. The full factorial experimental design and the resulted response was shown in Table 4.4. Response

was analyzed by examining fitting a model, interpreting the model graphically, finding the best point, and validating the model. The aim of this objective was to investigate the effect of incubation temperature (°C) from 25 °C to 35 °C, initial pH medium from pH 4 to pH 8, agitation speed (rpm) from 100 rpm to 150 rpm and concentration of glucose (g/L) in the OPF juice from 20 g/L to 30 g/L on the production of methyl 3-(3,5-di-tert-butyl-4-hydroxyphenyl) propionate from OPF juice.

Table 4.4 Experimental result for production of methyl 3-(3,5-di-tert-butyl-4-hydroxyphenyl) propionate

Run	Factor 1: initial pH medium	Factor 2: Incubation temperature °C	Factor 3: Agitation speed (rpm)	Factor 4: glucose concentration (g/L)	Methyl 3-(3,5-di-tert-butyl-4-hydroxyphenyl) propionate production (% of area)
1	4	25	100	20	0.19
2	8	25	100	20	0.21
3	4	35	100	20	0.07
4	8	35	100	20	0.14
5	4	25	150	20	0.05
6	8	25	150	20	0.07
7	4	35	150	20	0.03
8	8	35	150	20	0.04
9	4	25	100	30	0.11
10	8	25	100	30	0.24
11	4	35	100	30	0.15
12	8	35	100	30	0.19
13	4	25	150	30	0.10
14	8	25	150	30	0.13
15	4	35	150	30	0.08
16	8	35	150	30	0.09

To study the variables that defined the experimental process, full factorial (2^4) experimentations were carried out, in two levels, which was at high level and low level. The experimental effects were designed in accordance to Table 3.3 in Chapter 3 which showed the values of the factors selected in this study. This factorial design had resulted in sixteen tests with all possible combination of X1, X2, X3 and X4. The production of methyl 3-(3,5-di-tert-butyl-4-hydroxyphenyl) propionate (Y) was measured for each of these tests as shown in Table 4.4. The production of methyl 3-(3,5-di-tert-butyl-4-hydroxyphenyl) propionate by *C. fimbriata* from OPF juice were done according to the parameters (factors) as shown in Table 4.4.

With reference to Table 4.4, 16 fermentation runs, were carried out with different levels of initial pH medium, incubation temperature, agitation speed and total glucose in OPF juice. As observed, run 10 and 2 showed the highest methyl 3-(3,5-di-tert-butyl-4-hydroxyphenyl) propionate production with the value of 0.24% of chromatogram area and 0.21% of chromatogram area respectively. Methyl 3-(3,5-di-tert-butyl-4-hydroxyphenyl) propionate production at 0.24% of chromatogram area was done at a condition where the initial pH medium value was 8, incubation temperature at 25 °C, total glucose in OPF juice was 30 g/L and the agitation speed of 100 rpm.

Meanwhile for methyl 3-(3,5-di-tert-butyl-4-hydroxyphenyl) propionate production at 0.21% of chromatogram area, it was done at the condition where the initial pH medium value was 8, incubation temperature at 25 °C, total glucose in OPF juice was 20 g/L and agitation speed of 100 rpm. The lowest methyl 3-(3,5-di-tert-butyl-4-hydroxyphenyl) propionate was at run 7 where it comprised of higher agitation speed of 150 rpm, higher incubation temperature at 35 °C, and the acidity of the initial pH medium was at pH 4. Thus, it could be said that *C. fimbriata* could not grow and tolerate at higher agitation speed, higher incubation temperature and high acidic of medium.

4.2.1 Model fitting

The analysis of experimental data by a complete 16 full factorial design was systematically conducted using Design Expert version 7.1. (State-Ease, Inc., Minneapolis, MN). The percent contribution came from adding up the total sum of squares and then taking each term's sum of squares and dividing by the total to get a percentage (Anderson et al., 2009). Meanwhile, percent contribution was calculated and

tabulated in Table 4.5. The interaction terms for the model were chosen based on percent contribution, however main effects bypass this process due to model hierarchy. Interaction terms with percent contribution more than 1 % were chosen for the regression model. This factorial design had resulted in sixteen tests with all possible combination of X_1, X_2, X_3 and X_4. The production of methyl 3-(3,5-di-tert-butyl-4-hydroxyphenyl) propionate (Y) was measured for each of these tests as shown in Table 4.4. The interaction model terms were X_1X_3, X_2X_4 and X_3X_4. A first-order model with all possible interactions was chosen to fit the experiment as shown in Equation (4.1)

$$Y = X_0 + X_1 - X_2 - X_3 - X_4 - X_1X_3 + X_2X_4 + X_3X_4 \quad (4.1)$$

The final equation, after putting values of all coefficients, was as follows:

$$Y = 0.64750 + 0.04X_1 - 0.0145X_2 - 1.97500E\text{-}003X_3 - 0.017250X_4 - 2.37500E\text{-}004X_1X_3 + 4.25000E\text{-}004X_2X_4 + 6.50000E\text{-}005X_3X_4 \quad (4.2)$$

Where X_1 was the initial pH medium, X_2 was the incubation temperature, X_3 was the agitation speed, X_4 was the total glucose in OPF juice, respectively. X_1, X_2, X_3 and X_4 were the main effect while X_1X_3, X_2X_4 and X_3X_4 were the interaction effect.

Table 4.5 The percentage contribution of each main factors and their interaction.

Effect list	**Contribution (%)**
X_1 initial pH medium	11.15
X_2 Incubation temperature	9.84
X_3 Agitation speed	51.61
X_4 Total glucose in OPF juice	8.61
X_1X_2	0.50
X_1X_3	3.70
X_1X_4	0.83
X_2X_3	0.83
X_2X_4	2.96
X_3X_4	1.73

With reference to Table 4.5, it was shown that X_3 (Agitation speed) contributed the most to the production of methyl 3-(3,5-di-tert-butyl-4-hydroxyphenyl) propionate as much as 51.61 %. The rate of the agitation speed influenced the extent of mixing in the shake flasks system and affected the nutrient availability as well (Venugopal et al., 2007). Agitation speed had affected many enzymes activities in different strains of bacteria and fungi (Darah et al., 2011; Kalaichelvan, 2012) as well as microalgae (Sobczuk et al., 2006). Lower agitation speed, which resulted in insufficient oxygen in the culture medium affected the microbial growth, whereas higher agitation speeds sometimes also lowered the production of microorganism (Seth and Chand, 2000).

Initial pH medium was the second factor that contributed to the production of methyl 3-(3,5-di-tert-butyl-4-hydroxyphenyl) propionate with the percentage of 11.15 %. In this study, the fermentation was done at different initial pH medium. The values of pH studied in this experiment were 4 and 8. Fungus generally grew maximally over a certain range of the initial pH of the medium and would not grow at high and low extremes under given conditions. Previous reports had shown that the *C. fimbriata* grew very well at pH 6 (Soares et al., 2000).

The relative size of effects was visually demonstrated by Pareto Chart in Figure 4.6. For main effect, an effect was said to be positive when an increase to its high level will cause an increase in the response while the negative effect was when an increase in its high level would result in a decrease in the response. The positive effects were colored in orange and the negative ones in blue in all the Pareto chart. In this study, the negative effects were agitation speed (C) and incubation temperature (B), meanwhile for the positive effects were initial pH medium (A) and glucose concentration (D). Increasing the negative effects such as agitation speed, might be resulted in the decrease of the production of methyl 3-(3,5-di-tert-butyl-4-hydroxyphenyl) propionate, this was because it involved the submerged fermentations. The system involved the agitation to mix and shear in the fermentation, which could make nutrients and oxygen mix fully in the fermentation broth, but when the agitation speed was too high, it could damage fragile microorganisms and affect the production process. Meanwhile, increasing the positive effects such as initial pH medium, might be resulted in the increase of the production of the compound. The hydrogen and hydroxyl ion concentration might have a direct effect on microorganisms. From the previous study, the maximum growth of *C. fimbriata* was

a pH 7.5 and this study agrees relatively well with this study by using pH 8 for highest initial pH medium.

For interactions, the positive effect was that both factors were a chance to the same level (low or high), the response will increase. The negative effect was that when both factors were changing to the opposite level (one at its low and other at its high), the response will increase (Martendal et al., 2007). Effects of t-value limit (black line) were considered statistically significant at 95% confidence level whereas effects below t-value limit were not likely to be significant. For any model with a small global p-value, Bonferroni's corrected t-test were performed on the individual terms in the model to justify individual terms in models selected by forward selection (Mee, 2009). The effect on Bonferroni's corrected t-value limit (red line) was almost certainly significant (Anderson et al., 2009). A quick analysis was performed on the selected effects using Pareto chart to statistically check for significance of the selected effects at 95% confidence level. All the selected effects (X_1, X_2, X_3, X_4, X_1X_3, X_2X_4 and X_3X_4) shown to be significant at both t-value limit and Bonferroni's corrected t-value limit.

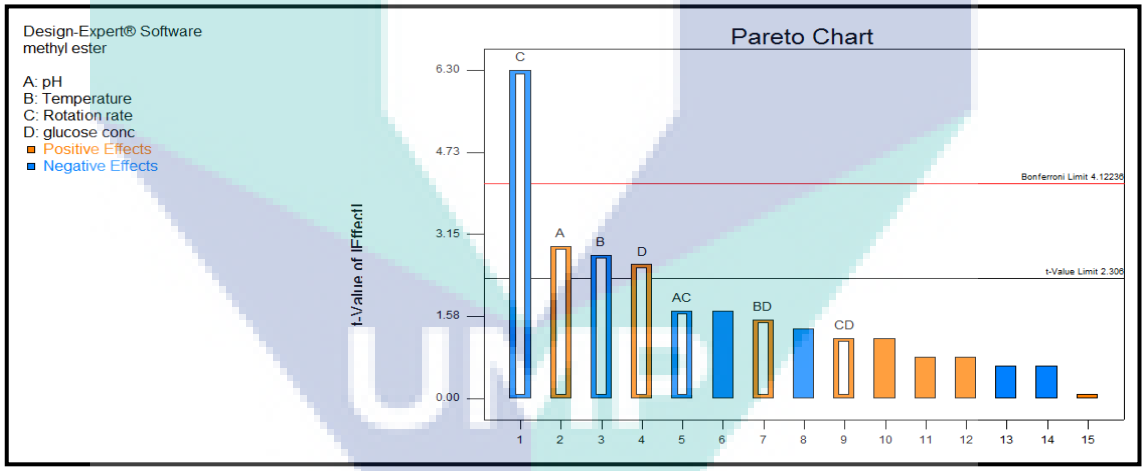


Figure 4.6 Pareto chart of effects of methyl 3-(3,5-di-tert-butyl-4-hydroxyphenyl) propionate production

4.2.2 Analysis of variance (Anova)

The models with selected effects were analyzed using analysis of variance (ANOVA) and found significant as shown in Table 4.6. The coefficient of determination (R^2) was the proportion of variation in the response attributed to the model. From Table

4.6, the F value of 9.84 with a probability value (Prob > F) of 0.0022 suggested that the model was significant and fitted well to the experimental data ($P < 0.05$) hence the model was valid for further studies for optimization experiment. The statistically significant variables at 95 % level of confidence were: ($R^2 = 0.8960$), where R squared was the correlation coefficient. Meanwhile, the p-value for each of the model terms suggest that X_1 (initial pH medium), X_2 (incubation temperature), X_3 (agitation speed) and X_4 (glucose in OPF juice) were the model terms that produce significant effect to the response. However, factor X_4 was less significant compared others factors in this screening model because of the p-value was almost approaching value 0.1. Thus, total glucose in OPF juice can be excluded from the model so that the model can be improved. The purpose of 2-level factorial design was to minimize the factor number. The design identified the factors that affect the response the most.

Table 4.6 Analysis of variance (ANOVA) analysis for 2^4 full factorial design (FFD)

Source	**Sum of square**	**Degree of freedom**	**Mean square**	**F value**	**p-value**	
Model	0.055	7	7.813E-003	9.84	0.0022	Significant
X_1-Initial pH medium	6.806E-003	1	6.806E-003	8.57	0.0190	
X_2-Incubation temperature	6.006E-003	1	6.006E-003	7.57	0.0250	
X_3-Agitation speed	0.032	1	0.032	39.69	0.0002	
X_4-Glucose in OPF juice	5.256E-003	1	5.256E-003	6.62	0.0330	
X_1X_3	2.256E-003	1	2.256E-003	2.84	0.1303	
X_2X_4	1.806E-003	1	1.806E-003	2.28	0.1699	
X_3X_4	1.056E-003	1	1.056E-003	1.33	0.2820	
Residual	6.350E-003	8	7.938E-004			
Cor total	0.061	15				

4.2.3 Effects on main factors on methyl 3-(3,5-di-tert-butyl-4-hydroxyphenyl) propionate production

All the main factors studied were statistically significant at 95 % confidence level towards on methyl 3-(3,5-di-tert-butyl-4-hydroxyphenyl) propionate production as shown by Pareto chart in Figure 4.6. Factor X_2 and X_3 was found to have negative effect and factor X_1 and X_4 were having a positive effect. When Incubation temperature and agitation speed was increased in its high level, the form production will decrease. Highest incubation temperature in this study was 35 °C, the *C. fimbriata* could not grow in incubation temperature above 30 °C because the optimum temperature for the growth of *C. fimbriata* was 18 °C – 30 °C (Johnson et al., 2005). Another negative effect was agitation speed. The highest agitation speed in this study was 150 rpm and it was not suitable for *C. fimbriata* to produce the compounds. Higher agitation speed will shear forces that could damage fragile microorganism and affect the production process. When higher incubation temperature and higher agitation speed were used, the fungus could not grow very well, thus the production of methyl 3-(3,5-di-tert-butyl-4-hydroxyphenyl) propionate will decrease.

The positive effects in this study were initial pH and total glucose concentration in this medium. When initial pH and total glucose concentration was increased in its high level, the form production will also increase. Higher initial pH medium in this study was pH 8. From the previous research by Sonyal (2010), the maximum growth of *C. fimbriata* was pH 7.5 and this research agreed relatively well with this study by using pH 8 for highest production of compounds. Another positive effect was total glucose in this medium and the total glucose using in this study was 30 g/L. Christen and Raimbault (1991) found that, the higher production of compounds was by using 30 g/L of glucose which was in good agreement with the results of this study. When initial pH medium and total glucose increased to its high level, the fungus could grow very well, thus the production of methyl 3-(3,5-di-tert-butyl-4-hydroxyphenyl) propionate will also increase.

There were six interactions found in this study as presented in Table 4.5. the highest percentage interaction was between Factor X_1, initial pH medium and Factor X_3, agitation speed with 3.70 %. The other two high value of interactions were between Factor X_2, incubation temperature with Factor X_4, total glucose in OPF juice and Factor

X_3, agitation speed with X_4, total glucose in OPF juice with 2.96 % and 1.73 % respectively. The other interactions with low percentage on contribution could be ignored because they gave very low effect towards the fermentation process.

If the pH was increased (alkali), this affects the shape of proteins, by disrupting the bonds in the protein. For the screening process, pH 4 and pH 8 were used in this study. From the previous research by Sonyal (2010), the maximum growth of *C. fimbriata* was pH 7.5 and this study strongly agrees with the study of using pH 8 for the highest production during screening. This phenomenon could be related to the fact that alkaline medium at the optimum value had a significant effect on microbial growth and cell activity.

Agitation speed was one of important factors because it was aerobic fermentation and it required the provision oxygen. Agitation could mainly cause mixing and shear in the fermentation process, which could make oxygen, heat and nutrients mix fully and be transferred efficiently in the fermentation broth and disperse the air into small bubbles to improve the gas-liquid contact area and prevent mycelia from clustering to favor of oxygen absorption (Mantzouridou et al, 2002). A very high agitation speed not only increased the power consumption, but also created heterogeneous mixing and shear forces that could damage fragile microorganisms and affect production process (Giavasis et al., 2006). On the other hand, when the agitation speed was too low, the viscosity of fermentation medium will be increased, leading to reduction in mass transfer efficiency (Bandaiphet and Prasertsan, 2006). For the screening process, 100 rpm and 150 rpm were used in this study. The research revealed that 100 rpm was the highest production during screening.

When alkaline medium met lower agitation speed, the fungus could grow very well, and lower agitation speed made the alkaline medium mixing with the nutrients very well to produce the higher production. According to the graph in Figure 4.7, it showed that the interaction between initial pH medium and agitation speed. As could be seen, methyl 3-(3,5-di-tert-butyl-4-hydroxyphenyl) propionate production was increased when initial pH medium was 8 with the decreased of agitation speed. The agitation speed that used in this design were 100 rpm and 150 rpm. At 150 rpm, the methyl 3-(3,5-di-tert-butyl-4-hydroxyphenyl) propionate production was decreased when initial pH medium

was pH 4 compared to pH 8. This interaction showed that the agitation speed at 100 rpm was more favorable for the fermentation compared to 150 rpm.

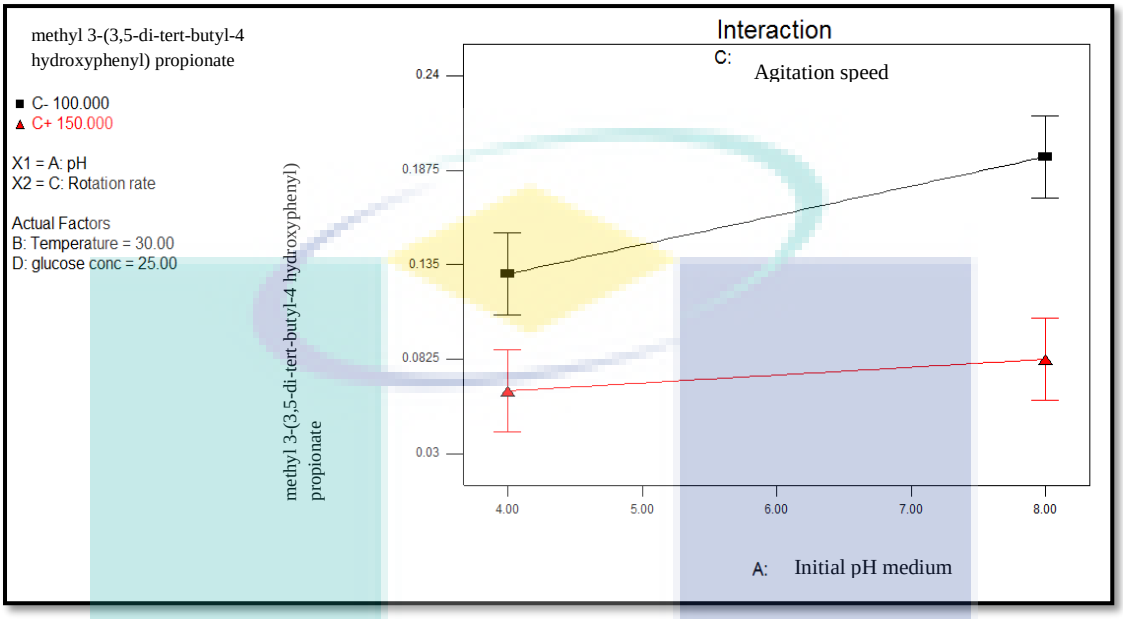


Figure 4.7 The initial pH medium effect and agitation speed (rpm) on the methyl 3-(3,5-di-tert-butyl-4-hydroxyphenyl) propionate production

Glucose was more rapidly absorbed at all concentrations than fructose. Therefore, the fungus appeared to have a higher affinity with glucose than fructose (Hamad, Alma, Ismael et al., 2014). From the previous study (Zahari et al., 2012), it stated that, the glucose was a dominant sugar in OPF juice, so the fungus was fully consuming the glucose during the fermentation. (Christen and Raimbault, 1991) found that, the higher production of compounds was when using 30 g/L of glucose which was in good agreement with the results of this study.

Growth and metabolism of microorganisms should proceed under the appropriate temperature. When the temperature exceeded the optimum growth temperature of a microorganism, inactivation and denaturation of enzymes will occur, resulted in microorganism death and finally in reduction of the fermentation cycle. Furthermore, the optimum temperatures for cell growth and metabolite accumulation were frequently different. The optimum temperature for the growth of *C. fimbriata* was 18 °C – 30 °C (Johnson et al., 2005). Consistent with findings by Johnson et al. (2005) where this study found that 25 °C showed the optimum temperature for *C. fimbriata* to produce methyl 3-(3,5-di-tert-butyl-4-hydroxyphenyl) propionate.

When the total glucose of 30 g/L in OPF juice was used and 25 °C of incubation temperature, the fungus could grow very well to produce higher production of methyl 3-(3,5-di-tert-butyl-4-hydroxyphenyl) propionate. According to the graph in Figure 4.8, it showed the interaction between total glucose concentration in OPF juice and temperature. As could be seen, methyl 3-(3,5-di-tert-butyl-4-hydroxyphenyl) propionate production was increased when the total glucose concentration in OPF juice was increased with the temperature that was set at 25 °C. At 35 °C, the methyl 3-(3,5-di-tert-butyl-4-hydroxyphenyl) propionate production was decreased with the decreased of total glucose concentration in OPF juice. This interaction showed that temperature at 25 °C was more favorable temperature for the fermentation compared to 35 °C.

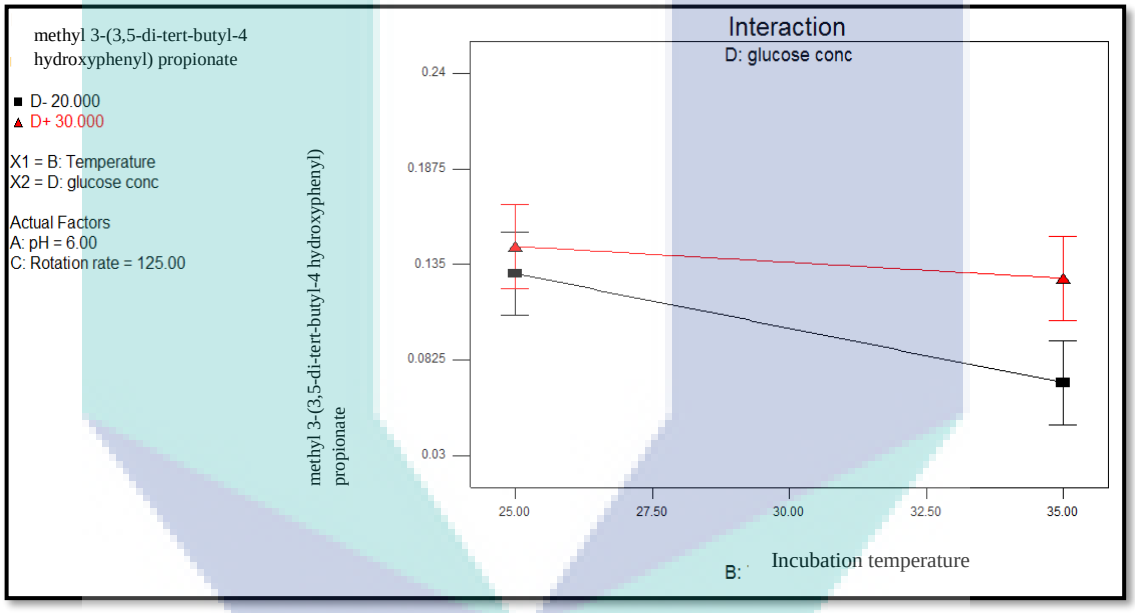


Figure 4.8 The glucose concentration in OPF juice effect and temperature (°C) on the methyl 3-(3,5-di-tert-butyl-4-hydroxyphenyl) propionate production

According to the graph in Figure 4.9, it showed the interaction between total glucose concentration in OPF juice and agitation speed. As could be seen, methyl 3-(3,5-di-tert-butyl-4-hydroxyphenyl) propionate production was increased when the total glucose concentration in OPF juice was increased with the agitation speed was set at 100 rpm. At 150 rpm, the methyl 3-(3,5-di-tert-butyl-4-hydroxyphenyl) propionate production was decreased with the decreasing of total glucose in OPF juice. This interaction showed that, the total glucose in OPF juice at 30 g/L was more favorable for the fermentation compared to 20 g/L. Further explanation on the interaction parameters

affecting the production of methyl 3-(3,5-di-tert-butyl-4-hydroxyphenyl) propionate in OPF juice by *C. fimbriata* will be further discussed in Section 4.3.2.

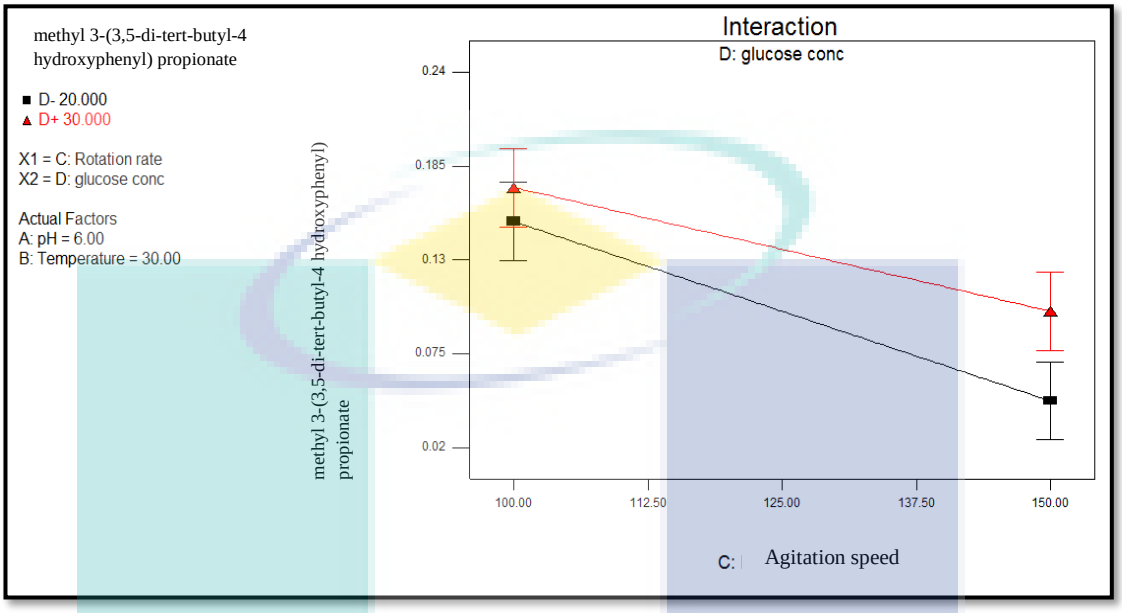


Figure 4.9 The glucose concentration in OPF juice effect and agitation speed (rpm) on the methyl 3-(3,5-di-tert-butyl-4-hydroxyphenyl) propionate production

4.2.4 Validation of experiment

In order to verify the experiments, triplicate of validation experiments was performed based on suggested condition in Design Expert 7.1. The experiments were performed according to the suggested best condition in Table 4.7 and the result was presented in Table 4.8. The validation experiments were conducted at the suggested best condition and the percentage error was calculated as low as 1.75 %, 6.43 % and 6.43 %. Based on the predicted and experimental results presented, the experimental values were in good agreement with the predicted values proposed by the model with an error less than 10 % and proved to be adequate model.

Table 4.7 Suggested best condition for factors in methyl 3-(3,5-di-tert-butyl-4-hydroxyphenyl) propionate production

Factors	Best condition
X_1-Initial pH medium	8
X_2-Incubation temperature	25 °C
X_3-Agitation speed	100 rpm
X_4-glucose in OPF juice	30 g/L

Table 4.8 Comparison between predicted and experimental value for best condition

Description	Run 1	Run 2	Run 3
Predicted value	0.21375	0.21375	0.21375
Experimental value	0.21	0.20	0.20
Error	1.75 %	6.43 %	6.43 %

4.2.5 Summary for screening

Based on the GC-SPME analysis, it showed that, the most favorable condition for methyl 3-(3,5-di-tert-butyl-4-hydroxyphenyl) propionate production were achieved at an initial pH medium of 8, agitation speed (100 rpm), incubation temperature (25°C) and 30 g/L of glucose present in the OPF juice. Methyl 3-(3,5-di-tert-butyl-4-hydroxyphenyl) propionate were produced at the retention time of 32.859 minute and the relative peak area was 0.24 % of chromatogram area by using GC-SPME. However, the main factors that affect methyl 3-(3,5-di-tert-butyl-4-hydroxyphenyl) propionate production were agitation speed and initial pH medium with value of 51.61 % and 11.15 %, respectively. The least affecting factors were temperature and total glucose in OPF juice with 9.84 % and 8.61 %, respectively. The highest contribution of interaction between initial pH medium and agitation speed as high as 3.70 % proved that these factors were the most important factors in the VOCs fermentation. For future objective, optimization on the parameters affecting methyl 3-(3,5-di-tert-butyl-4-hydroxyphenyl) propionate production from OPF juice by *C. fimbriata* were to be looked upon to improve the yield.

4.3 Optimization for production of methyl 3-(3,5-di-tert-butyl-4-hydroxyphenyl) propionate

CCD with total of 20 experiments, including 7 for factorial design, 7 for axial points and 6 repetitions at the central point, were performed. The CCD experimental design and the resulted response was shown in Table 4.9. Response was analyzed by examining fitting a model, interpreting the model graphically, finding the optimized point, and validating the model. From Table 4.9, it was apparent that the production of methyl 3-(3,5-di-tert-butyl-4-hydroxyphenyl) propionate was ranged between 0.04 % and 0.29 %. The maximum methyl 3-(3,5-di-tert-butyl-4-hydroxyphenyl) propionate production of 0.29 % was found at the centre point condition of initial pH medium was 8, agitation speed was 100 rpm and incubation temperature was 25 °C. Another five

centre points were performed to determine the experimental error required for the ANOVA as well as to observe the existence of curvature in the response surface plot later. Besides that, the centre point also to demonstrate approximately high methyl 3-(3,5-di-tert-butyl-4-hydroxyphenyl) propionate production comparable to the maximum one (Kim and Han, 2012).

Table 4.9 Experimental design using RSM with CCD and response for methyl 3-(3,5-di-tert-butyl-4-hydroxyphenyl) propionate production

Run no.	**Variables**			**Methyl 3-(3,5-di-tert-butyl-4-hydroxyphenyl) propionate production (%)**	
	Initial pH medium	**Incubation temperature (°C)**	**Agitation speed (rpm)**	**Experimental value**	**Predicted value**
1	6	20	70	0.05	0.06
2	6	20	130	0.12	0.13
3	10	20	70	0.16	0.15
4	10	20	130	0.17	0.17
5	6	30	70	0.12	0.12
6	6	30	130	0.18	0.19
7	10	30	70	0.19	0.18
8	10	30	130	0.20	0.19
9	8	25	40	0.04	0.05
10	8	25	160	0.14	0.13
11	4	25	100	0.12	0.10
12	12	25	100	0.18	0.20
13	8	15	100	0.05	0.05
14	8	35	100	0.13	0.13
15	8	25	100	0.26	0.26
16	8	25	100	0.28	0.26
17	8	25	100	0.29	0.26
18	8	25	100	0.24	0.26
19	8	25	100	0.24	0.26
20	8	25	100	0.23	0.26

4.3.1 Analysis of Variance (ANOVA)

Table 4.10 was the summary of the ANOVA results. The precision or fit of the model could be evaluated from ANOVA by referring to the model analysis and lack of fit test, as well as by using R^2 analysis. The ANOVA for optimization study was showed in Table 4.10. A model was considered significant if the p-values was less than 0.05 indicated that only a 5 % chance of noise could occur in the model. The previous research also mentioned that, the p-value was used to identify the significance of the effects of each linear, quadratic and interaction term toward the response (Tan et al., 2011). It was apparent from table that the model obtained was significant with p-value was <0.0001. Moreover, the model equation adequately defined the response as the p-value was low (< 0.0001).

From Table 4.10, it was clearly shown that, the model was statistically significant regarding highest F-value of 26.52, very low probability value ($p < 0.0001$) and sum of square of 0.10. It was also observed that all the linear (A, B and C) and quadratic (A^2, B^2 and C^2) coefficients were significant on methyl 3-(3,5-di-tert-butyl-4-hydroxyphenyl) propionate production as the p-values calculated for this factor was less than 0.05. Therefore, changes in these factors could significantly impacted the methyl 3-(3,5-di-tert-butyl-4-hydroxyphenyl) propionate production from OPF juice fermentation. Meanwhile, the all the interaction (AB, AC and BC) coefficients were insignificant, indicating that these terms had little impact on methyl 3-(3,5-di-tert-butyl-4-hydroxyphenyl) propionate production. The most significant effect of the linear coefficients was agitation speed (A) and incubation temperature (C) followed by initial pH medium (B). It was apparent from the table that the model obtained was significant with p-value was ($p < 0.0001$) while the p–value for FFD was ($p < 0.0022$) respectively. Based on p-value of RSM and FFD, it showed p-value' RSM was smaller than FFD, and it can be concluded that optimization using RSM had better production of the compound compared screening using FFD.

The lack of fit could be tested when the experimental design performed with reliable repetitions at least in its centre point (Bezerra et al., 2008). Those repetitions point also could be used in determining the pure error. Besides that, well-fitted model could be explained when it produced a significant regression and an insignificant lack of fit to the experimental data. In other words, the main part of variation observation must

be interpreted by the regression equation and remaining variation certainly due to the residuals. If there was significant lack of fit and low p-value, the response predictor was rejected (Nath and Chattopadhyay, 2007). The lack of fit for this model was not significant with p-value 0.8036. Thus, the model was well adapted to the response.

Table 4.10 ANOVA for the experimental results of CCD quadratic model

Source	**Sum of square**	**DF**	**Mean square**	**F value**	**P-value**	
Model	0.10	9	0.011	26.52	<0.0001	significant
A- Agitation speed	7.656E-003	1	7.656E-003	18.08	0.0017	
B- Initial pH medium	8.556E-003	1	8.556E-0.03	20.21	0.0012	
C- Incubation temperature	7.656E-003	1	7.656E-003	18.08	0.0017	
AB	1.513E-003	1	1.513E-003	3.57	0.0880	
AC	1.250E-005	1	1.250E-005	0.030	0.8670	
BC	6.125E-004	1	6.125E-004	1.45	0.2567	
A^2	0.043	1	0.043	102.17	<0.0001	
B^2	0.018	1	0.018	41.64	<0.0001	
C^2	0.043	1	0.043	102.17	<0.0001	
Residual	4.234E-003	10	4.234E-004			
Lack of fit	1.300E-003	5	2.600E-004	0.44	0.8036	Not significant
Pure error	2.933E-003	5	5.867E-004			
Cor total	0.11	19				
Standard deviation =0.021		PRESS = 0.015		Pred R^2 = 0.8573		
Mean = 0.17		R^2 = 0.9598		Adeq precision = 14.410		
C.V = 12.14		Adj R^2 = 0.9236				

Another important finding was that the R^2 value obtained in this model was 0.9598, which indicated that the model could explain 95.98 % variability of the response variable. The 0.9236 of adjusted R^2 was satisfactory and confirmed the significance of the model. This finding agreed with previous finding which suggested that R^2 above 0.80 was a good fit for a model (Yemiş and Mazza, 2011). In addition, the model with high value of R^2 showed a close agreement between theoretical values predicted by the model

and experimental data as shown in Figure 4.10. These statistical tests showed that the model was suitable to represent the data and able to explain a good description of the relationship between the process variables and response.

Previous research also point towards high R^2 values will represent a good model which is compatible with theoretical data (Lin et al., 2017). When the lack of fit was insignificant but the R2 value was high, it showed that the model was well adapted to the response (Fang et al., 2010). The actual vs. predicted and residuals vs. predicted responses were showed respectively in Figure 4.10 and Figure 4.11. The actual vs predicted response exhibit almost a linear relationship which predicts the reasonable precision of fitted empirical model. Residual vs. predicted responses in Figure 4.11 represent unusual structure and equally scattered points above and below the x-axis but all these points were between ± 3.0 which imply adequacy and reliability of proposed models. Hence, it could be resolved that developed models were adequate in predicting methyl 3-(3,5-di-tert-butyl-4-hydroxyphenyl) propionate production from OPF juice by *C. fimbriata*. methyl 3-(3,5-di-tert-butyl-4-hydroxyphenyl) propionate production from OPF juice by *C. fimbriata*.

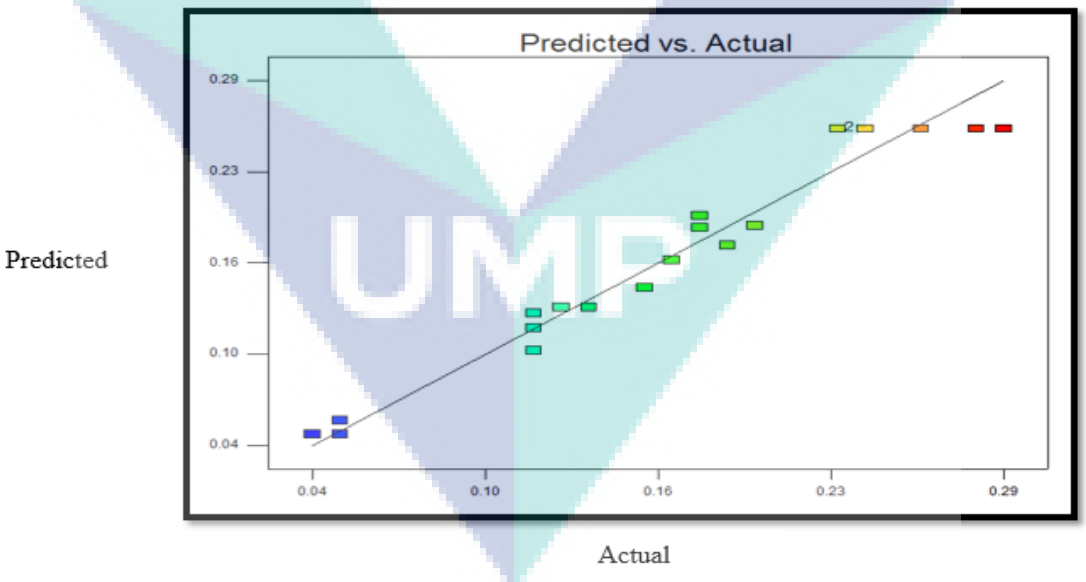


Figure 4.10 Actual vs predicted response of methyl 3-(3,5-di-tert-butyl-4-hydroxyphenyl) propionate response

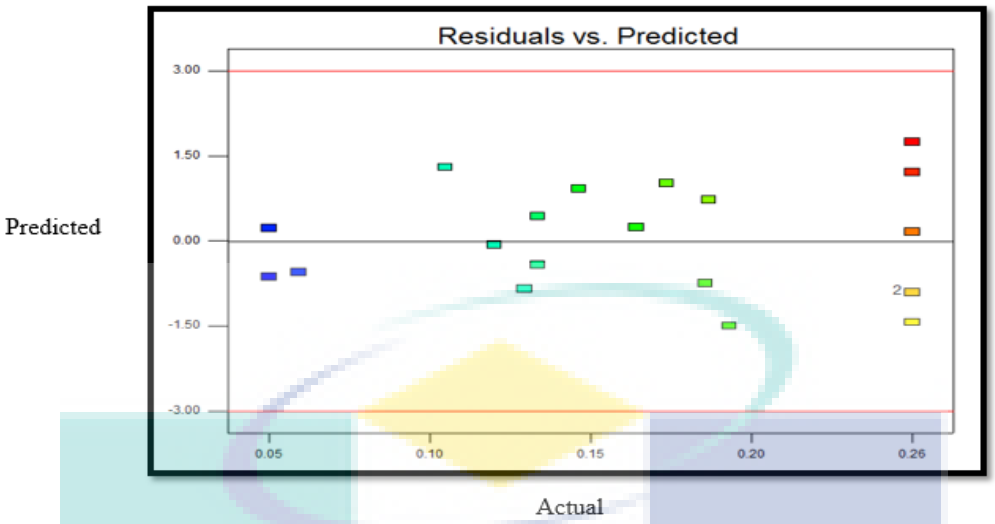


Figure 4.11 Residual plot for methyl 3-(3,5-di-tert-butyl-4-hydroxyphenyl) propionate response

The response surface quadratic model for methyl 3-(3,5-di-tert-butyl-4-hydroxyphenyl) propionate production in this study optimization study were presented in terms of coded factors as illustrated below in Equation (4.3)

$$Y = 0.26 + 0.022A + 0.023B + 0.022C - 0.014AB - 1.250E - 003AC - 8.750E - 003BC - 0.041A^2 - 0.026B^2 - 0.041C^2 \quad 4.3$$

Where Y was methyl 3-(3,5-di-tert-butyl-4-hydroxyphenyl) propionate production (%), A was agitation speed, B was initial pH medium and C was incubation temperature. The unknowns A, B and C were referred to the main effects while AB, AC, BC, A^2, B^2 and C^2 were the interaction effects that contribute in methyl 3-(3,5-di-tert-butyl-4-hydroxyphenyl) propionate process.

4.3.2 Parametric interaction effect

The 2D contour plot and 3D response surface were the graphical plots that represent the regression equation which provide the procedure to show the correlation between response and experimental level for each variables and interaction between two test variables (Qiao et al., 2009). 3D surface plot could visualize the effects of independent variables towards the response (Fang et al., 2010). The evaluation of the interaction between various factors using RSM quantified in terms of three-dimensional

response surface and contour lines. Figure 4.12, 4.13 and 4.14 were plotted to demonstrate the interactions among the three factors and to estimate methyl 3-(3,5-di-tert-butyl-4-hydroxyphenyl) propionate production over the independent variables. These plots demonstrated the effects of two factors on the response at a time and assisted in arbitration of degree of parametric on the desired responses. Three response surfaces were generated depending on three variables involved in the process.

Figure 4.12 showed three-dimensional response surface relationship between agitation speed and initial pH medium on the methyl 3-(3,5-di-tert-butyl-4-hydroxyphenyl) propionate production at the centre level of the fermentation. This figure clearly depicted the effect of initial pH medium from pH 6 to pH 10 and agitation speed from 70 rpm to 130 rpm on the methyl 3-(3,5-di-tert-butyl-4-hydroxyphenyl) propionate production from relative peak area 0.15 % of chromatogram area to relative peak area 0.24 % of chromatogram area. The plane exhibited an optimum point of methyl 3-(3,5-di-tert-butyl-4-hydroxyphenyl) propionate production at relative peak area 0.29 % by the interaction factors of initial pH medium and agitation speed which were pH 8 and 100 rpm respectively. The red regions in the figure showed the maximum production of methyl 3-(3,5-di-tert-butyl-4-hydroxyphenyl) propionate. The desirability of this design was 1.0. For the interaction between initial pH medium and agitation speed, the percentage of methyl 3-(3,5-di-tert-butyl-4-hydroxyphenyl) propionate increased and reached the maximum level when the initial pH medium was pH 8 and 100 rpm of agitation speed and the production formation was decreased beyond that.

When the pH is increased (alkali), the bonds in the protein will be disrupted and automatically change the shape of the protein. For the optimization process, pH 4, pH 6, pH 8, pH 10 and pH 12 were used in the study. From the previous research by Sonyal (2010), the maximum growth of *C. fimbriata* was pH 7.5 and this study agreed relatively well with this study by using pH 8 for the highest production during optimization process. This fermentation could be related to the fact that Ceratocystis sp. usually grew best in the environments that were slightly alkaline but when the alkaline medium was too high, the fungus could not adapt to the environment.

For the optimization process, 40 rpm, 70 rpm, 100 rpm, 130 rpm and 150 rpm were used in this study. The research found that 100 rpm was the highest production during optimization. A very high agitation could cause shear forces that could damage

fragile microorganisms and definitely affected the production process. When alkaline medium pH 8 and 100 rpm agitation speed, the fungus could grow very well, and the agitation speed made the alkaline medium mixing with the nutrients very well to produce higher production of methyl 3-(3,5-di-tert-butyl-4-hydroxyphenyl) propionate.

The three-dimensional response surface relationship between agitation speed and incubation temperature was illustrated in Figure 4.13. This figure clearly depicted the effect of agitation speed from 70 rpm to 130 rpm and incubation temperature from 20 °C to 30 °C on the methyl 3-(3,5-di-tert-butyl-4-hydroxyphenyl) propionate production from relative peak area 0.15 % of chromatogram area to relative peak area 0.24 % of chromatogram area. The plane showed an optimum point of methyl 3-(3,5-di-tert-butyl-4-hydroxyphenyl) propionate production at relative peak area 0.29 % by the interaction factors of agitation speed and incubation temperature which were 100 rpm and 25 °C respectively. The red regions in the figure showed the maximum production of methyl 3-(3,5-di-tert-butyl-4-hydroxyphenyl) propionate. The desirability of this design was 1.0.

For the interaction between incubation temperature and agitation speed, the percentage of methyl 3-(3,5-di-tert-butyl-4-hydroxyphenyl) propionate increased and reached the maximum level on 25 °C of incubation temperature and 100 rpm of agitation speed and the production formation was decreased beyond that. A very high agitation speed not only increased the power consumption, but also destroyed fragile microorganisms and affect the production process by creating heterogeneous mixing and shear forces (Giavasis et al., 2006). However, a very low agitation speed would lead to the increase of viscosity of fermentation medium, and also leading to a reduction in mass transfer efficiency (Bandaiphet and Prasertsan, 2006). For the optimization process, 40 rpm, 70 rpm, 100 rpm, 130 rpm and 150 rpm were used in this study. During optimization, the research revealed that 100 rpm was the highest production. This concluded that a high agitation speed could cause shear forces that could destroy fragile microorganisms and affected production process.

The optimum temperatures for cell growth and metabolite accumulation were frequently different. The optimum temperature for the growth of *C. fimbriata* was 18 °C – 30 °C (Johnson et al., 2005). Consistent with findings by Johnson et al. (2005) this study found that 25 °C showed the optimum temperature for *C. fimbriata* to produce methyl 3-(3,5-di-tert-butyl-4-hydroxyphenyl) propionate. For the optimization process,

15 °C, 20 °C, 25 °C, 30 °C and 35 °C were used in this study. The research revealed that 25 °C was the highest production during optimization. Too high incubation temperature leads to the inactivation and denaturation of enzymes, resulting in microorganism death and finally caused reduction of the fermentation cycle. When 25 °C of incubation temperature and 100 rpm agitation speed, the fungus could grow very well with the optimum temperature, and the agitation speed made the nutrients very well to produce higher production of methyl 3-(3,5-di-tert-butyl-4-hydroxyphenyl) propionate.

Figure 4.14 displayed three-dimensional response surface interactions between initial pH medium and incubation temperature. The figure clearly depicted the effect of initial pH medium from pH 6 to pH 10 and incubation temperature from 20 °C to 30 °C on the methyl 3-(3,5-di-tert-butyl-4-hydroxyphenyl) propionate production from relative peak area 0.15 % of chromatogram area to relative peak area 0.24 % of chromatogram area. The plane showed an optimum point of methyl 3-(3,5-di-tert-butyl-4-hydroxyphenyl) propionate production at relative peak area 0.29 % by the interaction factors of initial pH medium and incubation temperature which were pH 8 and 25 °C respectively. The red regions in the figure showed the maximum production of methyl 3-(3,5-di-tert-butyl-4-hydroxyphenyl) propionate. The desirability of this design was 1.0. For the interaction between incubation temperature and initial pH medium the percentage of methyl 3-(3,5-di-tert-butyl-4-hydroxyphenyl) propionate increased and reached the maximum level at 25 °C of incubation temperature and initial pH medium was pH 8 and the production formation was decreased beyond that.

The optimum temperature for the growth of *C. fimbriata* was 18 °C – 30 °C (Johnson et al., 2005). A finding by Johnson et al. (2005) found that 25 °C showed the optimum temperature for *C. fimbriata* to produce methyl 3-(3,5-di-tert-butyl-4-hydroxyphenyl) propionate. For the optimization process, 15 °C, 20 °C, 25 °C, 30 °C and 35 °C were used in this study. During optimization, the research revealed that 25 °C was the highest production. An extremely incubation temperature could cause inactivation and denaturation of enzymes, resulted in the death of the microorganism and reduction of the fermentation cycle.

If the pH is increased which was more alkaline, this affected the shape of proteins, by disrupting the bonds in the protein. For the optimization process, pH 4, pH 6, pH 8, pH 10 and pH 12 were used in study. From the previous research by Sonyal (2010), the

maximum growth of *C. fimbriata* was in the pH 7.5 and this study approved the study by using pH 8 for highest production during optimization process. This fermentation could be related to the fact that Ceratocystis sp. usually grew best in environments that were slightly alkaline but the fungus could not tolerate to the environment where the alkaline medium was too high. When medium slightly alkaline such as pH 8 and 25 °C of incubation temperature, the fungus could grow very well with the optimum temperature, to produce higher production of methyl 3-(3,5-di-tert-butyl-4-hydroxyphenyl) propionate.

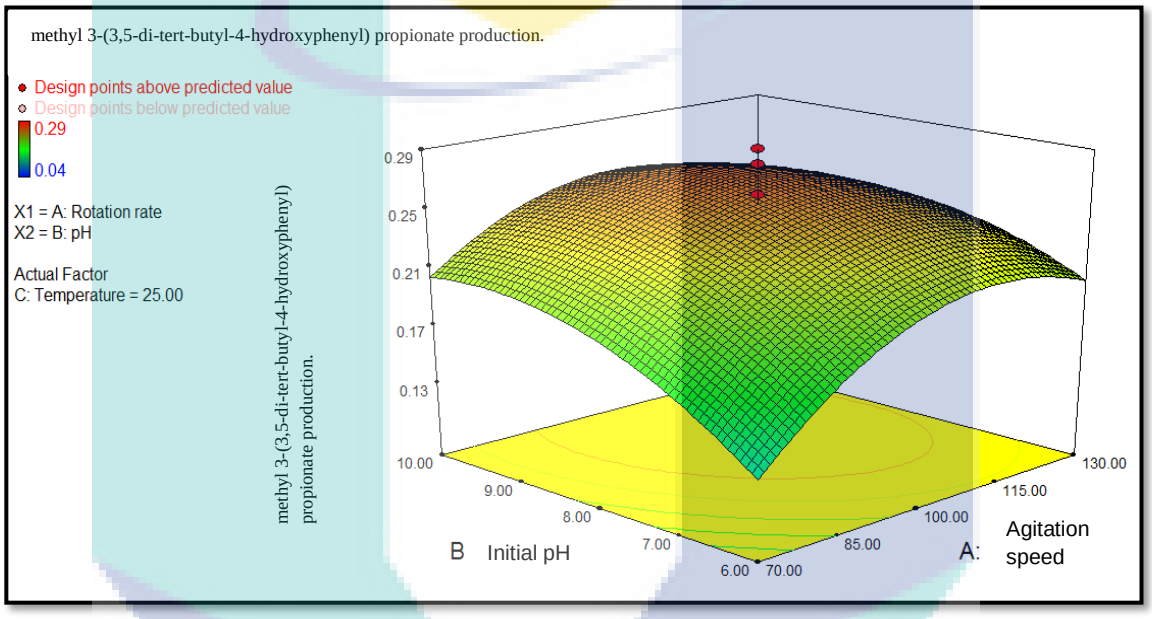


Figure 4.12 Response surface and contour plot showing the effect of initial pH medium and agitation speed on methyl 3-(3,5-di-tert-butyl-4-hydroxyphenyl) propionate production

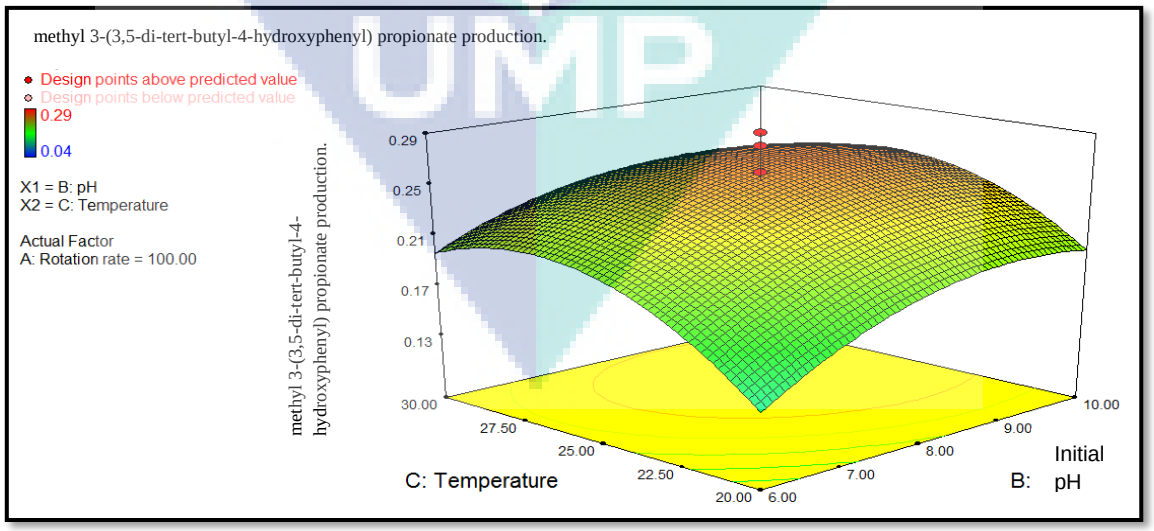


Figure 4.13 Response surface and contour plot showing the effect of incubation temperature and agitation speed on methyl 3-(3,5-di-tert-butyl-4-hydroxyphenyl) propionate production

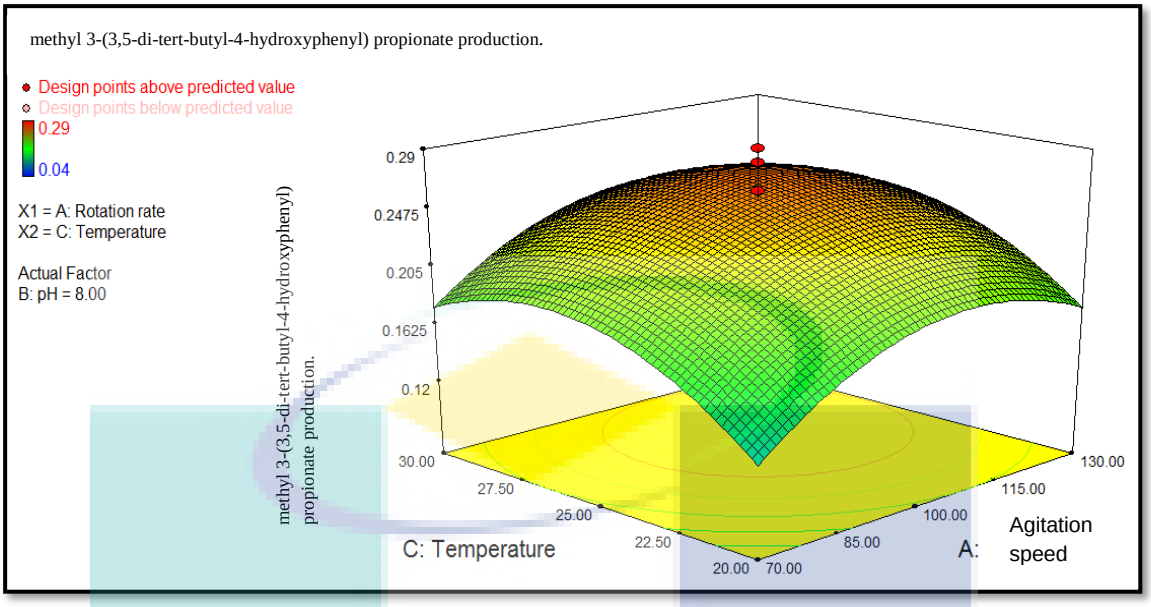


Figure 4.14 Response surface and contour plot showing the effect of incubation temperature and initial pH medium on methyl 3-(3,5-di-tert-butyl-4-hydroxyphenyl) propionate production

4.3.3 Validation of methyl 3-(3,5-di-tert-butyl-4-hydroxyphenyl) propionate optimization

Optimization might be carried out using mathematical (numerical) or graphical (contour plot) approaches. Graphical optimization was limited to cases in which there were only a few responses. Numerical optimization required defining an objective function (called a desirability or score function) that reflected the levels of each response in terms of minimum (zero) to maximum (one) desirability (Simon, 2003). Numerical optimization was performed with the goal to maximize the response and gave the following best solution as illustrated in Table 4.11 with predicted response at relative peak area 0.26 % of chromatogram area at desirability of 1.0. To further validate the accuracy of RSM prediction, an experiment was performed under the optimal condition obtained from Table 4.9.

This validation was also used to verify the accuracy of the model. The results were verified by triplicate experiments and were performed according to the suggested best condition in Table 4.11 and the result was presented in Table 4.12. The validation experiments were conducted at the suggested best conditions and the error from these runs were 3.85 %, 7.69 % and - 3.85 %. According to the predicted and experimental

results presented, the experimental values were in good agreement with the predicted values proposed by the model.

Table 4.11 Conditions for optimizing methyl 3-(3,5-di-tert-butyl-4-hydroxyphenyl) propionate factors

Factor	Condition
Initial pH medium	8
Incubation temperature (°C)	25 °C
Agitation speed (rpm)	100 rpm

Table 4.12 Comparison between predicted and experimental value for optimum condition

Description	Methyl 3-(3,5-di-tert-butyl-4-hydroxyphenyl) propionate production factors		
	Run 1	Run 2	Run 3
Predicted value	0.26 %	0.26 %	0.26 %
Experimental value	0.25 %	0.24 %	0.27 %
Error	3.85 %	7.69 %	-3.85 %

4.3.4 Optimization of methyl 3-(3,5-di-tert-butyl-4-hydroxyphenyl) propionate production

The model generated from experimental results (Table 4.9) represented the maximum methyl 3-(3,5-di-tert-butyl-4-hydroxyphenyl) propionate production was at the centre point conditions with incubation temperature at 25 °C, initial pH medium was pH 8 and agitation speed 100 rpm. At these conditions, the methyl 3-(3,5-di-tert-butyl-4-hydroxyphenyl) propionate production was up to relative peak area 0.29 % of chromatogram area.

Ester were the product of an enzyme-catalyzed condensation reaction between alcohol and acyl-CoA (Verstrepen et al., 2003). Other studies indicated that the compound such as acetate esters increased with temperature during fermentation (Dufour et al., 2008). Generally, increased fermentation temperatures in the range of 10–25°C lead to the increased ester production. According to (Peddie, 1990), when temperature was elevated, the concentration of esters produced during the fermentation was also increased due to increasing membrane fluidity. An increase in membrane fluidity may

allow more esters to diffuse into the medium. (Verstrepen et al., 2003) reported that up to 75% more esters were produced at 12°C than at 10°C. However, different esters might show different temperature dependencies. Some studies showed that ethyl acetate and phenyl ethyl acetate were produced maximally at 20°C, whereas maximal production of isoamyl acetate and ethyl caprylate occurred at 15°C (Rose and Harrison, 2012). This temperature dependency was not valid for all yeast strains, and some strains might show different temperature dependencies for the production of certain volatile esters (Ramos-Jeunehomme, 1991). In this study, the production rate and specific productivity parameters were carried out at 25 °C. (Oliveira et al., 2015) found that, the optimum incubation temperature for *C. fimbriata* ranged from 24 °C to 26 °C. This finding was consistent with the findings of past studies by Sonyal, where fungus grew well in abundance in the incubation temperature range of 20 °C to 35˚C (Sonyal, 2010).

The fungus generally grew maximally over a certain range of initial pH of the medium and would fail to grow at high and low extremes under given conditions. The optimum initial pH medium was varied depending upon the strain of microorganism and substrate used. A study was found by (Sonyal, 2010), who observed that the maximum growth of *C. fimbriata* was a pH 7.5, which was on par with pH 7.0 and pH 8.00 which was the finding was consistent with this study by using pH 8 for production of VOCs. On the other hand, the production of VOCs by *C. fimbriata* from coffee husk was investigated by (Medeiros et al., 2006) and they found that the optimum initial pH medium was 6. Besides that, studies performed by (Christen et al., 1997; Christen and Raimbault, 1991; Christen et al., 1994) revealed that the optimum initial pH medium was 6 for the production of VOCs. As could be seen in Figure 4.12 and Figure 4.14, the acidic medium at pH 6 showed lower production of VOCs compared to alkaline medium at pH 8. This phenomenon could be related to the fact that alkaline medium at the optimum value had a considerable effect on microbial growth and cell activity.

The optimum agitation speed by *C. fimbriata* for aroma production discovered by (Christen and Raimbault, 1991; Christen et al., 1994) was 150 rpm and 180 rpm. The majority of fermentation processes were aerobic and therefore require oxygen. The oxygen demanded on fermentation process was normally satisfied by aeration and agitating the fermentation broth. An additional beneficial effect of agitation was to diminish the size of mycelial aggregates, making oxygen more easily accessible to the

cells (Jafari et al., 2007). Martin and Bailey (1985) observed that higher agitation speed caused an increase in mycelial biomass concentration associated with high frequency of filamentous mycelia but a decrease in the pellet size of mycelium. As could be observed in Figure 4.13, the production of methyl 3-(3,5-di-tert-butyl-4-hydroxyphenyl) propionate was increasing when the agitation speed increased from 70 rpm until 100 rpm but decreased when the agitation speed was from 100 rpm to 130 rpm. The optimum agitation speed was varied depending upon the strain of microorganism and substrate used.

4.3.5 Summary for the optimization

The main factors chosen for CCD design were incubation temperature, initial pH medium and agitations speed. Using RSM based on CCD model, optimum conditions for methyl 3-(3,5-di-tert-butyl-4-hydroxyphenyl) propionate production from OPF juice by *C. fimbriata* were; incubation temperature was at 25 °C, initial pH medium was pH 8 and agitation speed was 100 rpm respectively. The CCD design exemplified a quadratic polynomial model with the coefficients of determines (R^2) for the response was 0.9598. However, the model had shown the lack of fit was not significant where lack of fit was 0.8036 not significant relative to the pure error. The non-significant lack of fit was positive because it demonstrated a good fit of the model to the data. A good fit means that the generated models adequately explained the variation of data. The interactions between the factors could be observed that methyl 3-(3,5-di-tert-butyl-4-hydroxyphenyl) propionate production increased with increasing of incubation temperature, agitation speed and initial pH medium and reached to the maximum point which were the optimum conditions for the fermentation process. The maximum methyl 3-(3,5-di-tert-butyl-4-hydroxyphenyl) propionate production was observed at relative peak area 0.29 % of chromatogram area.

CHAPTER 5

CONCLUSION AND RECOMMENDATION

5.1 Conclusion

In summary, OPF juice can be utilized in the production of VOCs via biosynthesis using *C. fimbriata*. Overall, the research findings of this study could contribute to the proper management of waste disposal from the oil palm industry. The utilization of renewable biomass such as OPF as fermentation feedstock could reduce the huge volume of biomass generated from the oil palm plantation, which directly reduced the negative impact of oil palm biomass to the environment. Therefore, fully utilization of OPF was not only beneficial in terms of value-added products such as methyl 3-(3,5-di-tert-butyl-4-hydroxyphenyl) propionate, but it also can reduce the environmental pollution problems due to its large accumulation in nature. Furthermore, other VOCs that produced by waste substrate such as ester, alcohol, phenol, ketone, aldehyde can be also produced by OPF juice.

In this study, the highest VOCs' group that produced from OPF juice by *C. fimbriata* was ester which is comparable to the other study. As for example, other studies showed that the compounds from ester group such as ethyl acetate were produced by coffee husk and a mixture of cassava bagasse and soybean waste. Meanwhile, other study showed that the mixture of apple pomace, cassava bagasse and soybean waste showed the higher aroma intensity (ester) compared to other compounds. It is worth to mention that, OPF juice can be used as a potential feedstock for production of ester since it also produced a similar compound as compared to the other biowaste. Moreover, OPF is an oil palm biomass which is produced as by-products of the palm oil industry. Utilization of OPF as an alternative and renewable source of raw material for the production of methyl 3-(3,5-di-tert-butyl-4-hydroxyphenyl) propionate can reduce the dependence on

food crops such as cassava, apple pomace, soybean and amaranth grain. As a conclusion, under the optimized condition, the highest methyl 3-(3,5-ditert-butyl-4-hydroxyphenyl) propionate production was obtained at the relative peak area was 0.29 % of chromatogram area and it proved that, the OPF juice was successfully produce methyl 3-(3,5-ditert-butyl-4-hydroxyphenyl) propionate.

5.2 Recommendation

Further investigation and experimentation into this study is recommended. There are several recommendations for future studies based on this study.

1) This present study does not include the analysis of using Gas Chromatography Flame Ionization Detector or GC-FID. GC-FID is a very common analytical technique that is widely used in the petrochemical, pharmaceutical and natural gas markets. GC-FID also is most useful for the detection of organic compounds; it is generally insensitive to carbonyl, alcohol and amine functionalities as well as halogens and noncombustible gases such as water and carbon dioxide. In this study, GC-MS SPME was used for identifying the qualitative of VOCs in fermentation process but by using GC-FID, it can be identified the quantitative of VOCs.

2) A further study by using kinetic study would be interesting. Kinetic study is a method to explain the reaction mechanism of certain process. Using the kinetic study after optimization study will explain the overall reaction process by developing the mathematical model that can be used to study the influence of the variables to the production of VOCs.

3) A future study investigating the performance of *C. fimbriata* will be very interesting. It can be achieved by comparing *C. fimbriata* with other fungus that produce VOCs such as *Saccharomyces spp., Williopsis saturnus, Geotrichum klebahni, Kluyveromyces lactis* and *Sporidiobolus salmonicolor*. If the performance of *C. fimbriata* is comparable with other fungus, it is suggested to use *C. fimbriata* due to the higher production of VOCs compared to the other fungus.

4) In addition, as an extension to this study, further development on fermentation strategy in the larger scale and precise control of fermentation parameters shall be conducted in near future in order to obtain high production of VOCs and a cost-effective process that is comparable to conventional VOCs produced from petroleum industries.

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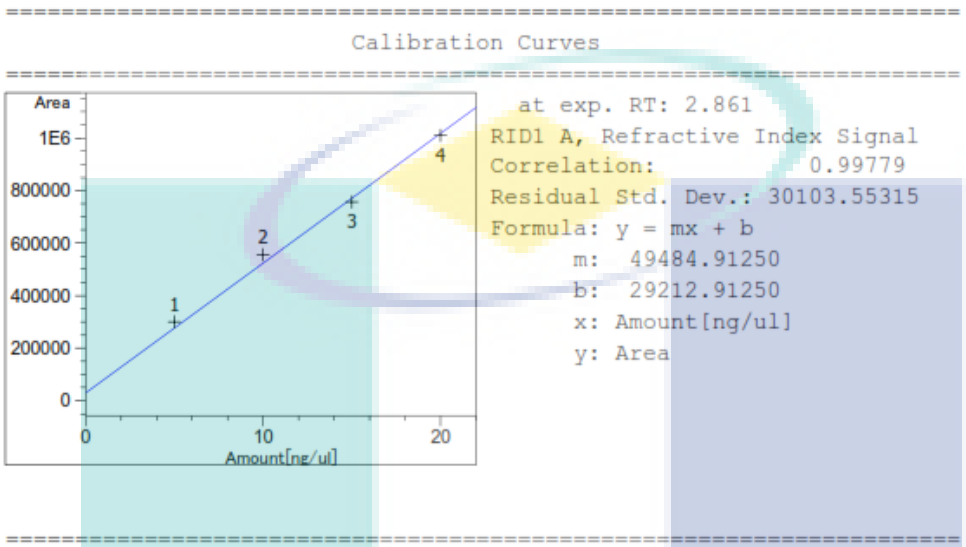
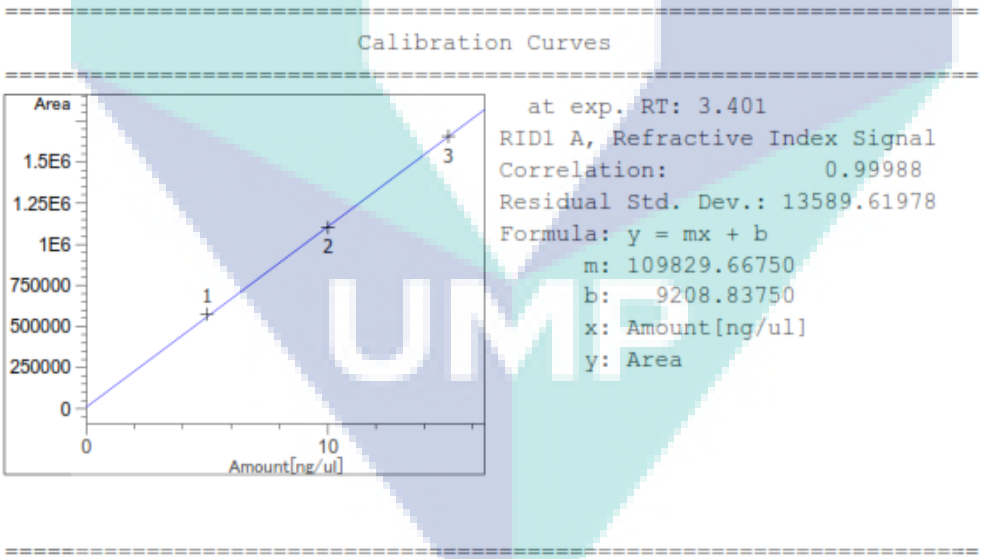
APPENDIX A
HPLC STANDARD CURVE

A-1 Sucrose standard curve

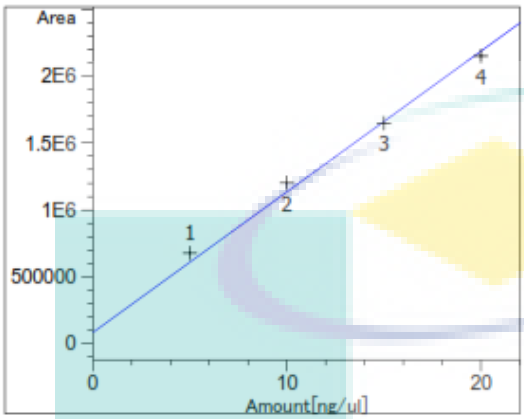


A-2 Glucose standard curve



A-3 Fructose standard curve

Calibration Curves



at exp. RT: 3.680
RID1 A, Refractive Index Signal
Correlation: 0.99685
Residual Std. Dev.: 76605.99161
Formula: y = mx + b
m: 105401.77750
b: 81227.45000
x: Amount[ng/ul]
y: Area

APPENDIX B

SCREENING AND OPTIMIZATION USING GC-MS SPME ANALYSIS

B-1 Results for screening using full factorial analysis

```
Data Path : D:\Data\nangmkb15009\DEC2016\
Data File : SAMPLE 9 I.D
Acq On    : 24 Jan 2017  18:31
Operator  : NANG
Sample    : SAMPLE 9
Misc      :
ALS Vial  : 1   Sample Multiplier: 1

Search Libraries:    C:\Database\NIST05a.L              Minimum Quality:    0

Unknown Spectrum:   Apex
Integration Events: ChemStation Integrator - autoint1.e
```

Pk#	RT	Area%	Library/ID	Ref#	CAS#	Qual
1	1.360	24.52	C:\Database\NIST05a.L			
			Hexane	1790	000110-54-3	91
			Hexane	1792	000110-54-3	91
			Hexane	1791	000110-54-3	72
2	1.917	29.84	C:\Database\NIST05a.L			
			Heptane	3886	000142-82-5	95
			Heptane	3885	000142-82-5	94
			Heptane	3887	000142-82-5	91
3	3.901	1.16	C:\Database\NIST05a.L			
			Cyclotrisiloxane, hexamethyl-	73123	000541-05-9	90
			Cyclotrisiloxane, hexamethyl-	73122	000541-05-9	72
			Benzo[h]quinoline, 2,4-dimethyl-	62243	000605-67-4	45
4	6.126	0.22	C:\Database\NIST05a.L			
			Propane	78	000074-98-6	4
			Propane	77	000074-98-6	4
			Acetaldehyde	71	000075-07-0	3
5	6.960	0.20	C:\Database\NIST05a.L			
			3-Butyn-1-ol	502	000927-74-2	5
			Urea, methyl-	756	000598-50-5	4
			Acetaldehyde	71	000075-07-0	3
6	8.661	1.40	C:\Database\NIST05a.L			
			1-Octen-3-ol	12049	003391-86-4	90
			1-Octen-3-ol	12054	003391-86-4	72
			1-Octen-3-ol	12053	003391-86-4	59
7	10.421	0.95	C:\Database\NIST05a.L			
			1-Hexanol, 2-ethyl-	13229	000104-76-7	74
			1-Hexanol, 2-ethyl-	13237	000104-76-7	74
			1-Hexanol, 2-ethyl-	13235	000104-76-7	63
8	11.817	0.44	C:\Database\NIST05a.L			
			4-Aminobutanoic acid	4443	000056-12-2	9
			Pentanol, 5-amino-	4486	002508-29-4	9
			Pentadecylamine	76641	002570-26-5	9
9	12.732	2.50	C:\Database\NIST05a.L			
			Phenylethyl Alcohol	9611	000060-12-8	83
			Toluene	2398	000108-88-3	35
			Phenylethyl Alcohol	9612	000060-12-8	30
10	14.053	0.19	C:\Database\NIST05a.L			
			Ethyl 4-chloro-3-trifluoromethylca rbanilate	102997	018585-06-3	3
			N-(2-Thioethyl) N'-methyl urea	14482	072545-68-7	2
			Ethylene glycol bis(3-aminopropyl) ether	40698	002997-01-5	2
11	15.026	0.08	C:\Database\NIST05a.L			
			3-Butyn-1-ol	502	000927-74-2	4
			1-Pentanamine	1873	000110-58-7	4
			1-Butanamine, 2-methyl-	1879	000096-15-1	4

```
 12  15.604  0.37 C:\Database\NIST05a.L
                  Benzaldehyde, 3,4-dimethyl-          14819 005973-71-7 81

MIX CHEMICAL.M Wed Jan 24 20:57:42 2017
```

```
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 Data File : SAMPLE 9 I.D
 Acq On    : 24 Jan 2017  18:31
 Operator  : NANG
 Sample    : SAMPLE 9
 Misc      :
 ALS Vial  : 1   Sample Multiplier: 1

 Search Libraries:   C:\Database\NIST05a.L            Minimum Quality:    0

 Unknown Spectrum:   Apex
 Integration Events: ChemStation Integrator - autoint1.e

Pk#     RT Area%          Library/ID                  Ref#     CAS#   Qual

                  Benzaldehyde, 3,5-dimethyl-        14817 005779-95-3 76
                  2,6-Dimethylbenzaldehyde           14806 001123-56-4 74

 13  16.149 14.11 C:\Database\NIST05a.L
                  Ethyl(dimethyl)methoxysilane        8510 052686-75-6 40
                  1,1,1-Tris(chloromethyl)ethane     39128 003922-27-8 33
                  Silane, (2-methoxyethyl)trimethyl- 13932 018173-63-2 33

 14  18.107  0.57 C:\Database\NIST05a.L
                  Nonanoic acid                      28885 000112-05-0 74
                  3,4-Altrosan                       30997 1000129-76-4 50
                  .alpha.-D-Glucopyranoside, methyl  53106 000097-30-3 38



 15  19.257  0.26 C:\Database\NIST05a.L
                  Cyclotrisiloxane, hexamethyl-      73123 000541-05-9 72
                  Benzo[h]quinoline, 2,4-dimethyl-   62243 000605-67-4 59
                  2,4,6-Cycloheptatrien-1-one, 3,5-b 91822 1000161-21-8 50
                  is-trimethylsilyl-

 16  19.925  0.17 C:\Database\NIST05a.L
                  Propanoic acid, 2-methyl-, 2,2-dim 68388 074367-33-2 38
                  ethyl-1-(2-hydroxy-1-methylethyl)p
                  ropyl ester
                  Ether, hexyl pentyl                37797 032357-83-8 27
                  Oxalic acid, hexyl neopentyl ester 87735 1000309-73-1 22

 17  21.263  0.65 C:\Database\NIST05a.L
                  5-Fluoro-8-quinolinol              31621 000387-97-3 46
                  Benzonitrile, 2-amino-5-nitro-     31544 017420-30-3 43
                  6-tert-Butyl-2,4-dimethylphenol    41645 001879-09-0 43

 18  22.643  0.71 C:\Database\NIST05a.L
                  Methanol, (dimethylsilylene)bis-,  60275 002917-61-5 73
                  diacetate
                  1-Cyclohexyldimethylsilyloxy-2-met 67034 1000282-20-7 46
                  hylpropane
                  1-Cyclohexyldimethylsilyloxybutane 67024 1000281-95-6 37

 19  23.349  0.18 C:\Database\NIST05a.L
                  n-Dodecyl acetate                  77271 000112-66-3 14
                  1-Tetradecanol                     67322 000112-72-1 11
                  1-Heptadecanol                     96333 001454-85-9 11

 20  24.044 19.42 C:\Database\NIST05a.L
                  Phenol, 2,4-bis(1,1-dimethylethyl) 61449 000096-76-4 97
                  Phenol, 2,4-bis(1,1-dimethylethyl) 61438 000096-76-4 96
                  Phenol, 2,5-bis(1,1-dimethylethyl) 61441 005875-45-6 94



 21  25.424  0.20 C:\Database\NIST05a.L
                  Dodecanoic acid                    57057 000143-07-7 38
                  Butyraldehyde, semicarbazone       12403 013183-21-6 32
                  Dodecanoic acid                    57054 000143-07-7 25

 22  28.681  0.15 C:\Database\NIST05a.L
                  Silane, [[4-[1,2-bis[(trimethylsil 180820 056114-62-6 38
                  yl)oxy]ethyl]-1,2-phenylene]bis(ox
                  y)]bis[trimethyl-
                  Benzeneacetic acid, .alpha.,3,4-tr 182236 037148-65-5 37
                  is[(trimethylsilyl)oxy]-, trimethy

MIX CHEMICAL.M Wed Jan 24 20:57:42 2017
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Acq On : 24 Jan 2017 18:31
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Sample : SAMPLE 9
Misc :
ALS Vial : 1 Sample Multiplier: 1

Search Libraries: C:\Database\NIST05a.L Minimum Quality: 0

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Integration Events: ChemStation Integrator - autoint1.e

Pk#	RT	Area%	Library/ID	Ref#	CAS#	Qual
			lsilyl ester			
			1,2-Benzenediol, 4-(2-amino-1-hydr	180735	056145-09-6	35
			oxyethyl)-, tetrakis(trimethylsily			
			l) deriv., (R)-			
23	29.157	0.01	C:\Database\NIST05a.L			
			Ethyne, fluoro-	76	002713-09-9	2
			Ethyne, fluoro-	75	002713-09-9	2
			Ethylene oxide	74	000075-21-8	2
24	30.452	0.27	C:\Database\NIST05a.L			
			Chromium, (.eta.5-2,4-cyclopentadi	108675	101860-22-4	64
			en-1-yl)[(1,2,5,6-.eta.)-1,5-hexad			
			iene](trimethylphosphine)-			
			6-Methylphenanthridine	52077	003955-65-5	53
			2-Mercapto-4-phenylthiazole	52226	002103-88-0	53
25	32.174	0.08	C:\Database\NIST05a.L			
			7,9-Di-tert-butyl-1-oxaspiro(4,5)d	109508	082304-66-3	90
			eca-6,9-diene-2,8-dione			
			7,9-Di-tert-butyl-1-oxaspiro(4,5)d	109509	082304-66-3	47
			eca-6,9-diene-2,8-dione			
			Pyrido[3,2-d]pyrimidine-2,4(1H,3H)	60441	016953-81-4	35
			-dione, 1,3,6-trimethyl-			
26	32.859	0.24	C:\Database\NIST05a.L			
			Benzenepropanoic acid, 3,5-bis(1,1	119794	006386-38-5	94
			-dimethylethyl)-4-hydroxy-, methyl			
			ester			
			1,3,5-Triazine, 2-allylamino-4-ter	119490	1000259-03-3	47
			t-butylamino-6-(4-morpholyl)-			
			2-Nitrophenoxathiin-10,10-dioxide	109815	105583-03-7	38
27	35.067	0.01	C:\Database\NIST05a.L			
			Formamide, N,N-dimethyl-	726	000068-12-2	4
			Formamide, N,N-dimethyl-	728	000068-12-2	4
			Formamide, N,N-dimethyl-	727	000068-12-2	4

B-2 Results of optimization using central composite design

```
Data Path : D:\Data\nangmkb15009\DEC2016\
Data File : SAMPLE 17 I.D
Acq On    : 14 Mei 2017  20:31
Operator  : NANG
Sample    : SAMPLE 17
Misc      :
ALS Vial  : 1   Sample Multiplier: 1

Search Libraries:   C:\Database\NIST05a.L          Minimum Quality:   0

Unknown Spectrum:   Apex
Integration Events: ChemStation Integrator - autoint1.e

Pk#     RT  Area%          Library/ID                    Ref#     CAS#   Qual
-----------------------------------------------------------------------------
 1   1.360 24.52 C:\Database\NIST05a.L
                 Hexane                                 1790 000110-54-3 91
                 Hexane                                 1792 000110-54-3 91
                 Hexane                                 1791 000110-54-3 72

 2   1.917 29.84 C:\Database\NIST05a.L
                 Heptane                                3886 000142-82-5 95
                 Heptane                                3885 000142-82-5 94
                 Heptane                                3887 000142-82-5 91

 3   3.901  1.16 C:\Database\NIST05a.L
                 Cyclotrisiloxane, hexamethyl-         73123 000541-05-9 90
                 Cyclotrisiloxane, hexamethyl-         73122 000541-05-9 72
                 Benzo[h]quinoline, 2,4-dimethyl-      62243 000605-67-4 45

 4   6.126  0.22 C:\Database\NIST05a.L
                 Propane                                  78 000074-98-6  4
                 Propane                                  77 000074-98-6  4
                 Acetaldehyde                             71 000075-07-0  3

 5   6.960  0.20 C:\Database\NIST05a.L
                 3-Butyn-1-ol                            502 000927-74-2  5
                 Urea, methyl-                           756 000598-50-5  4
                 Acetaldehyde                             71 000075-07-0  3

 6   8.661  2.36 C:\Database\NIST05a.L
                 1-Octen-3-ol                          12049 003391-86-4 90
                 1-Octen-3-ol                          12054 003391-86-4 72
                 1-Octen-3-ol                          12053 003391-86-4 59

 7  10.421  0.95 C:\Database\NIST05a.L
                 1-Hexanol, 2-ethyl-                   13229 000104-76-7 64
                 1-Hexanol, 2-ethyl-                   13237 000104-76-7 64
                 1-Hexanol, 2-ethyl-                   13235 000104-76-7 53

 8  11.817  0.44 C:\Database\NIST05a.L
                 4-Aminobutanoic acid                   4443 000056-12-2  9
                 Pentanol, 5-amino-                     4486 002508-29-4  9
                 Pentadecylamine                       76641 002570-26-5  9

 9  12.742  2.40 C:\Database\NIST05a.L
                 Phenylethyl Alcohol                    9611 000060-12-8 83
                 Toluene                                2398 000108-88-3 35
                 Phenylethyl Alcohol                    9612 000060-12-8 30

10  14.053  0.19 C:\Database\NIST05a.L
                 Ethyl 4-chloro-3-trifluoromethylca    102997 018585-06-3  3
                 rbanilate
                 N-(2-Thioethyl) N'-methyl urea         14482 072545-68-7  2
                 Ethylene glycol bis(3-aminopropyl)     40698 002997-01-5  2
                  ether

11  15.026  0.08 C:\Database\NIST05a.L
                 3-Butyn-1-ol                            502 000927-74-2  4
                 1-Pentanamine                          1873 000110-58-7  4
                 1-Butanamine, 2-methyl-                1879 000096-15-1  4

12  15.614  0.37 C:\Database\NIST05a.L
                 Benzaldehyde, 3,4-dimethyl-           14819 005973-71-7 81

MIX CHEMICAL.M Wed Mei 14 22:57:42 2017
```

```
Data Path : D:\Data\nangmkb15009\DEC2016\
Data File : SAMPLE 17 I.D
Acq On    : 14 Mei 2017  20:31
Operator  : NANG
Sample    : SAMPLE 17
Misc      :
ALS Vial  : 1   Sample Multiplier: 1

Search Libraries:   C:\Database\NIST05a.L            Minimum Quality:    0

Unknown Spectrum:   Apex
Integration Events: ChemStation Integrator - autoint1.e
```

Pk#	RT	Area%	Library/ID	Ref#	CAS#	Qual
			Benzaldehyde, 3,5-dimethyl-	14817	005779-95-3	76
			2,6-Dimethylbenzaldehyde	14806	001123-56-4	74
13	16.149	14.11	C:\Database\NIST05a.L			
			Ethyl(dimethyl)methoxysilane	8510	052686-75-6	40
			1,1,1-Tris(chloromethyl)ethane	39128	003922-27-8	33
			Silane, (2-methoxyethyl)trimethyl-	13932	018173-63-2	33
14	18.107	0.63	C:\Database\NIST05a.L			
			Nonanoic acid	28885	000112-05-0	74
			3,4-Altrosan	30997	1000129-76-4	50
			.alpha.-D-Glucopyranoside, methyl	53106	000097-30-3	38
15	19.257	0.26	C:\Database\NIST05a.L			
			Cyclotrisiloxane, hexamethyl-	73123	000541-05-9	72
			Benzo[h]quinoline, 2,4-dimethyl-	62243	000605-67-4	59
			2,4,6-Cycloheptatrien-1-one, 3,5-b	91822	1000161-21-8	50
			is-trimethylsilyl-			
16	19.925	0.17	C:\Database\NIST05a.L			
			Propanoic acid, 2-methyl-, 2,2-dim	68388	074367-33-2	38
			ethyl-1-(2-hydroxy-1-methylethyl)p			
			ropyl ester			
			Ether, hexyl pentyl	37797	032357-83-8	27
			Oxalic acid, hexyl neopentyl ester	87735	1000309-73-1	22
17	21.263	0.65	C:\Database\NIST05a.L			
			5-Fluoro-8-quinolinol	31621	000387-97-3	46
			Benzonitrile, 2-amino-5-nitro-	31544	017420-30-3	43
			6-tert-Butyl-2,4-dimethylphenol	41645	001879-09-0	43
18	22.643	0.71	C:\Database\NIST05a.L			
			Methanol, (dimethylsilylene)bis-,	60275	002917-61-5	53
			diacetate			
			1-Cyclohexyldimethylsilyloxy-2-met	67034	1000282-20-7	46
			hylpropane			
			1-Cyclohexyldimethylsilyloxybutane	67024	1000281-95-6	37
19	23.349	0.18	C:\Database\NIST05a.L			
			n-Dodecyl acetate	77271	000112-66-3	14
			1-Tetradecanol	67322	000112-72-1	11
			1-Heptadecanol	96333	001454-85-9	11
20	24.047	18.42	C:\Database\NIST05a.L			
			Phenol, 2,4-bis(1,1-dimethylethyl)	61449	000096-76-4	96
			Phenol, 2,4-bis(1,1-dimethylethyl)	61438	000096-76-4	95
			Phenol, 2,5-bis(1,1-dimethylethyl)	61441	005875-45-6	94
21	25.424	0.20	C:\Database\NIST05a.L			
			Dodecanoic acid	57057	000143-07-7	38
			Butyraldehyde, semicarbazone	12403	013183-21-6	32
			Dodecanoic acid	57054	000143-07-7	25
22	28.681	0.15	C:\Database\NIST05a.L			
			Silane, [[4-[1,2-bis[(trimethylsil	180820	056114-62-6	38
			yl)oxy]ethyl]-1,2-phenylene]bis(ox			
			y)]bis[trimethyl-			
			Benzeneacetic acid, .alpha.,3,4-tr	182236	037148-65-5	37
			is[(trimethylsilyl)oxy]-, trimethy			

```
MIX CHEMICAL.M Wed Mei 14 22:57:42 2017
```

Data File : SAMPLE 17 I.D
Acq On : 14 Mei 2017 20:31
Operator : NANG
Sample : SAMPLE 17
Misc :
ALS Vial : 1 Sample Multiplier: 1

Search Libraries: C:\Database\NIST05a.L Minimum Quality: 0

Unknown Spectrum: Apex
Integration Events: ChemStation Integrator - autoint1.e

Pk#	RT	Area%	Library/ID	Ref#	CAS#	Qual
			lsilyl ester			
			1,2-Benzenediol, 4-(2-amino-1-hydr	180735	056145-09-6	
			35 oxyethyl)-, tetrakis(trimethylsily			
			l) deriv., (R)-			
23	29.157	0.01	C:\Database\NIST05a.L			
			Ethyne, fluoro-	76	002713-09-9	2
			Ethyne, fluoro-	75	002713-09-9	2
			Ethylene oxide	74	000075-21-8	2
24	30.452	0.27	C:\Database\NIST05a.L			
			Chromium, (.eta.5-2,4-cyclopentadi	108675	101860-22-4	64
			en-1-yl)[(1,2,5,6-.eta.)-1,5-hexad			
			iene](trimethylphosphine)-			
			6-Methylphenanthridine	52077	003955-65-5	53
			2-Mercapto-4-phenylthiazole	52226	002103-88-0	53
25	32.174	0.18	C:\Database\NIST05a.L			
			7,9-Di-tert-butyl-1-oxaspiro(4,5)d	109508	082304-66-3	90
			eca-6,9-diene-2,8-dione			
			7,9-Di-tert-butyl-1-oxaspiro(4,5)d	109509	082304-66-3	47
			eca-6,9-diene-2,8-dione			
			Pyrido[3,2-d]pyrimidine-2,4(1H,3H)	60441	016953-81-4	35
			-dione, 1,3,6-trimethyl-			
26	32.853	0.29	C:\Database\NIST05a.L			
			Benzenepropanoic acid, 3,5-bis(1,1	119794	006386-38-5	96
			-dimethylethyl)-4-hydroxy-, methyl			
			ester			
			1,3,5-Triazine, 2-allylamino-4-ter	119490	1000259-03-3	37
			t-butylamino-6-(4-morpholyl)-			
			2-Nitrophenoxathiin-10,10-dioxide	109815	105583-03-7	28
27	35.067	0.01	C:\Database\NIST05a.L			
			Formamide, N,N-dimethyl-	726	000068-12-2	4
			Formamide, N,N-dimethyl-	728	000068-12-2	4
			Formamide, N,N-dimethyl-	727	000068-12-2	4

APPENDIX C
FACTORIAL ANALYSIS

Response 1 methyl ester

ANOVA for selected factorial model

Analysis of variance table [Partial sum of squares - Type III]

Source	Sum of Squares	df	Mean Square	F Value	p-value Prob > F	
Model	0.055	7	7.813E-003	9.84	0.0022	significant
A-pH	*6.806E-003*	*1*	*6.806E-003*	*8.57*	*0.0190*	
B-Temperature	*6.006E-003*	*1*	*6.006E-003*	*7.57*	*0.0250*	
C-Rotation rate	*0.032*	*1*	*0.032*	*39.69*	*0.0002*	
D-glucose con	*5.256E-003*	*1*	*5.256E-003*	*6.62*	*0.0330*	
AC	*2.256E-003*	*1*	*2.256E-003*	*2.84*	*0.1303*	
BD	*1.806E-003*	*1*	*1.806E-003*	*2.28*	*0.1699*	
CD	*1.056E-003*	*1*	*1.056E-003*	*1.33*	*0.2820*	
Residual	6.350E-003	8	7.938E-004			
Cor Total	0.061	15				

The Model F-value of 9.84 implies the model is significant. There is only a 0.22% chance that a "Model F-Value" this large could occur due to noise.

Values of "Prob > F" less than 0.0500 indicate model terms are significant.
In this case A, B, C, D are significant model terms.
Values greater than 0.1000 indicate the model terms are not significant.
If there are many insignificant model terms (not counting those required to support hierarchy), model reduction may improve your model.

Std. Dev.	0.028	R-Squared	0.8960
Mean	0.12	Adj R-Squared	0.8050
C.V. %	23.85	Pred R-Squared	0.5839
PRESS	0.025	Adeq Precision	10.353

The "Pred R-Squared" of 0.5839 is not as close to the "Adj R-Squared" of 0.8050 as one might normally expect. This may indicate a large block effect or a possible problem with your model and/or data. Things to consider are model reduction, response tranformation, outliers, etc.

"Adeq Precision" measures the signal to noise ratio. A ratio greater than 4 is desirable. Your ratio of 10.353 indicates an adequate signal. This model can be used to navigate the design space.

Factor	Coefficient Estimate	df	Standard Error	95% CI Low	95% CI High	VIF
Intercept	0.12	1	7.043E-003	0.10	0.13	
A-pH	0.021	1	7.043E-003	4.383E-003	0.037	1.00
B-Temperature	-0.019	1	7.043E-003	-0.036	-3.133E-003	1.00
C-Rotation rate	-0.044	1	7.043E-003	-0.061	-0.028	1.00
D-glucose conc	0.018	1	7.043E-003	1.883E-003	0.034	1.00
AC	-0.012	1	7.043E-003	-0.028	4.367E-003	1.00
BD	0.011	1	7.043E-003	-5.617E-003	0.027	1.00
CD	8.125E-003	1	7.043E-003	-8.117E-003	0.024	1.00

Final Equation in Terms of Coded Factors:

methyl ester =
+0.12
+0.021 * A
-0.019 * B
-0.044 * C
+0.018 * D
-0.012 * A * C
+0.011 * B * D
+8.125E-003 * C * D

Final Equation in Terms of Actual Factors:

methyl ester =
+0.64750
+0.040000 * pH
-0.014500 * Temperature
-1.97500E-003 * Rotation rate
-0.017250 * glucose conc
-2.37500E-004 * pH * Rotation rate
+4.25000E-004 * Temperature * glucose conc
+6.50000E-005 * Rotation rate * glucose conc

Figure C1 ANOVA for factorial analysis

APPENDIX D

CENTRAL COMPOSITE DESIGN

Sequential Model Sum of Squares [Type I]

Source	Sum of Squares	df	Mean Square	F Value	p-value Prob > F	
Mean vs Total	0.57	1	0.57			
Linear vs Mean	0.024	3	7.956E-003	1.56	0.2371	
2FI vs Linear	2.137E-003	3	7.125E-004	0.12	0.9486	
Quadratic vs 2FI	0.075	3	0.025	59.10	< 0.0001	Suggested
Cubic vs Quadra	1.281E-003	4	3.203E-004	0.65	0.6470	Aliased
Residual	2.952E-003	6	4.920E-004			
Total	0.68	20	0.034			

*"Sequential Model Sum of Squares [Type I]": Select the highest order polynomial where the additional terms are significant and the model is not aliased.

Figure D1 Fit summary of optimization study

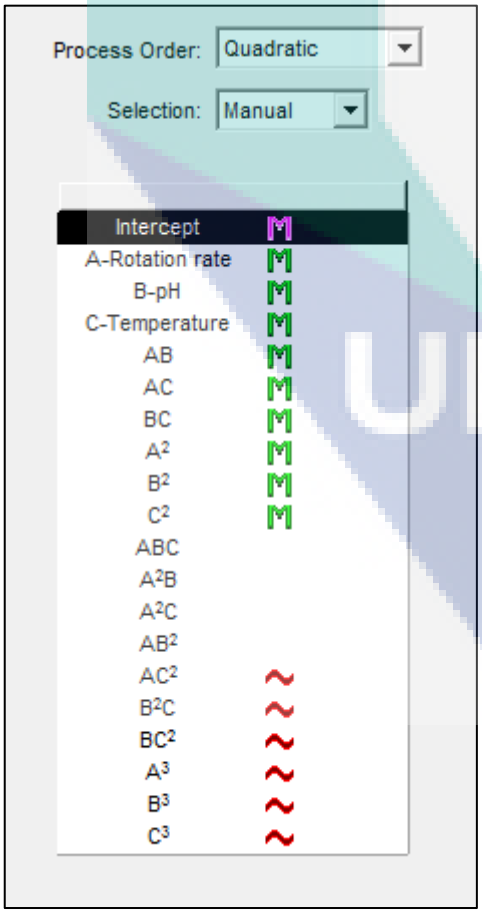

Figure D2 CCD model

Response 1 methyl ester

ANOVA for Response Surface Quadratic Model

Analysis of variance table [Partial sum of squares - Type III]

Source	Sum of Squares	df	Mean Square	F Value	p-value Prob > F	
Model	0.10	9	0.011	26.52	< 0.0001	significant
A-Rotation rate	*7.656E-003*	*1*	*7.656E-003*	*18.08*	*0.0017*	
B-pH	*8.556E-003*	*1*	*8.556E-003*	*20.21*	*0.0012*	
C-Temperature	*7.656E-003*	*1*	*7.656E-003*	*18.08*	*0.0017*	
AB	*1.513E-003*	*1*	*1.513E-003*	*3.57*	*0.0880*	
AC	*1.250E-005*	*1*	*1.250E-005*	*0.030*	*0.8670*	
BC	*6.125E-004*	*1*	*6.125E-004*	*1.45*	*0.2567*	
A^2	*0.043*	*1*	*0.043*	*102.17*	*< 0.0001*	
B^2	*0.018*	*1*	*0.018*	*41.64*	*< 0.0001*	
C^2	*0.043*	*1*	*0.043*	*102.17*	*< 0.0001*	
Residual	4.234E-003	10	4.234E-004			
Lack of Fit	*1.300E-003*	*5*	*2.600E-004*	*0.44*	*0.8036*	*not significant*
Pure Error	*2.933E-003*	*5*	*5.867E-004*			
Cor Total	0.11	19				

The Model F-value of 26.52 implies the model is significant. There is only a 0.01% chance that a "Model F-Value" this large could occur due to noise.

Values of "Prob > F" less than 0.0500 indicate model terms are significant.
In this case A, B, C, A^2, B^2, C^2 are significant model terms.
Values greater than 0.1000 indicate the model terms are not significant.
If there are many insignificant model terms (not counting those required to support hierarchy), model reduction may improve your model.

The "Lack of Fit F-value" of 0.44 implies the Lack of Fit is not significant relative to the pure error. There is a 80.36% chance that a "Lack of Fit F-value" this large could occur due to noise. Non-significant lack of fit is good -- we want the model to fit.

Std. Dev.	0.021	R-Squared	0.9598
Mean	0.17	Adj R-Squared	0.9236
C.V. %	12.14	Pred R-Squared	0.8573
PRESS	0.015	Adeq Precision	14.410

The "Pred R-Squared" of 0.8573 is in reasonable agreement with the "Adj R-Squared" of 0.9236.

"Adeq Precision" measures the signal to noise ratio. A ratio greater than 4 is desirable. Your ratio of 14.410 indicates an adequate signal. This model can be used to navigate the design space.

Factor	Coefficient Estimate	df	Standard Error	95% CI Low	95% CI High	VIF
Intercept	0.26	1	8.207E-003	0.24	0.28	
A-Rotation rate	0.022	1	5.144E-003	0.010	0.033	1.00
B-pH	0.023	1	5.144E-003	0.012	0.035	1.00
C-Temperature	0.022	1	5.144E-003	0.010	0.033	1.00
AB	-0.014	1	7.275E-003	-0.030	2.459E-003	1.00
AC	-1.250E-003	1	7.275E-003	-0.017	0.015	1.00
BC	-8.750E-003	1	7.275E-003	-0.025	7.459E-003	1.00
A^2	-0.041	1	4.103E-003	-0.051	-0.032	1.08
B^2	-0.026	1	4.103E-003	-0.036	-0.017	1.08
C^2	-0.041	1	4.103E-003	-0.051	-0.032	1.08

Figure D3 ANOVA for optimization

Final Equation in Terms of Actual Factors:

methyl ester =

-2.31834

+0.011988 * Rotation rate

+0.16226 * pH

+0.095163 * Temperature

-2.29167E-004 * Rotation rate * pH

-8.33333E-006 * Rotation rate * Temperature

-8.75000E-004 * pH * Temperature

-4.60859E-005 * Rotation rate^2

-6.61932E-003 * pH^2

-1.65909E-003 * Temperature^2

Final Equation in Terms of Coded Factors:

methyl ester =

+0.26

+0.022 * A

+0.023 * B

+0.022 * C

-0.014 * A * B

-1.250E-003 * A * C

-8.750E-003 * B * C

-0.041 * A^2

-0.026 * B^2

-0.041 * C^2

APPENDIX E
LIST OF PUBLICATION

JOURNALS

1. Nang Nor Azimah Long Nadzri, Mior Ahmad Khushairi Mohd Zahari, Saiful Nizam Tajuddin, Che Mohd Aizal Che Mohd (2017). Full factorial design for production of methyl 3-(3,5-di-tert-butyl-4-hydroxyphenyl) propionate using oil palm frond juice as a sole substrate by *Ceratocystis fimbriata*. Malaysian Applied Biology Journal. (Under review).

2. Nang Nor Azimah Long Nadzri, Mior Ahmad Khushairi Mohd Zahari, Saiful Nizam Tajuddin, Che Mohd Aizal Che Mohd (2017). Production of volatile organic compounds using oil palm frond juice as a sole substrate by *Ceratocystis fimbriata*. Malaysian Journal of Analytical Sciences. (Under review)

CONFERENCES

1. Nang Nor Azimah Long Nadzri, Mior Ahmad Khushairi Mohd Zahari, Saiful Nizam Tajuddin, Che Mohd Aizal Che Mohd (2017). Production of volatile organic compounds using oil palm frond juice as a sole substrate by *Ceratocystis fimbriata*.The 2nd International Conference on Separation Technology (ICOST 2017), 15th – 16th of April 2017, Pulai Springs Resort, Johor Bahru, Johor Darul Takzim.

2. Nang Nor Azimah Long Nadzri, Mior Ahmad Khushairi Mohd Zahari, Saiful Nizam Tajuddin, Che Mohd Aizal Che Mohd (2017). Full factorial design for production of methyl 3-(3,5-di-tert-butyl-4-hydroxyphenyl) propionate using oil palm frond juice as a sole substrate by *Ceratocystis fimbriata*. International Conference on Recent Advancements in Science and Technology (ICoRAST 2017), 7th – 8th of November 2017, Novotel Melaka, Malaysia.