OPTIMIZATION, SCALE-UP AND CHARACTERIZATION OF SESQUITERPENE FROM OIL PALM FROND JUICE

(PENGOPTIMUMAN, MENSKALA NAIK DAN PENCIRIAN SESQUITERPENE DARIPADA JUS PELEPAH KELAPA SAWIT)

DR. MIOR AHMAD KHUSHAIRI BIN MOHD ZAHARI

RESEARCH VOTE NO:
RDU150315

Fakulti Kej. Kimia & Sumber Asli
Universiti Malaysia Pahang

2017

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 Research Management Centre (RMC) and Department of Research and Innovation (DR&I) for providing me this research grant RDU150315.

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. Appreciation also goes to all staff of laboratory Faculty of Chemical and Natural Resources Engineering (FKKSA) for helping me along to complete this study. I want to thank Universiti Malaysia Pahang (UMP) for providing me the financial support.

My gratitude goes to the management and community of Lembaga Kemajuan Perusahaan Pertanian (LKPP) Lepar Besar, Gambang, Pahang for providing me raw material which is oil palm frond and to all my friends for their togetherness, kindness and moral support. Thanks for being with me through all the hardship during the project. Thank You.

I am indebted to all of you from the bottom of my heart. I am truly grateful for having such good people around me who gave endless support throughout the study even though it takes very challenging time.

ABSTRAK

Kulat menghasilkan pelbagai campuran fasa gas, sebatian berasaskan karbon yang dipanggil sebatian organik meruap (VOC) yang disebabkan oleh saiznya yang kecil dapat meresap melalui atmosfera dan tanah. VOC adalah pepejal dan cecair berasaskan karbon yang mudah memasuki fasa gas dengan menguap pada 0.01 kPa pada suhu kira-kira 20 ° C. *Ceratocystis fimbriata* adalah kulat yang mempunyai potensi untuk mensintesis ester, ia tumbuh dengan cepat dan menghasilkan pelbagai aroma (peach, nanas, pisang, sitrus dan rose) bergantung kepada keadaan persekitaran dan budaya yang digunakan dalam kajian ini. Tujuan kajian ini adalah untuk menyaring dan mengoptimumkan 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 spektroskopi jisim kromatografi gas (GC-MS) digunakan untuk memisahkan kawasan puncak relatif sebatian semasa penapaian. Pengoptimuman pengeluaran metil 3- (3,5-di-tert-butil-4-hidroksifenil) propionat dipengaruhi oleh beberapa faktor semasa tempoh penapaian. Siri reka bentuk eksperimen digunakan untuk menyaring dan mengoptimumkan pengeluaran sebatian itu. Dalam penyaringan 2^4 rekabentuk faktorial penuh telah digunakan untuk mencari faktor-faktor penting yang mempengaruhi pengeluaran metil 3- (3,5-di-tert-butil-4-hidroksifenil) propionat, yang merupakan suhu inkubasi (° C), medium pH awal, kelajuan agitasi (rpm) dan kepekatan glukosa (g / L) dalam jus OPF. Respon dalam penapisan dipasang dengan persamaan regresi linear berganda dan memperoleh korelasi (R^2 = 0. 0.8960) antara data percubaan 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 penting ($p < 0.0001$) untuk menghasilkan metil 3- (3,5-di-tert-butil-4-hidroksifenil) propionat. Respon tersebut dipasang dengan persamaan polinomial urutan kedua dengan korelasi tinggi (R^2 = 0.9598) di antara nilai yang diperhatikan dan yang diramalkan. Keputusan proses pengoptimuman menunjukkan bahawa pengeluaran 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 pengekalan adalah 32.80 minit dan kawasan puncak relatif 0.25% kawasan kromatogram dengan menggunakan GC-SPME. Kajian ini akan memberi garis panduan yang baik untuk menghasilkan sebatian metil 3- (3,5-di-tert-butil-4-hidroksifenil) propionat menggunakan jus OPF sebagai substrat tunggal oleh *Ceratocystis fimbriata*.

Key researchers:

DR. MIOR AHMAD KHUSHAIRI BIN MOHD ZAHARI
ASSOC. PROF. DR. SAIFUL NIZAM BIN TAJUDDIN
DR. AIZI NOR MAZILA BINTI RAMLI
DR. NINA SUHAITY BINTI AZMI

E-mail: ahmadkhushairi@ump.edu.my
Tel. No.: 09-5492837, 014-8290960

Vote No.: RDU150315

ABSTRACT

Fungi produce various mixtures of gas-phase, carbon-based compounds called volatile organic compounds (VOCs) that due to their small size can diffuse through the atmosphere and soils. 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. *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 was using in this study. The aim of this study 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 production of methyl 3-(3,5-di-tert-butyl-4-hydroxyphenyl) propionate 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 screening 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 (°C), initial pH medium, agitation speed (rpm) and concentration of glucose (g/L) in OPF juice. The responses in screening were fitted with a multiple linear regression equation and obtained a correlation (R^2 = 0. 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 found to be when the retention time was 32.80 minutes and the relative peak area was 0.25 % of chromatogram area by using GC-SPME. This study will provide good guideline to produce methyl 3-(3,5-di-tert-butyl-4-hydroxyphenyl) propionate compound using OPF juice as sole substrate by *Ceratocystis fimbriata.*

Key researchers:

DR. MIOR AHMAD KHUSHAIRI BIN MOHD ZAHARI
ASSOC. PROF. DR. SAIFUL NIZAM BIN TAJUDDIN
DR. AIZI NOR MAZILA BINTI RAMLI
DR. NINA SUHAITY BINTI AZMI

E-mail: ahmadkhushairi@ump.edu.my
Tel. No.: 09-5492837, 014-8290960

Vote No.: RDU150315

TABLE OF CONTENT

LIST OF TABLES

LIST OF FIGURES

LIST OF SYMBOLS

mL	millimetre
L	Liter
kg	kilogram
g	gram
°C	temperature
rpm	Rotation per minute
g/L	Gram per Litre
mL/min	Millimetre per minute
spores/mL	Spores per millimetre

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
$(NH_4)_2SO_4$	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 Research 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 oil palm plantation has shown an increasing number of area in Peninsular Malaysia; from 96,900 hectares in 1965 to 2.05 million hectares in 2000. In Sabah, about 38,433 hectares were planted with oil palm in 1970 and with rapid growth, the planted area rose to 1,000,777 hectares in 2000. Meanwhile, in Sarawak, the oil palm planted area expanded from 975 hectares in 1970 to 330,387 hectares in 2000. The largest area, in about 53.1% of the total area; was occupied by the private estate sector. The rest of the estates were owned by government (29.7%), state (8.0%) and small holdings (9.2%). The overall growth is forecast to be at 2.2% from 1991 - 2020. The Malaysian palm oil industry is primarily export-oriented. In 2000, about 9,081,011 tonnes of palm oil were produced. While 520,280 of palm kernel oil were produced in the same year (Zahari *et al.*, 2003). 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. Lately, Malaysian Agricultural Research and Development Institute (MARDI) has developed a new product for ruminants which is known as oil palm frond based ruminant pellet. The feeds based on oil palm frond, in cube-shaped, can be used as complete or balanced diet for fattening beef cattle as well as for intensive dairying in Malaysia and abroad (Zahari *et al.*, 2003).

Generally, some portions of OPF can be utilised as a source of animal food and pulp production. However, almost all of them have no practical way of utilization and become troublesome wastes (Sumathi *et al.*, 2008). 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 (M. A. 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). The potential of solid waste residues, such as oil palm frond (OPF), for the production of VOCs 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; F. W. 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 (Hari Krishna & 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; E. Vandamme, 1996; F. W. 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 & Stowers, 1991).

VOCs are carbon-based liquids and solids that readily enter the gas phase by vaporizing at 0.01 kPa at a temperature of approximately 20 °C(Pagans *et al.*, 2006). 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 & 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, 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 & 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.

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 OPF is a biomass from the oil palm plantation and it could be easy to obtain around Malaysia. Further study on the OPF juice had been done and (Zahari *et al.*, 2012) had justified the existence of glucose in the OPF juice. Furthermore, feedstock for microbial fermentation today is currently taken from edible food source, such as soy bean, malt and glucose that were also consumed by humans and animals. Competition on food consumption occurs between the needs for growth of human and animals and microbes may affect the food chain survival. Thus, studies on the potential of biomass to be utilized as a source of fermentable sugar is carried out to reduce the production cost and the dependence on the food crops.

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). 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, renewable and cheap fermentation feedstock for the production of VOCs. Hence this study was done in order to explore and clarify the potential of OPF juice as sustainable promising sources for VOCs production. This will reduce large volume of biomass generated from the oil plantation, which is one of the problematic issues related to the palm oil industry. The utilization of OPF as a source of fermentation substrate will also reduce the waste from agricultural sector which at the same time provide an alternative solution for the disposal problems that involve large volume production of oil palm waste from the palm oil industry. In addition, it is hope that the proposed study could also provide a better

approach for the oil palm settler to generate extra income, create new job opportunities as well as generating income to the country.

1.3 Objectives of study

The main objective of this research is to utilize sugars derived from OPF juice as a novel and renewable carbon source for the production of VOCs through fermentation using fungus strain of *Ceratocystis fimbriata* (*C. fimbriata*).

The specific objectives of the study are:

1) To study the growth profile of *Ceratocystis fimbriata* and to investigate its ability to produce VOCs by using OPF juice as a sole carbon source in the shake flask experiment.

2) To screen the parameters affecting methyl 3-(3,5-di-tert-butyl-4-hydroxyphenyl) propionate production such as effect of initial pH medium, agitation speed, incubation temperature and 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) by investigating the effect of incubation temperature (°C), initial pH medium and agitation speed (rpm).

1.4 Scopes of the study

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

a) The potential of OPF juice as sole substrate for the VOCs production during fermentation using *C. fimbriata* were investigated. The growths of fungi were monitored 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).

b) 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.*

c) Factors affecting the methyl 3-(3,5-di-tert-butyl-4-hydroxyphenyl) propionate 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 flaks as the fermentation system by using full factorial design method.

d) 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 are as follows; temperature (°C), initial pH medium and agitation speed (rpm).

e) The significant factors that influences VOCs production will be statistically analysed using the Design Expert Software Version 7.1.

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 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 VOCs 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 & 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 & 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 & Amerine, 1970; Rojas *et al.*, 2001; Romano & Marchese, 1998; P Romano *et al.*, 1997; Patrizia Romano *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* species 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*

Ceratocystis fimbriata (*C. fimbriata*) is a fungal plant pathogen that attacks a variety of temperate and tropical plants (Engelbrecht & 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* species (Paulin-Mahady *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 particulary 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 (E. J. 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 & 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 fuel 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. was reported by (Brigido, 2000; Brown & Hammond, 2003; Kobayashi *et al.*, 2008; G. 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 & Hammond, 2003; Kłosowski & Czupryński, 2006; Kobayashi *et al.*, 2008), *Staphylococcus* (Talon *et al.*, 1998), *Ceratocystis fimbriata* (Soares *et al.*, 2000) and *Ceratocysis moniliformis* (Bluemke & 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 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).

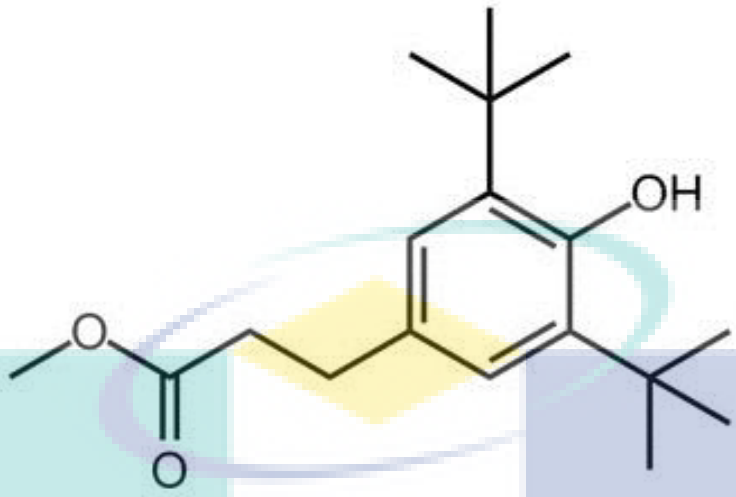


Figure 2. 1 Methyl 3-(3,5-di-tert-butyl-4-hydroxyphenyl) propionate (Li et al., 2014)

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, fl oral and spice-like odor (Burdock, 2016). It naturally occurs in many products such as bitter almond, peach, apricot kernel, cheeses and black tea (Surburg & 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 & 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 & 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.

2.5.1 Gas chromatography mass spectrometry solid phase micro extraction (GC-MS SPME)

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 & 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 further more because of its many advantages in terms of sample preparation (Kataoka & Saito, 2011). Although a large number of SPME applications have been reported for biomedical analysis (Souza-Silva

et al., 2015), it also has great potential in analyzing environmental samples (Popiel & Sankowska, 2011; Saraji & 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 *ab*sorption 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 & 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 & 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; G. M. Pastore *et al.*, 1994; F. Welsh, 1994) and such aromas have been detected in cassava processing industries.

2.6.2 Production of VOCs from apple pomace

The industrial processing of apples is performed mainly for the production of juice, jelly, and pulp. Fruits that are not suitable for consumption *in natura* are processed, generating large amounts of residues. Apple pomace, the solid residue from juice production, represents around 30% of the original fruit and is generated during fruit pressing (Villas Bôas & Esposito, 2000). Large amounts of apple pomace are produced worldwide, and, being highly biodegradable, its disposal causes a serious environmental problem. In Brazil, about 800,000 tons of apple pomace are produced per year (Protas & Sanhueza, 2003), and it is mostly used as animal feed. This utilization is, however, limited due to a low protein and vitamin content, which means a low nutritional value.

Many researchers, looking for value-added products, have proposed the use of apple pomace for the production of enzymes (Berovič & Ostroveršnik, 1997; Favela-Torres *et al.*, 2006; Shrikot *et al.*, 2004; Zheng & Shetty, 2000a), organic acids (Shojaosadati & Babaeipour, 2002), protein-enriched feeds (Albuquerque *et al.*, 2006; Bhalla & Joshi, 1994; Devrajan *et al.*, 2004; Vendruscolo *et al.*, 2007), edible mushrooms (Worrall & Yang, 1992; Zheng & Shetty, 2000b), ethanol (Ngadi & 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 & Lu, 1999; Lu & Foo, 2000), and edible fibers (Grigelmo-Miguel & 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. More specifically, varieties of fermented soybean foods such as soy sauce, tempeh, Japanese miso and natto, Thai thua nao, Chinese sufu and Korean doenjang are included (Leejeerajumnean *et al.*, 2001; Steinkraus, 1991). Although the fermented soybean products of different countries have distinctive qualities, these products share several common characteristics such as base ingredients, fermentation and processing methods (Chung, 1999). Traditional Korean soybean paste (doenjang) is primarily made with

meju, which typically uses natural flora and soybeans as the basic ingredient; whereas Japanese miso is made with koji, which utilizes *Aspergillus oryzae* and soybean and grain ingredients (H.-K. Park *et al.*, 2003).

However, due to the unequal fermentation progression that occurs with natural micro-flora, the traditional method of making Korean soybean paste (doenjang) has been adapted for mass production. Thus, large manufacturing companies are producing commercial fermented soybean pastes using wheat koji inoculated with *A. oryzae.* However, there is increasing consumer interest for traditionally made soybean pastes that possess significant health effects (Z.-I. Shin *et al.*, 2001) as well as full, complex aroma characteristics. 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. The volatile component profiles of products vary with the micro-flora involved, as well as by the processing conditions (e.g. fermentation, drying, brining, or ageing) (Chung, 1999; Leejeerajumnean *et al.*, 2001; Sugawara, 1991). Some studies have examined the volatiles in Korean fermented soybean pastes prepared using different types of strains (J.-S. Park *et al.*, 1994; Seo *et al.*, 1996), as well as different extraction (H.-K. Park *et al.*, 2003; M. Shin & Joo, 1999) and ageing and mixing methods (Choi *et al.*, 1997).

2.6.4 Production of VOCs from amaranth grain

Species of the genus *Amaranthus* (L) are herbaceous plants distributed throughout the world. Both the seed and vegetative growth have been used for food. Nutritional evaluation of grain amaranth and forage indicate a high potential for use in animal and human diets (Alfaro *et al.*, 1987; Andrásofszky *et al.*, 1997; Pisarikova *et al.*, 2006; Szelenyi-Galantai & Zsolnai-Harszi, 1992). The dry matter of grain amaranth contains 12.6 to 18.0% of proteins, 5 to 8% of fat, 60 to 65% of saccharides, and 3 to 5% of crude

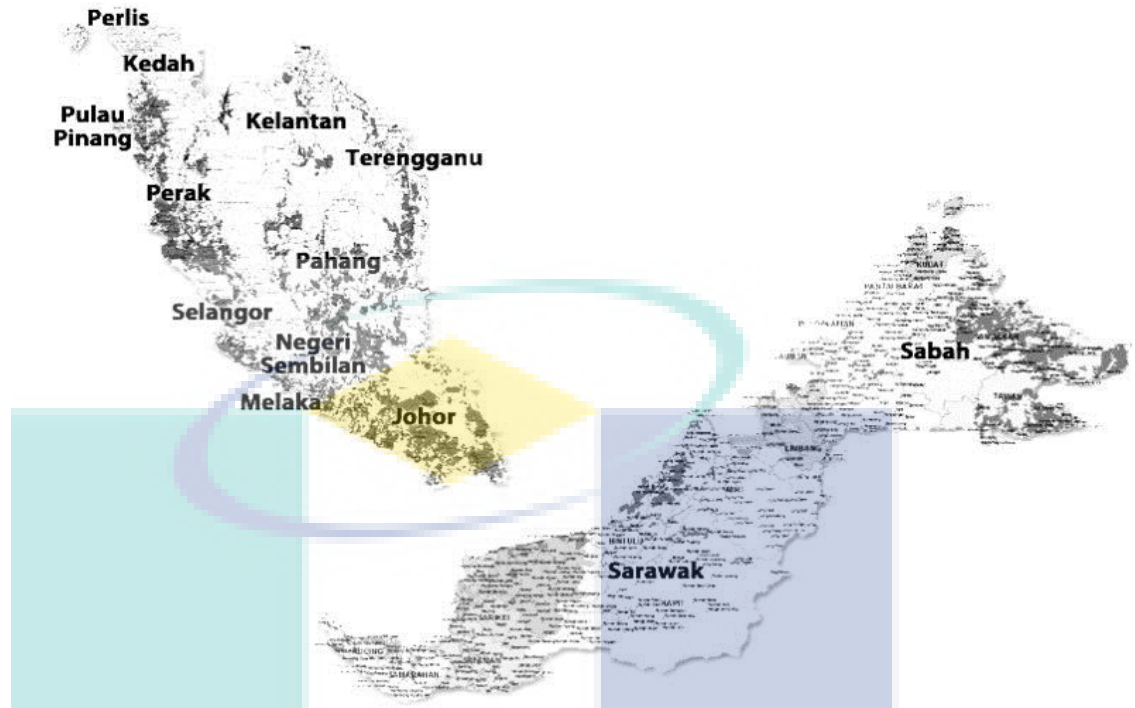
fibre (Yánez *et al.*, 1994). Grain amaranth is rich in lysine and sulphur amino acids. Amaranth oil is rich in unsaturated fatty acids, especially linoleic and oleic acids; the content of squalene (5 to 6%) is also important.

The nutritional value of the above-ground biomass depends on the growth stage of plants. The contents of crude protein ranged from 16.3 to 29.5%; crude fiber from 11.1 to 24.4%; fat from 2.0 to 3.0% and ash from 13.1 to 17.8% in dry matter (Zeman *et al.*, 1995). 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).



* The dark grey area refers to the distribution of oil palm plantations in Malaysia.

Figure 2. 2 The distribution of oil palm plantations in Malaysia (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 2.1. The volume and type of oil palm residues are expected to rapidly increase and will become a serious problem in the future.

Table 2. 1 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

Sources: (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 IW, 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 (Goh, Lee, *et al.*, 2010; Goh, Tan, *et al.*, 2010), absorbent for heavy metal ions in waste water (Salamatinia *et al.*, 2010), renewable sugar (Sabiha-Hanim *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.

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).

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 & Hahn-Hägerdal, 2000; Rumbold *et al.*, 2010).

2.8 Factors affecting production of VOCs

There were several other factors related to the biosynthesis of VOCs. 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 VOCs.

2.8.1 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 & Roberts, 1994; Carvalheiro *et al.*, 2005; Clark & Blanch, 1997; Dalsenter *et al.*, 2005; Dragone *et al.*, 2004; Hettenhaus, 1998; K. Evans, 2002; McMeekin *et al.*, 2002; Messens *et al.*, 2003; Salmon & Mauricio, 1994; Sanchez *et al.*, 2004). Besides that, temperature also can exert different effects on the growth and production phases of secondary metabolism (Rizk *et al.*, 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 & Raimbault, 1991; Christen *et al.*, 1994; Medeiros *et al.*, 2003; Soares *et al.*, 2000).

2.8.2 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 & 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 & 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.*

2.8.3 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 pellets (Darah & Ibrahim, 1996; Porcel *et al.*, 2005) that lead to cell destruction, thus lowering the production of microorganisms. 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 & Raimbault, 1991) and 180 rpm (Christen *et al.*, 1994).

2.8.4 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 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 & Raimbault, 1991) discover that 30 g/L of glucose was suitable for production of fruity aroma by using *C. fimbriata.*

2.9 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 is 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 & 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).

2.9.1 Factorial analysis

In an unoptimized VOCs production, there might exist factors or variables which do not have significant or any effect on VOCs process. Factorial analysis can be used to screen variables which are relevant to VOCs 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.

2.9.2 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 & Boyacı, 2007; Bezerra *et al.*, 2008; Nasrah *et al.*, 2017). The current research aimed to optimize VOCs 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 VOCs from OPF juice. For the preparation of OPF juice for biosynthesis of VOCs, 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 VOCs, pH of the OPF juice that was supplemented with synthetic medium was adjusted to 6 by adding sodium hydroxide (NaOH) 0.5 N and sterilized by autoclaving at the temperature of 121 °C for 20 minutes. 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 VOCs in 250 mL Erlenmeyer flask and was carried out at the temperature of 27 °C on a rotary shaker (Infors, Switzerland). The VOCs 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).

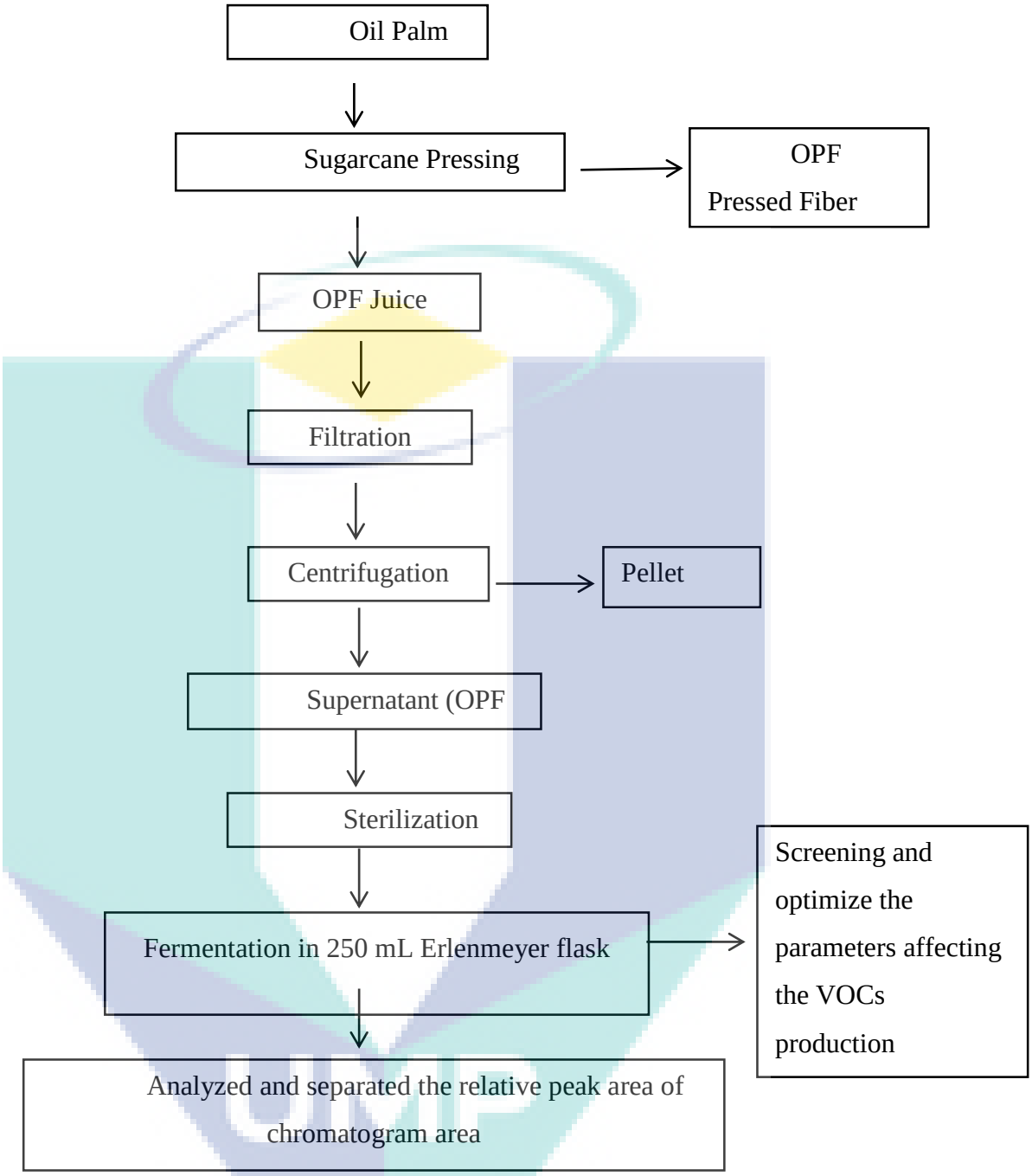


Figure 3. 1 Research methodology for production VOCs from OPF juice.

3.2 Preparation of 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 filtrate was stored in the freezer at the temperature -20 °C (Liebherr, Malaysia) before use (Zahari *et al.*, 2012).

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-NH_2 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.

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 10^8 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 N before autoclaved at 121 °C for 20 minutes. The trace element solution comprised of: Iron(III) nitrate nonahydrate ($Fe(NO_3)_3.9H_2O$) 723.8 mg/L; zinc sulfate heptahydrate ($ZnSO_4.7H_2O$) 439.8 mg/L; manganese(II) sulfate tetrahydrate ($MnSO_4.4H_2O$) 203.0 mg/L (Christen & 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 & 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). The MSM was prepared based on (Christen & Raimbault, 1991) and the MSM compositions are shown in Table 3.2. All compositions were mixed together, and the initial pH of medium was adjusted to 6.0 with NaOH 0.5 N before autoclaved. The trace element solution comprised of: Iron(III) nitrate nonahydrate ($Fe(NO_3)_3.9H_2O$) 723.8 mg/L; zinc sulfate heptahydrate ($ZnSO_4.7H_2O$) 439.8 mg/L; manganese(II) sulfate tetrahydrate ($MnSO_4.4H_2O$) 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 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.

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

Sources: (Christen & Raimbault, 1991)

3.7 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 & Raimbault, 1991). The CDW can be calculated from the Equation 3.1

$$\mathbf{CDW}\left(\frac{\mathbf{g}}{\mathbf{L}}\right) = (\mathbf{W2} - \mathbf{W1})\ \mathbf{g} \div \mathbf{100\ mL} \qquad \text{Equation 3. 1}$$

3.8 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.

3.9 Analytical procedure

The volatile compounds were detected by gas chromatography-mass spectroscop y solid phase micro extraction (GC-MS SPME). In this study, fiber DVB-CAR-PDMS (50/30 µm), which have been frequently employed for the extraction of the volatile fracti on from natural products, was tested to analyse the present of volatile compounds. The c oating was 1 cm long for the fiber. Before GC–MS analysis, the fiber was conditioned i

n the injector of the GC system, according to the instructions provided by the manufactu rer. 1 mL amount of sample was placed in a 4 mL flat-bottom headspace vial sealed wit h screw cap with PTFE/silicone septum (Agilent). The sample was heated for 45 minute s 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 headsp ace for 45 minutes during the extraction time. After sampling, the SPME fiber was imm ediately inserted into the GC injector and thermally desorbed. A desorption time of 1 mi nute at the temperature of 230 °C was used in the splitless mode. Before sampling, the fi ber was reconditioned for 5 minutes in the GC injector port at the temperature of 230 ° C (Pellati *et al.*, 2013).

3.10 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 2^k 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 2^4 full factorial design. This design was chosen in this study in order 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.

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

3.11 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
16	100	8	25
17	100	8	25
18	100	8	25
19	100	8	25
20	100	8	25

3.12 Fermentation procedure

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. Both medium had the following composition: urea, 0.75 g/L; $(NH_4)_2SO_4$, 2.25 g/L; KH_2PO_4, 1 g/L; $Ca(NO_3)_2.4H_2O$, 0.5 g/L; $MgSO_4.7H_2O$, 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) 1M 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.

3.13 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 3.2.

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

Equation 3. 2

CHAPTER 4

RESULTS AND DISCUSSION

4.1 Preliminary study

For the preliminary study, the ability of *Ceratocystis 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 was carried out at the temperature of 27 °C on a rotary shaker at 150 rpm for 8 days (Christen & Raimbault, 1991).

4.1.1 Extraction of OPF juice

Generally, the whole branch of OPF consists 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 pretreatment 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. The presence of sugar in OPF was expected due to the process of photosynthesis. Photosynthesis was the process by plants that converts carbon dioxide and water into glucose by using energy from 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 occurs.

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

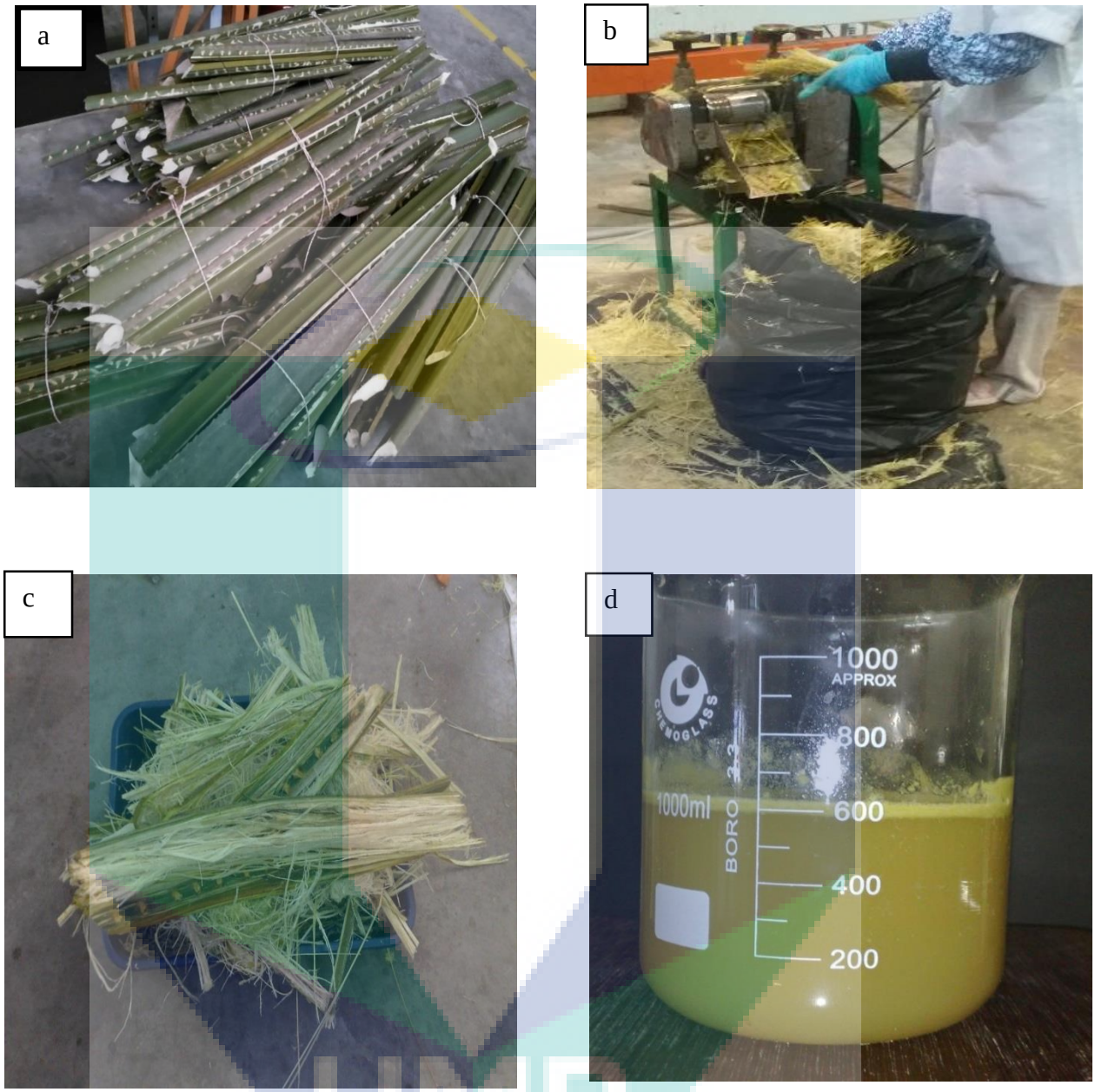
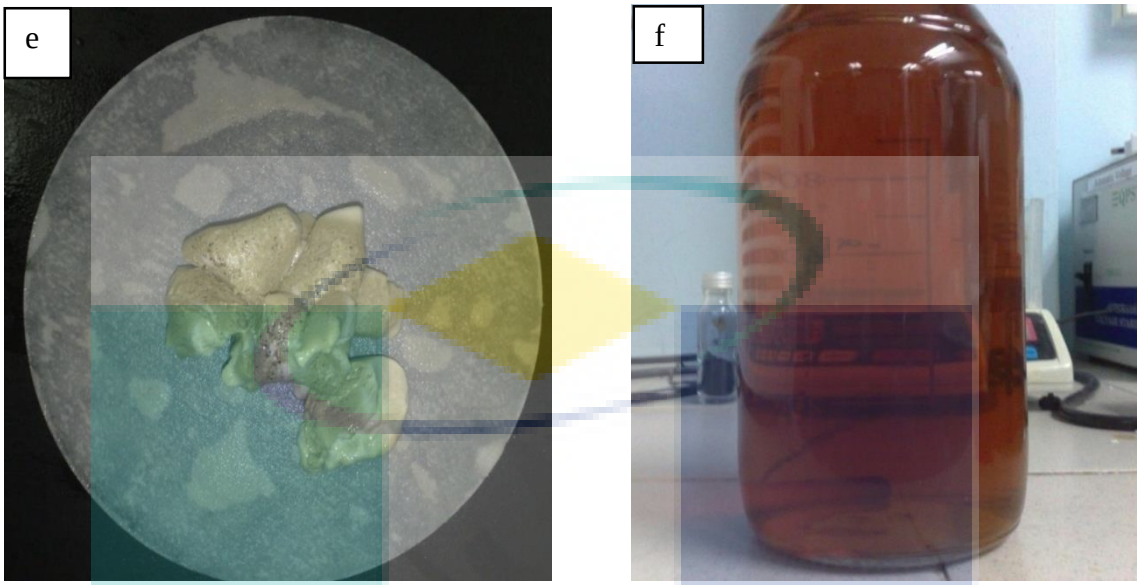


Figure 4. 1 (a) Freshly OPF without leaves, (b) Sugarcane pressing machine used in this study, (c) OPF after pressing, (d) Fresh OPF juice after pressing



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

4.1.2 Utilization of oil palm frond juice as a sole substrate

Based on the sugars content that showed in Section 4.1.1, it was suggested that OPF juice had potential to be used as fermentation substrate for the production of VOCs. However, there were some other factors that need to be considered before the OPF juice can be used as fermentation feedstock. Firstly, it was important to determine the presence of any inhibitory effect or impurities which may affect the cell growth and product formation. This can be proved through fermentation study. In addition, a good fermentation substrate should include some characteristics as follows; produce maximum yield of product or biomass per gram of substrate used, produce maximum concentration of product or biomass, permit the maximum rate of product formation, minimum yield of undesired product, cheap, consistent quality and should be available throughout the year.

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 can be considered as a potentially good fermentation substrate for the production of VOCs. However, further evaluation on potential of OPF juice as fermentation feedstock was yet to be clarified through fermentation study.

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

In order to study cell biomass and growth profile of *C. fimbriata*, the fermentation was carried out by using 100 mL of OPF juice supplemented with MSM in 250 mL Erlenmeyer flask and the initial pH medium was adjusted to 6.0 with (NaOH) 0.5 N before autoclaved 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 OPF juice that 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.

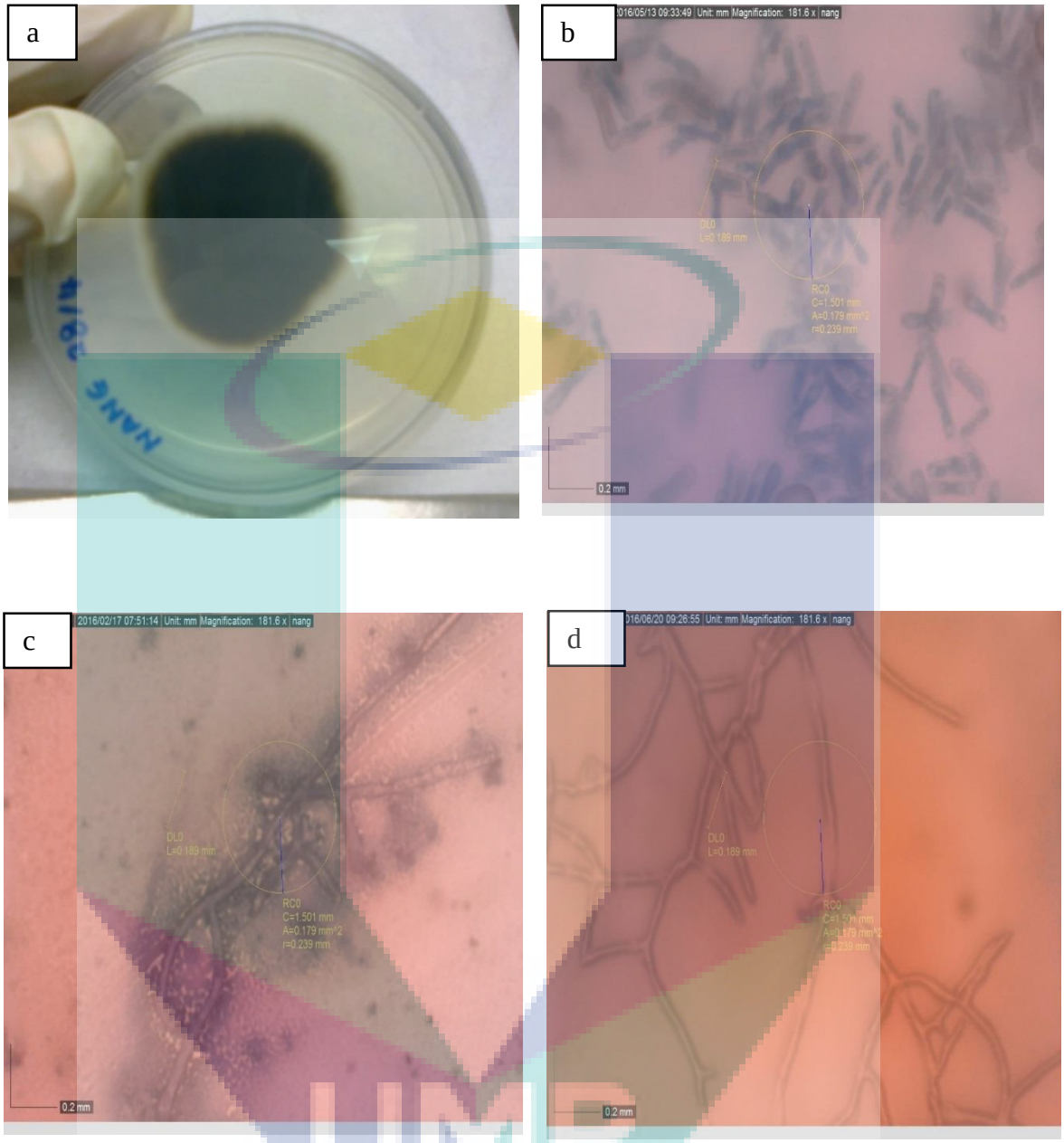


Figure 4. 2 (a) *C. fimbriata* after 7 days on PDA plate at incubation temperature of 30 °C, (b) *C. fimbriata* under microscope had cylindrical endoconidia (c) *C. fimbriata* had wide-mouth endoconidiophore with emerging doliiform endoconidium (d) *C. fimbriata* had flask-shape endoconidiophore producing cylindrical endoconidium

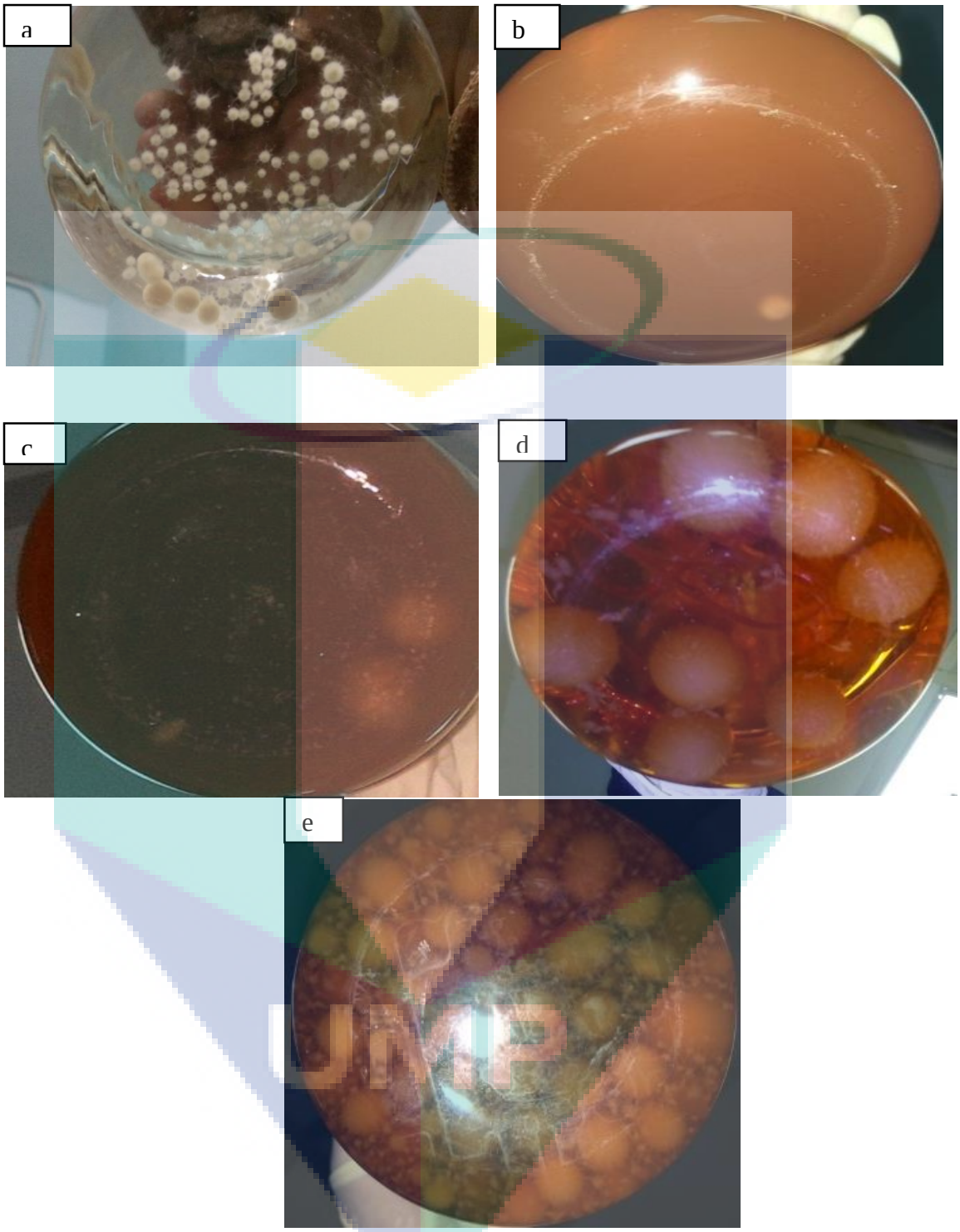


Figure 4. 3 (a) Seed culture of *C. fimbriata* in basal growth medium for the nine days on a rotary shaker at (150 rpm) in an aerobic condition, (b) The mycellium cell on the first day of fermentation, (c) The second day of fermentation, (d) third day of fermentation, (e) on the fourth day of fermentation.

In figure 4.3 (a), the seed culture was used as the inoculum in the fermentation of OPF juice. The seed culture was prepared at the temperature of 27 °C on a rotary shaker (150 rpm) and incubated for eight (8) days. The inoculum was then transferred to the fermentation medium which consisted of 30 g/L glucose in OPF juice supplemented with a MSM. 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 third days of fermentation, it had seven mycelium cells. On the fourth day until the end of fermentation, the fungus germinated and had grown into many myceliums of cells.

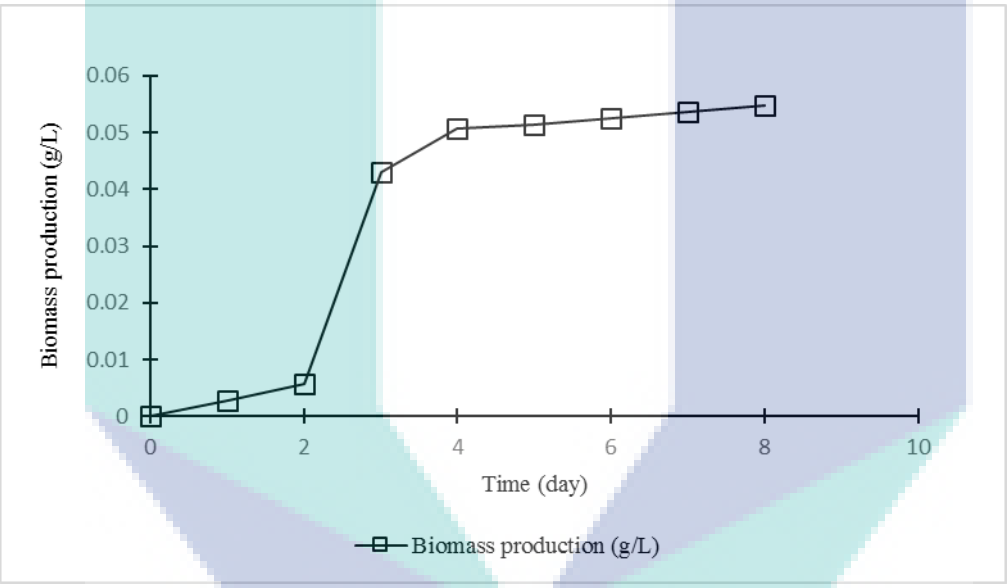


Figure 4. 4 Biomass production of *Ceratocystis fimbriata* in MSM supplemented with OPF juices as a sole substrate for 8 days of fermentation.

The results of the biomass produced by *Ceratocystis fimbriata* in MSM supplemented with OPF juice is shown in Figure 4.4. The biomass production was calculated from the first day until the eighth day of fermentation. As shown in Figure 4.4, it was apparent that the *Ceratocystis fimbriata* biomass has increased sharply from the second day to the fourth day and increased slowly thereafter. Based on the growth profile, the lag phase for *Ceratocystis 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 *Ceratocystis 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 *Ceratocystis fimbriata* decreased sharply from second day to the fourth day of fermentation period and decrease 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*.

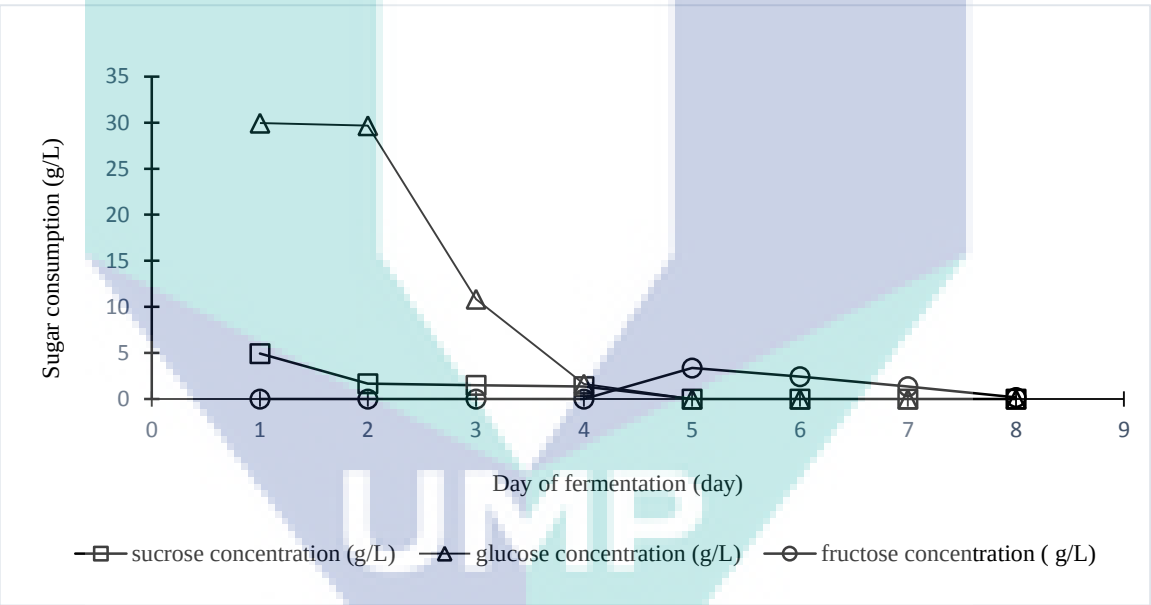


Figure 4. 5 Glucose consumption profile for eight day of fermentation period by *Ceratocystis fimbriata* in mineral salt medium supplemented with OPF juice.

4.1.4 VOCs production

From the fermentation of *Ceratocystis 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 was shown in Table 1. The volatile compounds identified

from fermentation of *Ceratocystis 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 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 does not play a predominant role in flavors but are known to contribute to the overall flavors quality and are precursors of fruit-like flavoring esters (Christen *et al.*, 1997). In microorganisms, all alcohol, except ethanol is formed by the reduction of α-keto acids which were derived from amino acids metabolism (F. W. 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. 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 correspond 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 day. With reference to the past study which had used coffee husk as a substrate, the major compound produced by *Ceratocystis fimbriata* were ethyl acetate, ethanol and acetaldehyde (Medeiros *et al.*, 2006). Others 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).

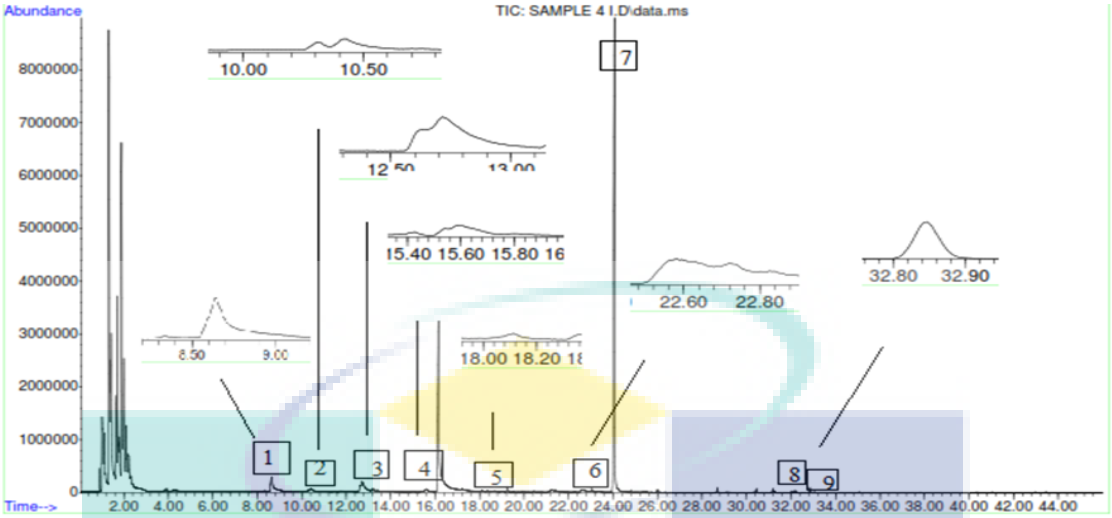


Figure 4. 6 (GC-MS) with (SPME) analysis produced 9 peaks on the fourth day of fermentation. Peak (1) retention time at 8.661, peak (2) retention time at 10.421, peak (3) retention time at 12.732 were the alcohol group; peak (6) retention time at 22.643 and peak (9) retention time at 32.858 were the 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 *Ceratocystis 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
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 *Ceratocystis fimbriata* using OPF juice as the sole carbon source for 8 days of fermentation (summarized from Table 4.2)

Chemical groups	Alcohols	Esters	Aldehydes	Phenols	Alkanes	Alkenes	Ketones	Others
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 compounds	11	7	6	10	3	2	2	4

4.1.5 Summary

Based on the results and discussion, it can be concluded that volatile organic compounds can be produced from the utilization of OPF juice as the carbon source by *Ceratocystis fimbriata*. With 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 *Ceratocystis 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 from the first day until the fifth day of the fermentation period. For future objective, further investigation on the parameters affecting methyl 3-(3,5-di-tert-butyl-4-hydroxyphenyl) propionate from OPF juice by *Ceratocystis fimbriata* were be look 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 (%)
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 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 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* cannot 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 comes 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, for 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 has 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)

$$Y = X_0 + X_1 - X_2 - X_3 - X_4 - X_1X_3 + X_2X_4 + X_3X_4 \quad \text{Equation 4. 1}$$

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

$$Y = 0.64750 + 0.04X_1 - 0.0145X_2 - 1.97500E\text{-}003X_3 - 0.017250X_4 - 2.37500E\text{-}004X_1X_3 + 4.25000E\text{-}004X_2X_4 + 6.50000E\text{-}005X_3X_4 \quad \text{Equation 4. 2}$$

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) contributes 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 has 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 resulting in insufficient oxygen in the culture medium affects the microbial growth, whereas higher agitation speeds sometimes also lowered the production of microorganism (Seth & 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 grows maximally over a certain range of the initial pH of the medium and will not grow at high and low extremes under

given conditions. Previous reports have shown that the *C. fimbriata* grow very well at pH 6 (Soares *et al.*, 2000).

The relative size of effects was visually demonstrated by Pareto Chart in Figure 4.7. 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 will result in a decrease in the response. For interactions, the positive effect was both factors were a chance to the same level (low or high), the response will increase. The negative effect was 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). The positive effects were colored in orange and the negative ones in blue in all the Pareto chart.

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.

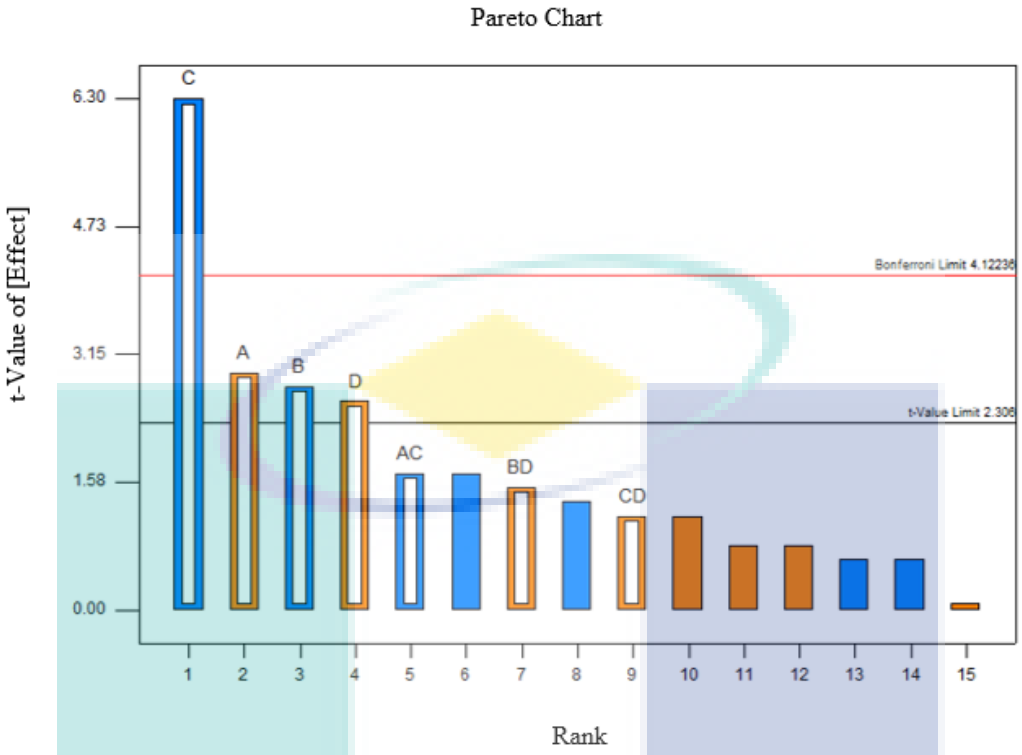


Figure 4. 7 Pareto chart of effects of methyl 3-(3,5-di-tert-butyl-4-hydroxyphenyl) propionate production.

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 is the correlation coefficient. The purpose of 2-level factorial design was to minimize the factor number. The design identifies 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				

Table 4. 7 Suggested best condition for factors in methyl 3-(3,5-di-tert-butyl-4-hydroxyphenyl) propionate production.

Factors	Best condition
X1-Initial pH medium	8
X2-Temperature	25 °C
X3-Agitation speed	100 rpm
X4-glucose in OPF juice	30 g/L

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 having a positive effect. 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 others two high values of interaction 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 can be ignored because they gave very low effect towards the fermentation process.

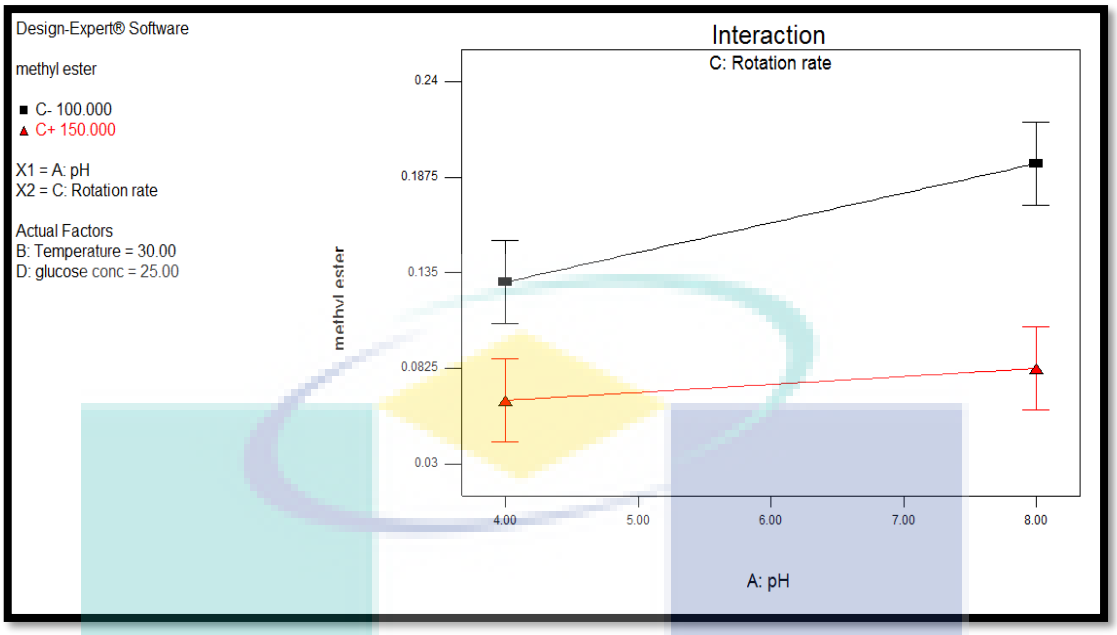


Figure 4. 8 The initial pH medium effect and agitation speed (rpm) on the methyl 3-(3,5-di-tert-butyl-4-hydroxyphenyl) propionate production.

According to the graph in Figure 4.8, it showed that the interaction between initial pH medium and agitation speed. As can 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.

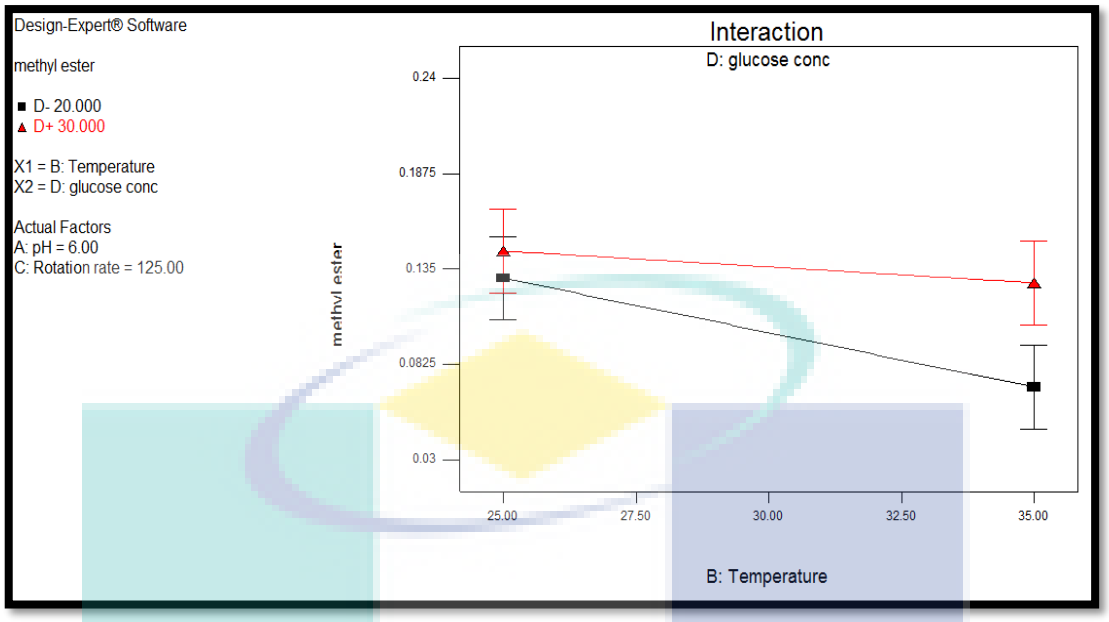


Figure 4. 9 The glucose concentration in OPF juice effect and temperature (°C) on the methyl 3-(3,5-di-tert-butyl-4-hydroxyphenyl) propionate production.

According to the graph in Figure 4.9, it showed the interaction between total glucose concentration in OPF juice and temperature. As can 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 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.

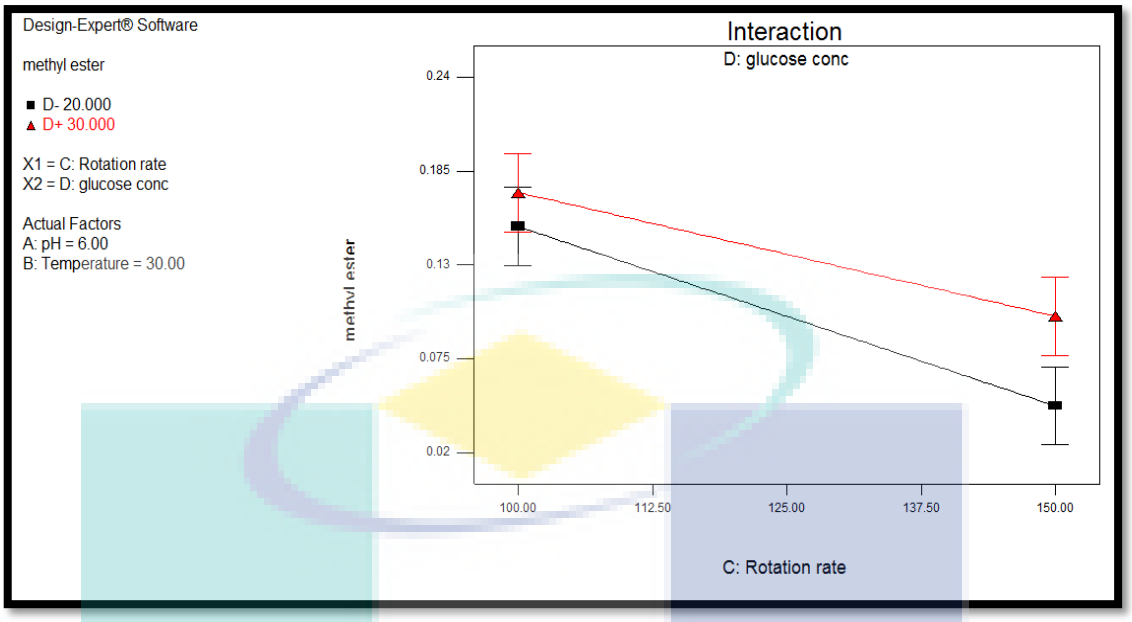


Figure 4. 10 The glucose concentration in OPF juice effect and agitation speed (rpm) on the methyl 3-(3,5-di-tert-butyl-4-hydroxyphenyl) propionate production.

According to the graph in Figure 4.10, it showed the interaction between total glucose concentration in OPF juice and agitation speed. As can 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.

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. 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

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 32.80 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 *Ceratocystis fimbriata* were to be look 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 center point condition of initial pH medium was 8, agitation speed was 100 rpm and incubation temperature was 25 °C. Another five center 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 center point also to demonstrated approximately high methyl 3-(3,5-di-tert-butyl-4-hydroxyphenyl) propionate production comparable to the maximum one (Kim & 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 Statistical Modelling and ANOVA

Table 4.10 was the summary of the ANOVA results. The precision or fit of the model can 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 indicates that only a 5 % chance of noise can 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 showed 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 significants 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 this factors could significantly impact 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).

The lack of fit can be tested when the experimental design performed with reliable repetitions at least in its center point (Bezerra *et al.*, 2008). Those repetitions point also can be used in determining the pure error. Besides that, well fitted model can 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 & 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 & 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 R^2 value is 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 4.11 and Figure 4.12. 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 4.12 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 can 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.*

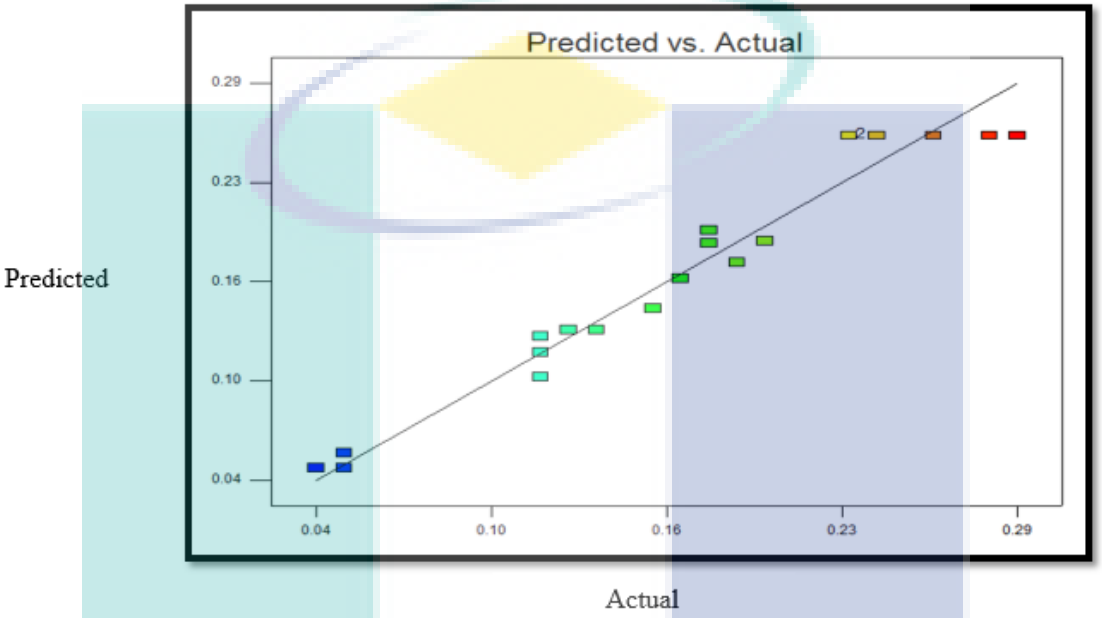


Figure 4. 11 Actual vs predicted response of methyl 3-(3,5-di-tert-butyl-4-hydroxyphenyl) propionate response.

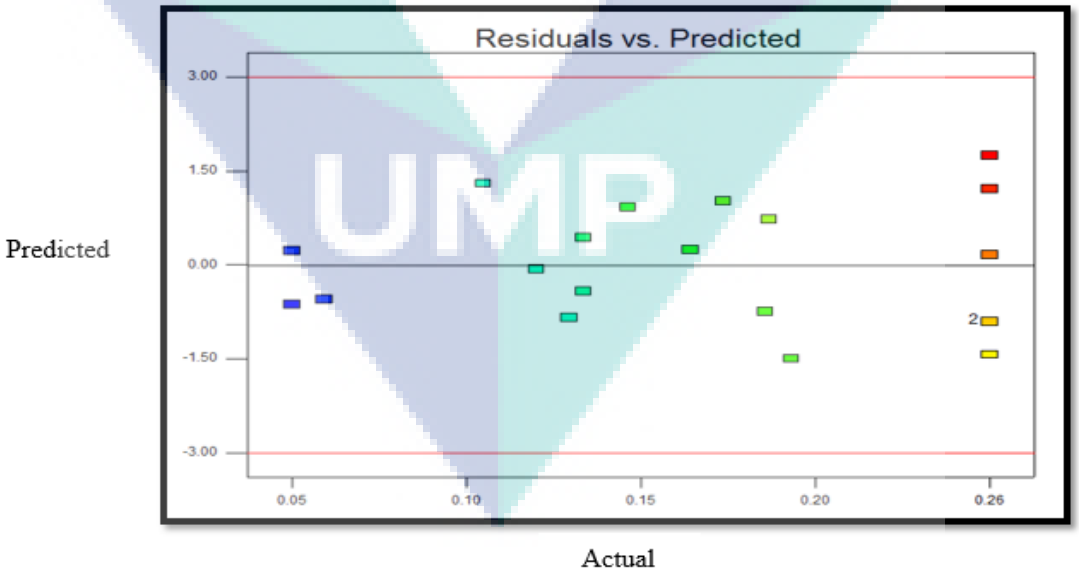


Figure 4. 12 Residual plot for methyl 3-(3,5-di-tert-butyl-4-hydroxyphenyl) propionate response

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)

$$\boldsymbol{Y = 0.26 + 0.022A + 0.023B + 0.022C - 0.014AB - 1.250E - 003AC - 8.750E - 003BC - 0.041A^2 - 0.026B^2 - 0.041C^2}$$ Equation 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 can visualize the effects of independent variables towards the response (Fang *et al.*, 2010). The evaluation of the interaction between various factors using RSM quantifies in terms of three-dimensional response surface and contour lines. Figure 4.13, 4.14 and 4.15 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 demonstrate the effects of two factors on the response at a time and assist in arbitration of degree of parametric on the desired responses. Three response surfaces were generated depending on three variables involved in the process.

Figure 4.13 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 center level of the fermentation. This figure clearly depicts 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 exhibits 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.

The three-dimensional response surface relationship between agitation speed and incubation temperature was illustrated in Figure 4.14. This figure clearly depicts 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.

Figure 4.15 display three-dimensional response surface interactions between initial pH medium and incubation temperature. The figure clearly depicts 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 exhibits 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.

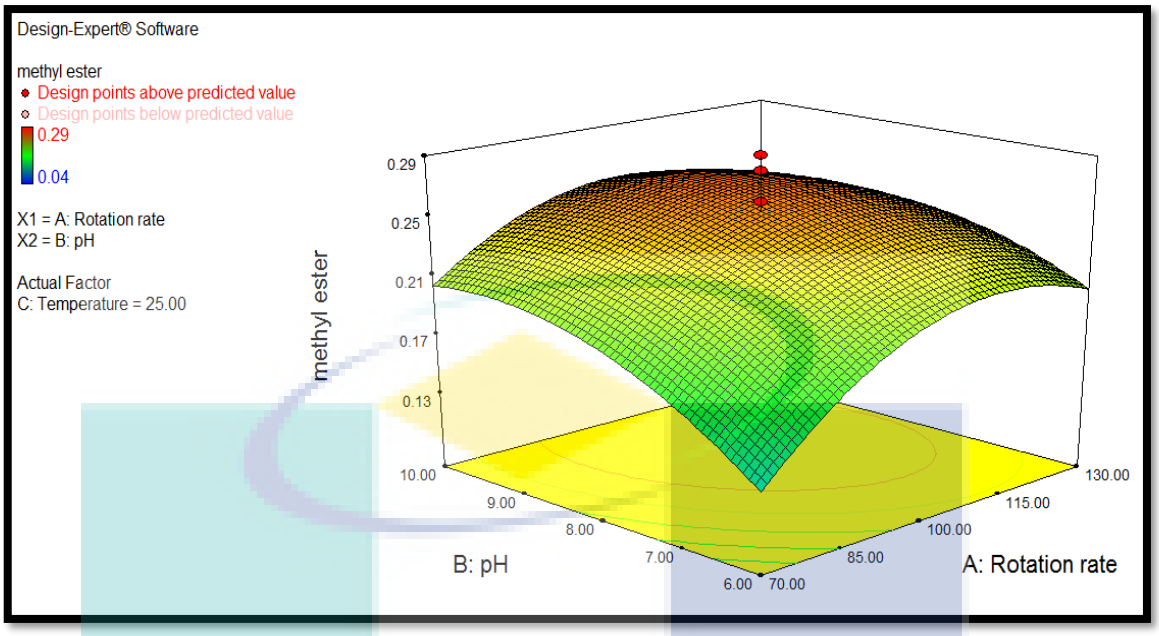


Figure 4. 13 Response surface and contour plot showing the effect of initial pH medium and agitation speed on methyl 3-(3,5-di-tert-butyl-4-hydroxyphenyl) propionate production.

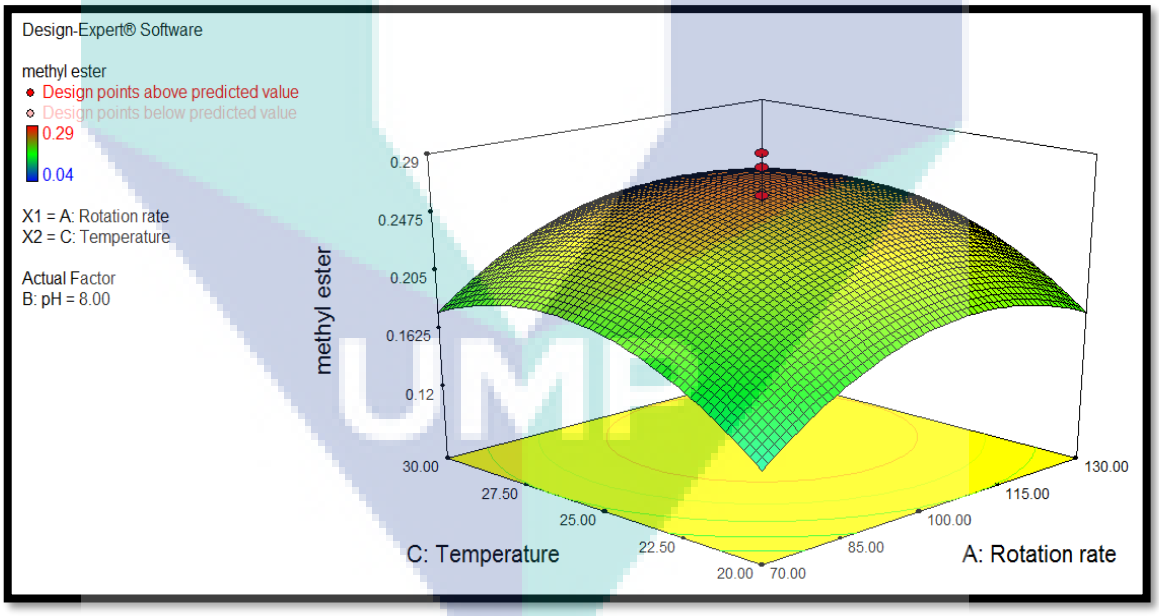


Figure 4. 14 Response surface and contour plot showing the effect of incubation temperature and agitation speed on methyl 3-(3,5-di-tert-butyl-4-hydroxyphenyl) propionate production.

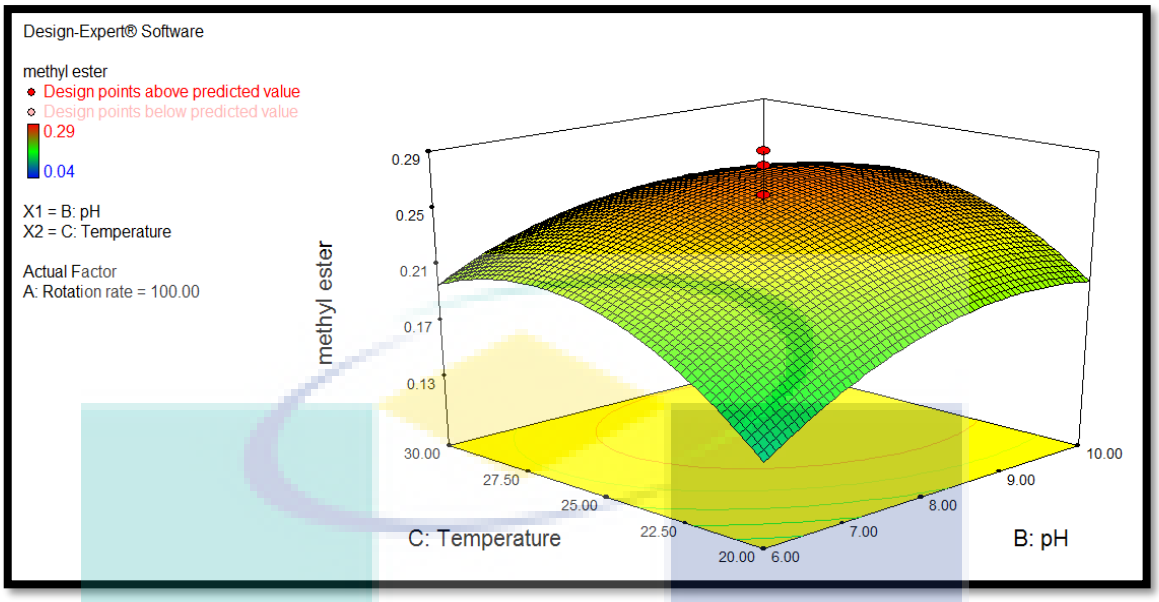


Figure 4. 15 Response surface and contour plot showing the effect of incubation temperature and initial pH medium on methyl 3-(3,5-di-tert-butyl-4-hydroxyphenyl) propionate production.

4.3.3 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 center 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. Optimum incubation temperature was also one of the pre-requisite for the growth and sporulation of the fungus. (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 findings of past studies by Sonyal, which fungus grew well in abundance in the incubation temperature range of 20 °C to 35˚C (Sonyal, 2010). Besides that, this result concurs with the findings of (Sastry *et al.*, 1989) which found high range of *C. paradoxa* growth between 12 °C to 34˚C. Similar results were obtained in the investigation of (Yadahalli, 2005). Study performed by (Darmasiwi & Ningsih, 2016), showed that the production of compounds could produce at incubation

temperature of 30 °C. The optimum incubation temperature was varied depending upon the strain microorganism and substrate were used.

The fungus generally grows maximally over a certain range of initial pH of the medium and will 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 & Raimbault, 1991; Christen *et al.*, 1994) revealed that the optimum initial pH medium was 6 for the production of VOCs. As can be seen in Figure 4.13 and Figure 4.15, the acidic medium at pH 6 showed lower production of VOCs compared to alkaline medium at pH 8. This phenomenon can 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 & 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 demand 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 & 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 can be observed in Figure 4.14, 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 from 100 rpm to 130 rpm. The optimum agitation speed was varied depending upon the strain of microorganism and substrate used.

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

Optimization may 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 reflects 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 %

4.3.5 Summary

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 exemplify 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 demonstrates 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 can be observed that methyl 3-(3,5-di-tert-butyl-4-hydroxyphenyl) propionate production increase with increasing of incubation temperature, agitation speed and initial pH medium and reach 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 0.25 % of chromatogram area.

CHAPTER 5

CONCLUSIONS AND RECOMMENDATIONS FOR FURTURE WORK

5.1 CONCLUSIONS

In summary, OPF juice can be utilized in the production of VOCs via biosynthesis using *C. fimbriata.* This study had shown the OPF that contain ± 53.82 g/L of total sugar and glucose was found as the major sugar component in OPF juice followed by sucrose. In addition, to determine the sugar content in OPF juice, HPLC analysis was used in this study. Meanwhile, to determine the relative peak area of chromatogram area of compounds that produce from fermentation, GC-MS SPME analysis was used. By using GC-MS SPME, several types of volatile compounds such as alcohol, phenol, esters, ketones and aldehyde can be potentially produced from OPF juice supplemented with MSM.

With 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 *Ceratocystis 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 it's production was consistent with glucose consumption from the first day until the fifth day of the fermentation period. It was produced at the retention time of 32.858 minutes and the relative peak area was 0.21 % of chromatogram area by using GC-MS SPME.

Besides, this research was done to screen the methyl 3-(3,5-di-tert-butyl-4-hydroxyphenyl) propionate production from OPF juice using *C. fimbriata*. Screening of four independent variables in methyl 3-(3,5-di-tert-butyl-4-hydroxyphenyl) propionate production was studied prior to the optimization methyl 3-(3,5-di-tert-butyl-4-hydroxyphenyl) propionate production. In order to achieve the maximum methyl 3-(3,5-di-tert-butyl-4-hydroxyphenyl) propionate production, a full factorial analysis 2^4 using

Design Expert version 7.1 (State-Ease, Inc., Minneapolis, MN) was employed to screen the best condition by varying the variables including total glucose concentration in OPF juice, incubation temperature, initial pH medium and agitation speed.

This study had found that the best conditions for methyl 3-(3,5-di-tert-butyl-4-hydroxyphenyl) propionate production obtained at 30 g/L of total glucose in OPF juice, initial pH medium was pH 8, incubation temperature was 25 °C and agitation speed was 100 rpm. The response was fitted with a multiple linear regression equation as shown in Equation (4.1). High correlation (R2 = 0.8960) between the experimental data and model data was obtained. The most contribution percentage in this study was agitation speed which was 51.61 %, initial pH medium was 11.15 %, incubation temperature was 9.84 % and total glucose in OPF juice was 8.61 % respectively.

From these factors, only three factors, which were incubation temperature, initial pH medium and agitation speed will be optimized in RSM by CCD. The contribution percentage of total glucose in OPF juice factor was not selected for next optimization process because it had the lowest percentage contribution compared to the other factors. The model obtained from full factorial was significant with p-value < 0.0022.

In this study, the CCD were used to optimize factors for methyl 3-(3,5-di-tert-butyl-4-hydroxyphenyl) propionate production from OPF juice using *C. fimbriata*. Optimization of three independent variables which were the incubation temperature, initial pH medium and agitation speed were employed using RSM. 20 experimental runs including 5 replicates of center point were designed by CCD. The optimal conditions obtained was pH 8 of initial pH medium, 25 °C of incubation temperature and 100 rpm of agitation speed produced relative peak area 0.25 % of chromatogram area which was close to the predicted value.

The model obtained from CCD was significant with p-value (<0.0001) and non-significant lack of fit. The CCD design exemplify a quadratic polynomial model with the coefficients of determines (R^2) for the response was 0.9598, implying a high correlation between the observed and predicted values. The 3D response surface plot that obtained from the model illustrates the presence of maximum point on the surface plot placed inside the experimental value.

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 VOCs production, but it also can reduce the environmental pollution problems due to its large accumulation in nature. 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 VOCs can reduce the dependence on food crops such as cassava, apple pomace, soybean and amaranth grain.

5.2 Recommendations

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.

REFERENCES

Abnisa, F., Arami-Niya, A., Daud, W. W., Sahu, J., & Noor, I. (2013). Utilization of oil palm tree residues to produce bio-oil and bio-char via pyrolysis. *Energy conversion and management, 76*, 1073-1082.

Agency, M. I. (2011). National Biomass Strategy 2020: New wealth creation for Malaysia's palm oil industry. Retrieved from https://innovation.my/

Alam, M. Z., Mamun, A. A., Qudsieh, I. Y., Muyibi, S. A., Salleh, H. M., & Omar, N. M. (2009). Solid state bioconversion of oil palm empty fruit bunches for cellulase enzyme production using a rotary drum bioreactor. *Biochemical Engineering Journal*, *46*(1), 61-64.

Alam, M. Z., Muyibi, S. A., Mansor, M. F., & Wahid, R. (2007). Activated carbons derived from oil palm empty-fruit bunches: Application to environmental problems. *Journal of Environmental Sciences, 19*(1), 103-108.

Albuquerque, P. M., Koch, F., Trossini, T. G., Esposito, E., & Ninow, J. L. (2006). Production of Rhizopus oligosporus Protein by Solid-State Fermentation of Apple Pomace. *Brazilian Archives of Biology and Technology*, *49*(I), 91.

Alfaro, M., Martinez, A., Ramirez, R., & Bressani, R. (1987). Yield and chemical composition of the vegetal parts of the amaranth (Amaranthus hypochondriacus, L.) at different physiological stages. *Archivos latinoamericanos de nutricion, 37*(1), 108-121.

Andersen, T. B., Cozzi, F., & Simonsen, H. T. (2015). Optimization of biochemical screening methods for volatile and unstable sesquiterpenoids using HS-SPME-GC-MS. *Chromatography, 2*(2), 277-292.

Anderson, M., Whitcomb, P., Kraber, S., & Adams, W. (2009). Handbook for experimenters. *Stat-Ease, Inc*.

Andrásofszky, E., Szöcs, Z., Fekete, S., & Jelenits, K. (1997). Evaluation of the nutritional value of the amaranth plant. I. Raw and heat-treated grain tested in experiments on growing rats. *Acta Veterinaria Hungarica*, *46*(1), 47-59.

Antony, J., & Roy, R. K. (1999). Improving the process quality using statistical design of experiments: a case study. *Quality Assurance: Good Practice, Regulation, and Law*, *6*(2), 87-95.

Ariffin, H., Abdullah, N., Umi Kalsom, M., Shirai, Y., & Hassan, M. (2006). Production and characterization of cellulase by Bacillus pumilus EB3. *Int. J. Eng. Technol, 3*(1), 47-53.

Asma IW, M. S., Zulkafli H, Othman S, Mori. (2010). *Malaysia oil palm biomass. Forest Research Institute Malaysia,*. Paper presented at the Regional workshop on UNEP/DTIE/IETC in collaboration with GEC,, Osaka Japan.

Bach, S. S., Bassard, J.-É., Andersen-Ranberg, J., Møldrup, M. E., Simonsen, H. T., & Hamberger, B. (2014). High-throughput testing of terpenoid biosynthesis candidate genes using transient expression in Nicotiana benthamiana. *Plant Isoprenoids: Methods and Protocols*, 245-255.

Baharuddin, A. S., Hock, L. S., Yusof, M., Rahman, N. A. A., Shah, U., Hassan, M. A., . . . Shirai, Y. (2010). Effects of palm oil mill effluent (POME) anaerobic sludge from 500 m 3 of closed anaerobic methane digested tank on pressed-shredded empty fruit bunch (EFB) composting process. *African Journal of Biotechnology, 9*(16), 2427-2436.

Baranyi, J., & Roberts, T. A. (1994). A dynamic approach to predicting bacterial growth in food. *International journal of food microbiology, 23*(3-4), 277-294.

Barka, N., Abdennouri, M., Boussaoud, A., Galadi, A., Baâlala, M., Bensitel, M., . . . Sadiq, M. (2014). Full factorial experimental design applied to oxalic acid photocatalytic degradation in TiO 2 aqueous suspension. *Arabian Journal of Chemistry, 7*(5), 752-757.

Baş, D., & Boyacı, İ. H. (2007). Modeling and optimization I: Usability of response surface methodology. *Journal of Food Engineering, 78*(3), 836-845.

Bauer, K., Garbe, D., & Surburg, H. (2008). *Common fragrance and flavor materials: preparation, properties and uses*: John Wiley & Sons.

Bengaly, K., Liang, J., Jelan, Z., Ho, Y., & Ong, H. (2010). Utilization of steam-processed oil palm (Elaeis guineensis) frond by ruminants in Malaysia: investigations for nitrogen supplementation. *African Journal of Agricultural Research, 5*(16), 2131-2136.

Berger, R., Neuhäuser, K., & Drawert, F. (1987). Biotechnological production of flavor compounds: III. High productivity fermentation of volatile flavors using a strain of Ischnoderma benzoinum. *Biotechnology and bioengineering, 30*(8), 987-990.

Berovič, M., & Ostroveršnik, H. (1997). Production of Aspergillus niger pectolytic enzymes by solid state bioprocessing of apple pomace. *Journal of Biotechnology, 53*(1), 47-53.

Bezerra, M. A., Santelli, R. E., Oliveira, E. P., Villar, L. S., & Escaleira, L. A. (2008). Response surface methodology (RSM) as a tool for optimization in analytical chemistry. *Talanta, 76*(5), 965-977.

Bhalla, T., & Joshi, M. (1994). Protein enrichment of apple pomace by co-culture of cellulolytic moulds and yeasts. *World journal of microbiology and biotechnology, 10*(1), 116-117.

Bigelis, R. (1992). Flavor metabolites and enzymes from filamentous fungi. *Food technology (USA).*

Bluemke, W., & Schrader, J. (2001). Integrated bioprocess for enhanced production of natural flavors and fragrances by Ceratocystis moniliformis. *Biomolecular Engineering, 17*(4), 137-142.

Bramorski, A., Soccol, C. R., Christen, P., & Revah, S. (1998). Fruity aroma production by Ceratocystis fimbriata in solid cultures from agro-industrial wastes. *Revista de Microbiologia, 29*(3).

Braunegg, G., Lefebvre, G., & Genser, K. F. (1998). Polyhydroxyalkanoates, biopolyesters from renewable resources: physiological and engineering aspects. *Journal of Biotechnology, 65*(2), 127-161.

Breheret, S., Talou, T., Rapior, S., & Bessière, J.-M. (1997). Monoterpenes in the aromas of fresh wild mushrooms (Basidiomycetes). *Journal of agricultural and food chemistry, 45*(3), 831-836.

Brigido, B. M. (2000). Produção de compostos volateis de aroma por novas linhagens de Neurospora sp.

Brown, A., & Hammond, J. (2003). Flavour control in small-scale beer fermentations. *Food and bioproducts processing, 81*(1), 40-49.

Burdock, G. A. (2016). *Fenaroli's handbook of flavor ingredients*: CRC press.

Bushman, Z. (2015). Ester Production in Fermentation. Retrieved from https://www.gastrograph.com/blogs/gastronexus/ester-production-in-fermentation.html

Campillo, N., Vinas, P., López-Garcıa, I., Aguinaga, N., & Hernández-Córdoba, M. (2004). Purge-and-trap capillary gas chromatography with atomic emission

detection for volatile halogenated organic compounds determination in waters and beverages. *Journal of Chromatography A, 1035*(1), 1-8.

Carvalheiro, F., Garrote, G., Parajó, J. C., Pereira, H., & Gírio, F. M. (2005). Kinetic Modeling of Breweryapos; s Spent Grain Autohydrolysis. *Biotechnology progress, 21*(1), 233-243.

Che Maail, C. M. H., Ariffin, H., Hassan, M. A., Shah, U. K. M., & Shirai, Y. (2014). Oil palm frond juice as future fermentation substrate: a feasibility study. *BioMed Research International, 2014*.

Cheetham, P. S. (1997). Combining the technical push and the business pull for natural flavours *Biotechnology of aroma compounds* (pp. 1-49): Springer.

Chiron, N., & Michelot, D. (2005). Odeurs des champignons: chimie et rôle dans les interactions biotiques-une revue. *Cryptogamie. Mycologie, 26*(4), 299-364.

Cho, I., Namgung, H.-J., Choi, H.-K., & Kim, Y.-S. (2008). Volatiles and key odorants in the pileus and stipe of pine-mushroom (Tricholoma matsutake Sing.). *Food Chemistry, 106*(1), 71-76.

Choi, M.-K., Sohn, K.-H., & Jeon, H.-J. (1997). Changes in odor characteristics of doenjang with different preparing methods and ripening periods. *Journal of the Korean Society of Food Culture, 12*(3), 265-274.

Christen, P., Meza, J., & Revah, S. (1997). Fruity aroma production in solid state fermentation by Ceratocystis fimbriata: influence of the substrate type and the presence of precursors. *Mycological Research, 101*(8), 911-919.

Christen, P., & Raimbault, M. (1991). Optimization of culture medium for aroma production byCeratocystis fimbriata. *Biotechnology letters, 13*(7), 521-526.

Christen, P., Villegas, E., & Revah, S. (1994). Growth and aroma production byCeratocystis fimbriata in various fermentation media. *Biotechnology letters, 16*(11), 1183-1188.

Chung, H. Y. (1999). Volatile components in fermented soybean (Glycine max) curds. *Journal of agricultural and food chemistry, 47*(7), 2690-2696.

Chung, H. Y., Fung, P. K., & Kim, J.-S. (2005). Aroma impact components in commercial plain sufu. *Journal of agricultural and food chemistry, 53*(5), 1684-1691.

Ciganek, M., Pisarikova, B., & Zraly, Z. (2007). Determination of volatile organic compounds in the crude and heat treated amaranth samples. *VETERINARNI MEDICINA-PRAHA-, 52*(3), 111.

Claeson, A. S., & Sunesson, A. L. (2005). Identification using versatile sampling and analytical methods of volatile compounds from Streptomyces albidoflavus grown on four humid building materials and one synthetic medium. *Indoor Air, 15*(s9), 41-47.

Clark, D. S., & Blanch, H. W. (1997). *Biochemical engineering*: CRC Press.

Comi, G., Romano, P., Cocolin, L., & Fiore, C. (2001). Characterization of Kloeckera apiculata strains from the Friuli regionin Northern Italy. *World journal of microbiology and biotechnology, 17*(4), 391-394.

Correa, A. D., Jokl, L., & Carlsson, R. (1986). Chemical constituents, in vitro protein digestibility, and presence of antinutritional substances in amaranth grains. *Archivos latinoamericanos de nutricion, 36*(2), 319-326.

Dahlan, I. (2000). Oil palm frond, a feed for herbivores. *Asian Australasian Journal of Animal Sciences, 13*, 300-303.

Dalsenter, F. D. H., Viccini, G., Barga, M. C., Mitchell, D. A., & Krieger, N. (2005). A mathematical model describing the effect of temperature variations on the kinetics of microbial growth in solid-state culture. *Process Biochemistry, 40*(2), 801-807.

Damasceno, S., Cereda, M., Pastore, G., & Oliveira, J. (2003). Production of volatile compounds by Geotrichum fragrans using cassava wastewater as substrate. *Process Biochemistry, 39*(4), 411-414.

Darah, I., & Ibrahim, C. (1996). Effect of agitation on production of lignin-degrading enzymes by Phanerochaete chrysosporium grown in shake-flask cultures. *Asia-Pacific Journal of Molecular Biology and Biotechnology, 4*(3), 174-182.

Darah, I., Sumathi, G., Jain, K., & Lim, S. (2011). Influence of agitation speed on tannase production and morphology of Aspergillus niger FETL FT3 in submerged fermentation. *Applied biochemistry and biotechnology, 165*(7-8), 1682-1690.

Darmasiwi, S., & Ningsih, O. C. (2016). Aromatic Compounds Production by Fungal Solid State Fermentation in Pandanus tectorius Fruits. *Biosciences Biotechnology Research Asia, 13*(1), 75-78.

Devrajan, A., Joshi, V. K., Gupta, K., Sheikher, C., & Lal, B. B. (2004). Evaluation of apple pomace based reconstituted feed in rats after solid state fermentation and ethanol recovery. *Brazilian Archives of Biology and Technology, 47*(1), 93-106.

Dewulf, J., Van Langenhove, H., & Wittmann, G. (2002). Analysis of volatile organic compounds using gas chromatography. *TrAC Trends in Analytical Chemistry, 21*(9), 637-646.

Diaconescu, R. M., Grigoriu, A.-M., Luca, C., & Georgescu, P. (2011). Study on the response surface modelling by central composite design and optimization of paper nanocoating. *Revista de Chimie, Bucureşti, 62*(5), 522.

Dragone, G., Silva, D. P., & e Silva, J. B. d. A. (2004). Factors influencing ethanol production rates at high-gravity brewing. *LWT-Food Science and Technology, 37*(7), 797-802.

Eiceman, G. A., Gardea-Torresdey, J., Overton, E., Carney, K., & Dorman, F. (2004). Gas chromatography. *Analytical chemistry, 76*(12), 3387-3394.

Engelbrecht, C. J. B., & Harrington, T. C. (2005). Intersterility, morphology and taxonomy of Ceratocystis fimbriata on sweet potato, cacao and sycamore. *Mycologia, 97*(1), 57-69.

Fang, H., Zhao, C., & Song, X.-Y. (2010). Optimization of enzymatic hydrolysis of steam-exploded corn stover by two approaches: Response surface methodology or using cellulase from mixed cultures of Trichoderma reesei RUT-C30 and Aspergillus niger NL02. *Bioresource technology, 101*(11), 4111-4119.

Farbood, M. I. (1991). Micro-organisms as a novel source of flavour compounds: Portland Press Limited.

Favela-Torres, E., Volke-Sepúlveda, T., & Viniegra-González, G. (2006). Production of Hydrolytic Depolymerising Pectinases. *Food Technology & Biotechnology, 44*(2).

Fernández, M., Ubeda, J., & Briones, A. (2000). Typing of non-Saccharomyces yeasts with enzymatic activities of interest in wine-making. *International journal of food microbiology, 59*(1), 29-36.

Foo, L. Y., & Lu, Y. (1999). Isolation and identification of procyanidins in apple pomace. *Food Chemistry, 64*(4), 511-518.

Fraatz, M. A., & Zorn, H. (2011). Fungal flavours *Industrial Applications* (pp. 249-268): Springer.

Fujita, E. M., Harshfield, G., & Sheetz, L. (2003). Performance audits and laboratory comparisons for SCOS97-NARSTO measurements of speciated volatile organic compounds. *Atmospheric environment, 37*, 135-147.

Fung, D.-R., Chuang, J.-J., Huang, Z.-J., & Chen, C.-Y. (2014). Method for making hindered phenolic antioxidant: Google Patents.

Gatto, V. J., Elnagar, H. Y., Cheng, C. H., & Adams, J. R. (2011). Preparation of sterically hindered hydroxyphenylcarboxylic acid esters: Google Patents.

Goh, C. S., Lee, K. T., & Bhatia, S. (2010). Hot compressed water pretreatment of oil palm fronds to enhance glucose recovery for production of second generation bio-ethanol. *Bioresource technology, 101*(19), 7362-7367.

Goh, C. S., Tan, H. T., Lee, K. T., & Mohamed, A. R. (2010). Optimizing ethanolic hot compressed water (EHCW) cooking as a pretreatment to glucose recovery for the production of fuel ethanol from oil palm frond (OPF). *Fuel Processing Technology, 91*(9), 1146-1151.

Górecki, T., Yu, X., & Pawliszyn, J. (1999). Theory of analyte extraction by selected porous polymer SPME fibres. *Analyst, 124*(5), 643-649.

Grigelmo-Miguel, N., & Martín-Belloso, O. (1999). Comparison of dietary fibre from by-products of processing fruits and greens and from cereals. *LWT-Food Science and Technology, 32*(8), 503-508.

Hari Krishna, S., & Karanth, N. (2002). Response Surface Modeling of Lipase-Catalyzed Isoamyl Propionate Synthesis. *Journal of food science, 67*(1), 32-36.

Hassan, Ishida, M., Shukri, I. M., & Tajuddin, Z. A. (1996). Oil-palm fronds as a roughage feed source for ruminants in Malaysia. *Extension bulletin. Food and Fertilizer Technology Center for the Asian and Pacific Region*, 1-8.

Hassan, M. A., Shirai, Y., Umeki, H., Yamazumi, H., Jin, S., Yamamoto, S., . . . Hashimoto, K. (1997). Acetic Acid Separation from Anaerobically Treated Palm Oil Mill Effluent by Ion Exchange Resins for the Production of Polyhydroxyaikanoate by Alcaligenes eutrophus. *Bioscience, biotechnology, and biochemistry, 61*(9), 1465-1468.

Hellen, H., Hakola, H., Pirjola, L., Laurila, T., & Pystynen, K.-H. (2006). Ambient air concentrations, source profiles, and source apportionment of 71 different C2− C10 volatile organic compounds in urban and residential areas of Finland. *Environmental science & technology, 40*(1), 103-108.

Hettenhaus, J. R. (1998). *Ethanol fermentation strains: present and future requirements for biomass to ethanol commercialization*. Retrieved from

Hunt, J. (1956). Taxonomy of the genus Ceratocystis. *Lloydia, 19*, 1-59.

Hussin, M. H., Rahim, A. A., Ibrahim, M. N. M., & Brosse, N. (2013). Physicochemical characterization of alkaline and ethanol organosolv lignins from oil palm (Elaeis guineensis) fronds as phenol substitutes for green material applications. *Industrial crops and products, 49*, 23-32.

Imeri, A., Flores, R., Elias, L., & Bressani, R. (1987). Effect of processing and amino acids supplementation on the protein quality of amaranth (Amaranthus caudatus). *Archivos latinoamericanos de nutricion, 37*(1), 161-173.

Jafari, A., Sarrafzadeh, M., Alemzadeh, I., & Vosoughi, M. (2007). Effect of stirrer speed and aeration rate on the production of glucose oxidase by Aspergillus niger. *Journal of Biological Sciences, 7*(2), 270-275.

Johnson, J. A., Harrington, T. C., & Engelbrecht, C. (2005). Phylogeny and taxonomy of the North American clade of the Ceratocystis fimbriata complex. *Mycologia, 97*(5), 1067-1092.

K. Evans, A. M., J. Hamilton, M. Zhang. (2002). *Effect of corn stover hydrolysate and temperature on fermentation performance of selected yeast strains*. Paper presented at the Proceedings of the 24th Biotechnology for Fuels and Chemicals Symposium,, Gatlinburg, TN, USA.

Kalaichelvan, P. (2012). Production and optimization of Pectinase from Bacillus sp. MFW7 using cassava waste. *Asian Journal of Plant Science and Research, 2*(3), 369-375.

Kataoka, H., & Saito, K. (2011). Recent advances in SPME techniques in biomedical analysis. *Journal of pharmaceutical and biomedical analysis, 54*(5), 926-950.

KAWAMOTO, H., MOHAMED, W. Z., SHUKUR, N. I. M., ALI, M. S. M., ISMAIL, Y., & OSHIO, S. (2001). Palatability, digestibility and voluntary intake of processed oil palm fronds in cattle. *Japan Agricultural Research Quarterly: JARQ, 35*(3), 195-200.

Khanna, S., & Srivastava, A. K. (2005). Recent advances in microbial polyhydroxyalkanoates. *Process Biochemistry, 40*(2), 607-619.

Kim, I., & Han, J.-I. (2012). Optimization of alkaline pretreatment conditions for enhancing glucose yield of rice straw by response surface methodology. *biomass and bioenergy, 46*, 210-217.

Kłosowski, G., & Czupryński, B. (2006). Kinetics of acetals and esters formation during alcoholic fermentation of various starchy raw materials with application of yeasts Saccharomyces cerevisiae. *Journal of Food Engineering, 72*(3), 242-246.

Kobayashi, M., Shimizu, H., & Shioya, S. (2008). Beer volatile compounds and their application to low-malt beer fermentation. *Journal of bioscience and bioengineering, 106*(4), 317-323.

Koller, M., Atlić, A., Dias, M., Reiterer, A., & Braunegg, G. (2010). Microbial PHA production from waste raw materials *Plastics from bacteria* (pp. 85-119): Springer.

Korpi, A., Järnberg, J., & Pasanen, A.-L. (2009). Microbial volatile organic compounds. *Critical reviews in toxicology*, *39*(2), 139-193.

Kot-Wasik, A., Dębska, J., & Namieśnik, J. (2004). Monitoring of organic pollutants in coastal waters of the Gulf of Gdańsk, Southern Baltic. *Marine pollution bulletin, 49*(3), 264-276.

Ku, K.-L., Chen, T.-P., & Chiou, R.-Y. (2000). Apparatus used for small-scale volatile extraction from ethanol-supplemented low-salt miso and GC− MS characterization of the extracted flavors. *Journal of agricultural and food chemistry, 48*(8), 3507-3511.

Kunkee, R., & Amerine, M. (1970). Yeasts in wine-making. *The yeasts, 3*, 5-72.

Lanza, E., Ko, K. H., & Palmer, J. K. (1976). Aroma production by cultures of Ceratocystis moniliformis. *Journal of agricultural and food chemistry*, *24*(6), 1247-1250.

Latrasse, A., Dameron, P., Hassani, H., & Staron, T. (1987). Formation of a fruity aroma by Geotrichum candidum. *Sciences des Aliments, 7*, 637-645.

Lazic, Z. R. (2006). *Design of experiments in chemical engineering: a practical guide*: John Wiley & Sons.

Lee, W.-H., Azizan, M. N., & Sudesh, K. (2004). Effects of culture conditions on the composition of poly (3-hydroxybutyrate-co-4-hydroxybutyrate) synthesized by Comamonas acidovorans. *Polymer Degradation and Stability, 84*(1), 129-134.

Leejeerajumnean, A., Duckham, S. C., Owens, J. D., & Ames, J. M. (2001). Volatile compounds in bacillus-fermented soybeans. *Journal of the Science of Food and Agriculture, 81*(5), 525-529.

Li, X., Wang, Z.-G., Chen, H.-H., & Liu, S.-G. (2014). The antioxidant methyl 3-(3, 5-di-tert-butyl-4-hydroxyphenyl) propionate. *Acta Crystallographica Section C: Structural Chemistry, 70*(11), 1050-1053.

Lin, Q., Li, H., Ren, J., Deng, A., Li, W., Liu, C., & Sun, R. (2017). Production of xylooligosaccharides by microwave-induced, organic acid-catalyzed hydrolysis of different xylan-type hemicelluloses: Optimization by response surface methodology. *Carbohydrate polymers, 157*, 214-225.

Lomascolo, A., Stentelaire, C., Asther, M., & Lesage-Meessen, L. (1999). Basidiomycetes as new biotechnological tools to generate natural aromatic flavours for the food industry. *TRENDS in Biotechnology, 17*(7), 282-289.

Loo, C.-Y., & Sudesh, K. (2007). Biosynthesis and native granule characteristics of poly (3-hydroxybutyrate-co-3-hydroxyvalerate) in Delftia acidovorans. *International journal of biological macromolecules, 40*(5), 466-471.

Lu, Y., & Foo, L. Y. (2000). Antioxidant and radical scavenging activities of polyphenols from apple pomace. *Food Chemistry, 68*(1), 81-85.

Mahlia, T., Abdulmuin, M., Alamsyah, T., & Mukhlishien, D. (2001). An alternative energy source from palm wastes industry for Malaysia and Indonesia. *Energy conversion and management, 42*(18), 2109-2118.

Mangani, F., Maione, M., & Palma, P. (2003). 4 GC-MS Analysis of Halocarbons in the Environment. *Advances in chromatography, 42*, 139-240.

Martendal, E., Budziak, D., & Carasek, E. (2007). Application of fractional factorial experimental and Box-Behnken designs for optimization of single-drop microextraction of 2, 4, 6-trichloroanisole and 2, 4, 6-tribromoanisole from wine samples. *Journal of Chromatography A, 1148*(2), 131-136.

Martin, A. M., & Bailey, V. I. (1985). Growth of Agaricus campestris NRRL 2334 in the form of pellets. *Applied and Environmental Microbiology, 49*(6), 1502-1506.

Mason, R. L., Gunst, R. F., & Hess, J. L. (2003). *Statistical design and analysis of experiments: with applications to engineering and science* (Vol. 474): John Wiley & Sons.

Masoodi, F., Sharma, B., & Chauhan, G. (2002). Use of apple pomace as a source of dietary fiber in cakes. *Plant Foods for Human Nutrition (Formerly Qualitas Plantarum), 57*(2), 121-128.

Matich, A. J., Rowan, D. D., & Guenther, C. (2008). Deceit and deception in volatile analysis. *Chemistry in New Zealand, 73*, 88-91.

McMeekin, T., Olley, J., Ratkowsky, D., & Ross, T. (2002). Predictive microbiology: towards the interface and beyond. *International journal of food microbiology, 73*(2), 395-407.

Medeiros, Christen, P., Roussos, S., Gern, J. C., & Soccol, C. R. (2003). Coffee residues as substrates for aroma production by Ceratocystis fimbriata in solid state fermentation. *Brazilian Journal of Microbiology, 34*(3), 245-248.

Medeiros, Pandey, A., Christen, P., Fontoura, P. S., de Freitas, R. J., & Soccol, C. R. (2001). Aroma compounds produced by Kluyveromyces marxianus in solid state fermentation on a packed bed column bioreactor. *World journal of microbiology and biotechnology*, *17*(8), 767-771.

Medeiros, Pandey, A., Freitas, R. J., Christen, P., & Soccol, C. R. (2000). Optimization of the production of aroma compounds by Kluyveromycesmarxianus in solid-state fermentation using factorial design and response surface methodology. *Biochemical Engineering Journal*, *6*(1), 33-39.

Medeiros, Pandey, A., Vandenberghe, L. P., Pastore, G. M., & Soccol, C. R. (2006). Production and recovery of aroma compounds produced by solid-state fermentation using different adsorbents. *Food Technology and Biotechnology*, *44*(1), 47-51.

Mee, R. (2009). *A comprehensive guide to factorial two-level experimentation*: Springer Science & Business Media.

Merfort, I. (2002). Review of the analytical techniques for sesquiterpenes and sesquiterpene lactones. *Journal of Chromatography A*, *967*(1), 115-130.

Messens, W., Verluyten, J., Leroy, F., & De Vuyst, L. (2003). Modelling growth and bacteriocin production by Lactobacillus curvatus LTH 1174 in response to temperature and pH values used for European sausage fermentation processes. *International journal of food microbiology*, *81*(1), 41-52.

Montgomery, D. C. (2017). *Design and analysis of experiments*: John Wiley & Sons.

Mori, Y., Kiuchi, K., & Tabei, H. (1983). Flavor components of miso: basic fraction. *Agricultural and biological chemistry*, *47*(7), 1487-1492.

Morimoto, M., Atsuko, M., Atif, A., Ngan, M., Fakhru'l-Razi, A., Iyuke, S., & Bakir, A. (2004). Biological production of hydrogen from glucose by natural anaerobic microflora. *International Journal of Hydrogen Energy*, *29*(7), 709-713.

MPOB. (2010). Foreword from the Chairman of the Malaysian Palm Oil Board. Retrieved from www.mpob.gov.my

MPOB. (2011a). Annual and Forecast of Crude Palm Oil Production (Tonnes) 2010 & 2011. Retrieved from econ.mpob.gov.my/economy/EID_web.htm

MPOB. (2011b). Oil Palm Planted Areas as at September 2011. Retrieved from econ.mpob.gov.my/economy/area/Area_category.pdf

Mumtaz, T., Abd-Aziz, S., Rahman, N., Yee, P. L., Shirai, Y., & Hassan, M. (2008). Pilot-scale recovery of low molecular weight organic acids from anaerobically

treated palm oil mill effluent (POME) with energy integrated system. *African Journal of Biotechnology*, *7*(21).

Mumtaz, T., Yahaya, N. A., Abd-Aziz, S., Yee, P. L., Shirai, Y., & Hassan, M. A. (2010). Turning waste to wealth-biodegradable plastics polyhydroxyalkanoates from palm oil mill effluent–a Malaysian perspective. *Journal of Cleaner Production, 18*(14), 1393-1402.

Nasrah, N. S. M., Zahari, M. A. K. M., Masngut, N., & Ariffin, H. (2017). *Statistical Optimization for Biobutanol Production by Clostridium acetobutylicum ATCC 824 from Oil Palm Frond (OPF) Juice Using Response Surface Methodology*. Paper presented at the MATEC Web of Conferences.

Nath, A., & Chattopadhyay, P. (2007). Optimization of oven toasting for improving crispness and other quality attributes of ready to eat potato-soy snack using response surface methodology. *Journal of Food Engineering, 80*(4), 1282-1292.

Ngadi, M., & Correia, L. (1992a). Kinetics of solid-state ethanol fermentation from apple pomace. *Journal of Food Engineering, 17*(2), 97-116.

Ngadi, M., & Correia, L. (1992b). Solid state ethanol fermentation of apple pomace as affected by moisture and bioreactor mixing speed. *Journal of food science, 57*(3), 667-670.

Niinemets, Ü., Loreto, F., & Reichstein, M. (2004). Physiological and physicochemical controls on foliar volatile organic compound emissions. *Trends in plant science, 9*(4), 180-186.

Oliveira, L., Guimarães, L., Ferreira, M., Nunes, A., Pimenta, L., & Alfenas, A. (2015). Aggressiveness, cultural characteristics and genetic variation of Ceratocystis fimbriata on Eucalyptus spp. *Forest Pathology, 45*(6), 505-514.

Ortíz-Castro, R., Contreras-Cornejo, H. A., Macías-Rodríguez, L., & López-Bucio, J. (2009). The role of microbial signals in plant growth and development. *Plant signaling & behavior, 4*(8), 701-712.

Paganini, C., Nogueira, A., Silva, N. C., & Wosiacki, G. (2005). Utilization of apple pomace for ethanol production and food fiber obtainment. *Ciência e Agrotecnologia, 29*(6), 1231-1238.

Pagans, E., Font, X., & Sánchez, A. (2006). Emission of volatile organic compounds from composting of different solid wastes: abatement by biofiltration. *Journal of hazardous materials, 131*(1), 179-186.

Palmqvist, E., & Hahn-Hägerdal, B. (2000). Fermentation of lignocellulosic hydrolysates. II: inhibitors and mechanisms of inhibition. *Bioresource technology*, *74*(1), 25-33.

Pandey, A., Soccol, C. R., Nigam, P., Soccol, V. T., Vandenberghe, L. P., & Mohan, R. (2000). Biotechnological potential of agro-industrial residues. II: cassava bagasse. *Bioresource technology*, *74*(1), 81-87.

Park, H.-K., Gil, B., & Kim, J.-K. (2003). Characteristic flavor compounds of commercial soybean paste. *Food Science and Biotechnology*, *12*(6), 607-611.

Park, J.-S., Lee, M.-Y., Kim, K.-S., & Lee, T.-S. (1994). Volatile flavor components of soybean paste (doenjang) prepared from different types of strains. *Korean Journal of Food Science and Technology*, *26*(3), 255-260.

Pastore, G., Park, Y., & Min, D. (1994). Production of fruity aroma by Neurospora from beiju. *Mycological Research*, *98*(11), 1300-1302.

Pastore, G. M., Sato, H. H., Yang, T.-S., Park, Y. K., & Min, D. B. (1994). Production of fruity aroma by newly isolated yeast. *Biotechnology letters*, *16*(4), 389-392.

Paul, J. S., Tiwari, K., & Jadhav, S. (2015). Long term preservation of commercial important fungi in glycerol at 4 C. *International Journal of Biological Chemistry*, *9*(2), 79-85.

Paulin-Mahady, A. E., Harrington, T. C., & McNew, D. (2002). Phylogenetic and taxonomic evaluation of Chalara, Chalaropsis, and Thielaviopsis anamorphs associated with Ceratocystis. *Mycologia*, *94*(1), 62-72.

Pawliszyn, J. (2000). Theory of solid-phase microextraction. *Journal of chromatographic science*, *38*(7), 270-278.

Pellati, F., Prencipe, F. P., & Benvenuti, S. (2013). Headspace solid-phase microextraction-gas chromatography–mass spectrometry characterization of propolis volatile compounds. *Journal of pharmaceutical and biomedical analysis*, *84*, 103-111.

Petronilho, S., Coimbra, M. A., & Rocha, S. M. (2014). A critical review on extraction techniques and gas chromatography based determination of grapevine derived sesquiterpenes. *Analytica chimica acta*, *846*, 8-35.

Pisarikova, B., Zraly, Z., Kracmar, S., Trckova, M., & Herzig, I. (2006). The use of amaranth (genus Amaranthus L.) in the diets for broiler chickens. *VETERINARNI MEDICINA-PRAHA-*, *51*(7), 399.

Popiel, S., & Sankowska, M. (2011). Determination of chemical warfare agents and related compounds in environmental samples by solid-phase microextraction with gas chromatography. *Journal of Chromatography A, 1218*(47), 8457-8479.

Porcel, E. R., López, J. C., Pérez, J. S., Sevilla, J. F., & Chisti, Y. (2005). Effects of pellet morphology on broth rheology in fermentations of Aspergillus terreus. *Biochemical Engineering Journal, 26*(2), 139-144.

Protas, J. d. S., & Sanhueza, R. V. (2003). *Produção integrada de frutas: o caso da maçã no Brasil*: Embrapa Uva e Vinho.

Purwanto, L., Ibrahim, D., & Sudrajat, H. (2009). Effect of agitation speed on morphological changes in Aspergillus niger hyphae during production of tannase. *World J. Chem, 4*(1), 34-38.

Qiao, D., Hu, B., Gan, D., Sun, Y., Ye, H., & Zeng, X. (2009). Extraction optimized by using response surface methodology, purification and preliminary characterization of polysaccharides from Hyriopsis cumingii. *Carbohydrate polymers, 76*(3), 422-429.

Rahman, S., Choudhury, J., & Ahmad, A. (2006). Production of xylose from oil palm empty fruit bunch fiber using sulfuric acid. *Biochemical Engineering Journal, 30*(1), 97-103.

Rasat, M. S. M., Wahab, R., Sulaiman, O., Moktar, J., Mohamed, A., Tabet, T. A., & Khalid, I. (2011). Properties of composite boards from oil palm frond agricultural waste. *BioResources, 6*(4), 4389-4403.

Rizk, M., Abdel-Rahman, T., & Metwally, H. (2007). Factors affecting growth and antifungal activity of some Streptomyces species against Candida albicans. *International Journal of food, agriculture and environment, 5*(3-4), 446-449.

Rojas, V., Gil, J. V., Piñaga, F., & Manzanares, P. (2001). Studies on acetate ester production by non-Saccharomyces wine yeasts. *International journal of food microbiology, 70*(3), 283-289.

Romano, P., & Marchese, R. (1998). Metabolic characterization of Kloeckera apiculata strains from star fruit fermentation. *Antonie van Leeuwenhoek, 73*(4), 321-325.

Romano, P., Suzzi, G., Comi, G., Zironi, R., & Maifreni, M. (1997). Glycerol and other fermentation products of apiculate wine yeasts. *Journal of Applied Microbiology, 82*(5), 615-618.

Romano, P., Suzzi, G., Domizio, P., & Fatichenti, F. (1997). Secondary products formation as a tool for discriminating non-Saccharomyces wine strains. *Antonie van Leeuwenhoek, 71*(3), 239-242.

Roslan, A., Yee, P., Shah, U., Aziz, S., & Hassan, M. (2011). Production of bioethanol from rice straw using cellulase by local Aspergillus sp. *Int. J. Agric. Res, 6*(2), 188-193.

Rosli, W. W., Law, K., Zainuddin, Z., & Asro, R. (2004). Effect of pulping variables on the characteristics of oil-palm frond-fiber. *Bioresource technology, 93*(3), 233-240.

Rossi, S., Vandenberghe, L., Pereira, B., Gago, F., Rizzolo, J., Pandey, A., . . . Medeiros, A. (2009). Improving fruity aroma production by fungi in SSF using citric pulp. *Food research international, 42*(4), 484-486.

Rozman, H., Kumar, R., Khalil, H. A., Abusamah, A., Lim, P., & Ismail, H. (1997). Preparation and properties of oil palm frond composite based on methacrylic silane and glycidyl methacrylate. *European polymer journal, 33*(3), 225-230.

Rubiolo, P., Cagliero, C., Cordero, C., Liberto, E., Sgorbini, B., & Bicchi, C. (2014). Gas chromatography in the analysis of flavours and fragrances *Practical Gas Chromatography* (pp. 717-743): Springer.

Rumbold, K., van Buijsen, H. J., Gray, V. M., van Groenestijn, J. W., Overkamp, K. M., Slomp, R. S., . . . Punt, P. J. (2010). Microbial renewable feedstock utilization: a substrate-oriented approach. *Bioengineered bugs, 1*(5), 359-366.

Sabiha-Hanim, S., Noor, M. A. M., & Rosma, A. (2011). Effect of autohydrolysis and enzymatic treatment on oil palm (Elaeis guineensis Jacq.) frond fibres for xylose and xylooligosaccharides production. *Bioresource technology, 102*(2), 1234-1239.

Salamatinia, B., Kamaruddin, A. H., & Abdullah, A. Z. (2010). Regeneration and reuse of spent NaOH-treated oil palm frond for copper and zinc removal from wastewater. *Chemical engineering journal, 156*(1), 141-145.

Salmon, J., & Mauricio, J. (1994). Relationship between sugar uptake kinetics and total sugar consumption in different industrialSaccharomyces cerevisiae strains during alcoholic fermentation. *Biotechnology letters, 16*(1), 89-94.

Sanchez, S., Bravo, V., Moya, A., Castro, E., & Camacho, F. (2004). Influence of temperature on the fermentation of D-xylose by Pachysolen tannophilus to produce ethanol and xylitol. *Process Biochemistry, 39*(6), 673-679.

Santos, F., & Galceran, M. (2002). The application of gas chromatography to environmental analysis. *TrAC Trends in Analytical Chemistry, 21*(9), 672-685.

Saraji, M., & Ghani, M. (2015). Hollow fiber liquid–liquid–liquid microextraction followed by solid-phase microextraction and in situ derivatization for the determination of chlorophenols by gas chromatography-electron capture detection. *Journal of Chromatography A, 1418*, 45-53.

Sastry, M., Patil-Kulkarni, B., Hegde, R., & Hiremath, P. (1989). Studies on the disease caused by Thielaviopsis state of Ceratocystis paradoxa (Dade) Moreau on arecanut, coconut and sugarcane 1. Morphological, cultural and cross inoculation studies of three isolates of Ceratocystis paradoxa. *Mysore Journal of Agricultural Sciences, 23*(1), 44-48.

Schindler, J. (1982). Terpenoids by microbial fermentation. *Industrial & Engineering Chemistry Product Research and Development, 21*(4), 537-539.

Schrader, J. (2007). Microbial flavour production *Flavours and fragrances* (pp. 507-574): Springer.

Senemaud, C. (1988). *Les champignons filamenteux producteurs d'aromes fruités: études de faisabilité sur substrats agro-industriels.* Dijon.

Seo, J.-S., Chang, H.-G., Ji, W.-D., & Lee, E.-J. (1996). Aroma components of traditional Korean soy sauce and soybean paste fermented with the same meju. *Journal of Microbiology and Biotechnology, 6*(4), 278-285.

Seth, M., & Chand, S. (2000). Biosynthesis of tannase and hydrolysis of tannins to gallic acid by Aspergillus awamori—optimisation of process parameters. *Process Biochemistry, 36*(1), 39-44.

Shendell, D. G., Winer, A. M., Stock, T. H., Zhang, L., Zhang, J. J., Maberti, S., & Colome, S. D. (2004). Air concentrations of VOCs in portable and traditional classrooms: results of a pilot study in Los Angeles County. *Journal of Exposure Science and Environmental Epidemiology, 14*(1), 44.

Shimizu, H., Tamura, S., Ishihara, Y., Shioya, S., & Suga, K. (1994). Control of molecular weight distribution and mole fraction in poly (-D (-)-3-hydroxyalkanoate)(PHA) production by Mcaligenes eutrophus. *Y. Doi, 6*, 389-394.

Shin, M., & Joo, K. (1999). Fractionated volatile flavor components of soybean paste by dynamic headspace method. *J. Korean Soc. Food Sci. Nutr, 28*, 305-311.

Shin, Z.-I., Yu, R., Park, S.-A., Chung, D. K., Ahn, C.-W., Nam, H.-S., . . . Lee, H. J. (2001). His-His-Leu, an angiotensin I converting enzyme inhibitory peptide derived from Korean soybean paste, exerts antihypertensive activity in vivo. *Journal of agricultural and food chemistry*, *49*(6), 3004-3009.

Shirey, R. E., & Mindrup, R. F. (1999). SPME-adsorption versus absorption: which fiber is best for your application. *Supelco: Bellefonte, PA, USA*.

Shojaosadati, S., & Babaeipour, V. (2002). Citric acid production from apple pomace in multi-layer packed bed solid-state bioreactor. *Process Biochemistry*, *37*(8), 909-914.

Shrikot, C., Sharma, N., & Sharma, S. (2004). Apple pomace: An alternative substrate for xylanase production by na alkalophilic Bacillus macerans by using solid-state fermentation. *Journal of Microbial World*, *6*, 20-26.

Shuit, S. H., Tan, K. T., Lee, K. T., & Kamaruddin, A. (2009). Oil palm biomass as a sustainable energy source: A Malaysian case study. *Energy*, *34*(9), 1225-1235.

Simon, M. (2003). *Concrete mixture optimization using statistical methods*. Retrieved from

Soares, M., Christen, P., Pandey, A., & Soccol, C. R. (2000). Fruity flavour production by Ceratocystis fimbriata grown on coffee husk in solid-state fermentation. *Process Biochemistry*, *35*(8), 857-861.

Sobczuk, T. M., Camacho, F. G., Grima, E. M., & Chisti, Y. (2006). Effects of agitation on the microalgae Phaeodactylum tricornutum and Porphyridium cruentum. *Bioprocess and Biosystems Engineering*, *28*(4), 243.

Soccol, C. R., Medeiros, A. B., Vandenberghe, L. P., & Woiciechowski, A. L. (2007). Flavor Production by Solid and Liquid Fermentation. *Handbook of Food Products Manufacturing, 2 Volume Set*, 193.

Sonyal, S. (2010). *STUDIES ON POMEGRANATE WILT COMPLEX*. UNIVERSITY OF AGRICULTURAL SCIENCES, DHARWAD.

Souza-Silva, É. A., Reyes-Garcés, N., Gómez-Ríos, G. A., Boyacı, E., Bojko, B., & Pawliszyn, J. (2015). A critical review of the state of the art of solid-phase microextraction of complex matrices III. Bioanalytical and clinical applications. *TrAC Trends in Analytical Chemistry*, *71*, 249-264.

Splivallo, R., Novero, M., Bertea, C. M., Bossi, S., & Bonfante, P. (2007). Truffle volatiles inhibit growth and induce an oxidative burst in Arabidopsis thaliana. *New Phytologist*, *175*(3), 417-424.

Steele, D. B., & Stowers, M. D. (1991). Techniques for selection of industrially important microorganisms. *Annual Reviews in Microbiology*, *45*(1), 89-106.

Steinkraus, K. (1991). African alkaline fermented foods and their relation to similar foods in other parts of the world. *Traditional African foods: quality and nutrition. New York: Marcel Dekker*, 87-92.

Sugawara, E. (1991). Change in aroma components of miso with aging. *Nippon Shokuhin Kogyo Gakkaishi*, *38*(12), 1093-1097.

Sumathi, S., Chai, S., & Mohamed, A. (2008). Utilization of oil palm as a source of renewable energy in Malaysia. *Renewable and Sustainable Energy Reviews*, *12*(9), 2404-2421.

Surburg, H., & Panten, J. (2016). *Common fragrance and flavor materials: preparation, properties and uses*: John Wiley & Sons.

Szelenyi-Galantai, M., & Zsolnai-Harszi, I. (1992). Chemical and biological investigation of some seeds of the amaranth species and improvement of their protein utilisation by supplementation of maize and wheat. *Allattenyesztes es Takarmanyorzas*, *41*, 337-348.

Talon, R., Chastagnac, C., Vergnais, L., Montel, M., & Berdagué, J. (1998). Production of esters by Staphylococci. *International journal of food microbiology*, *45*(2), 143-150.

Tan, H. T., Lee, K. T., & Mohamed, A. R. (2011). Pretreatment of lignocellulosic palm biomass using a solvent-ionic liquid [BMIM] Cl for glucose recovery: An optimisation study using response surface methodology. *Carbohydrate polymers*, *83*(4), 1862-1868.

Telford, J. K. (2007). A brief introduction to design of experiments. *Johns Hopkins apl technical digest*, *27*(3), 224-232.

Tirillini, B., Verdelli, G., Paolocci, F., Ciccioli, P., & Frattoni, M. (2000). The volatile organic compounds from the mycelium of Tuber borchii Vitt. *Phytochemistry*, *55*(8), 983-985.

Tsurumi, R., Shiraishi, S., Ando, Y., Yanagida, M., & Takeda, K. (2001). Production of flavor compounds from apple pomace. *JOURNAL-JAPANESE SOCIETY OF FOOD SCIENCE AND TECHNOLOGY*, *48*(8), 564-569.

Uchiyama, S., Matsushima, E., Tokunaga, H., Otsubo, Y., & Ando, M. (2006). Determination of orthophthalaldehyde in air using 2, 4-dinitrophenylhydrazine-

impregnated silica cartridge and high-performance liquid chromatography. *Journal of Chromatography A, 1116*(1), 165-171.

Ujang, Z., Salmiati, S., & Salim, M. R. (2010). Microbial Biopolimerization Production from Palm Oil Mill Effluent (POME) *Biopolymers*: InTech.

van Stee, L. L., Udo, A. T., & Bagheri, H. (2002). Gas chromatography with atomic emission detection: a powerful technique. *TrAC Trends in Analytical Chemistry, 21*(9), 618-626.

Vandamme, E. (1996). Bacteria in front of the mirror-Biocosmetics via microbial synthesis. *Agro FOOD Industry Hi-Tech, 7*(4) 3-8).

Vandamme, E. J. (2003). Bioflavours and fragrances via fungi and their enzymes. *Fungal Diversity, 13*, 153-166.

Vendruscolo, F., Koch, F., de Oliveira Pitol, L., & Ninow, J. L. (2007). Produção de proteína unicelular a partir do bagaço de maçã utilizando fermentação em estado sólido. *Revista Brasileira de Tecnologia Agroindustrial, 1*(1).

Venugopal, T., Jayachandra, K., & Appaiah, A. (2007). Effect of aeration on the production of endopectinase from coffee pulp by a novel thermophilic fungi, Mycotypha sp. strain no. AKM1801. *Biotechnology, 6*(2), 245-250.

Villas Bôas, S., & Esposito, E. (2000). Bioconversão do bagaço de maçã: enriquecimento nutricional utilizando fungos para produção de um alimento alternativo de alto valor agregado. *Revista de Biotecnologia, Brasília, 1*(14), 38-42.

Wanrosli, W., Zainuddin, Z., Law, K., & Asro, R. (2007). Pulp from oil palm fronds by chemical processes. *Industrial crops and products, 25*(1), 89-94.

Welsh, F. (1994). Overview of bioprocess flavor and fragrance production. *Bioprocess production of flavor, fragrance, and color ingredients. Wiley, New York*, 1-17.

Welsh, F. W., Murray, W. D., Williams, R. E., & Katz, I. (1989). Microbiological and enzymatic production of flavor and fragrance chemicals. *Critical Reviews in Biotechnology, 9*(2), 105-169.

Worrall, J., & Yang, C. S. (1992). Shiitake and oyster mushroom production on apple pomace and sawdust. *HortScience, 27*(10), 1131-1133.

Wu, T., Mohammad, A. W., Jahim, J. M., & Anuar, N. (2006). Investigations on protease production by a wild-type Aspergillus terreus strain using diluted retentate of pre-filtered palm oil mill effluent (POME) as substrate. *Enzyme and Microbial Technology, 39*(6), 1223-1229.

Yacob, S., Shirai, Y., Hassan, M. A., Wakisaka, M., & Subash, S. (2006). Start-up operation of semi-commercial closed anaerobic digester for palm oil mill effluent treatment. *Process Biochemistry, 41*(4), 962-964.

Yadahalli, K. (2005). *Epidemiology and Management of Sett Rot [Ceratocystis paradoxa (De Seynes) Moreau] of Sugar Cane.*

Yamauchi, H., Obata, T., Amachi, T., & Hara, S. (1991). Production of characteristic odors by Neurospora. *Agricultural and biological chemistry, 55*(12), 3115-3116.

Yánez, E., Zacarías, I., Granger, D., Vásquez, M., & Estevez, A. M. (1994). Chemical and nutritional characterization of amaranthus (Amaranthus cruentus). *Archivos latinoamericanos de nutricion, 44*(1), 57-62.

Yee, P. L., Hassan, M. A., Shirai, Y., Wakisaka, M., & Karim, M. I. A. (2003). Continuous production of organic acids from palm oil mill effluent with sludge recycle by the freezing-thawing method. *Journal of chemical engineering of Japan, 36*(6), 707-710.

Yegemova, S., Bakaikina, N. V., Kenessov, B., Koziel, J. A., & Nauryzbayev, M. (2015). Determination of 1-methyl-1H-1, 2, 4-triazole in soils contaminated by rocket fuel using solid-phase microextraction, isotope dilution and gas chromatography–mass spectrometry. *Talanta, 143*, 226-233.

Yemiş, O., & Mazza, G. (2011). Acid-catalyzed conversion of xylose, xylan and straw into furfural by microwave-assisted reaction. *Bioresource technology, 102*(15), 7371-7378.

Yusoff, S. (2006). Renewable energy from palm oil–innovation on effective utilization of waste. *Journal of Cleaner Production, 14*(1), 87-93.

Zahari, Hassan, O. A., Wong, H., & Liang, J. (2003). Utilization of oil palm frond-based diets for beef and dairy production in Malaysia.

Zahari, Zakaria, M. R., Ariffin, H., Mokhtar, M. N., Salihon, J., Shirai, Y., & Hassan, M. A. (2012). Renewable sugars from oil palm frond juice as an alternative novel fermentation feedstock for value-added products. *Bioresource technology, 110*, 566-571.

Zain, S. M. S. M., Shaharudin, R., Kamaluddin, M. A., & Daud, S. F. (2017). Determination of hydrogen cyanide in residential ambient air using SPME coupled with GC–MS. *Atmospheric Pollution Research*.

Zakaria, M. R., Tabatabaei, M., Ghazali, F. M., Abd-Aziz, S., Shirai, Y., & Hassan, M. A. (2010). Polyhydroxyalkanoate production from anaerobically treated palm oil

mill effluent by new bacterial strain Comamonas sp. EB172. *World journal of microbiology and biotechnology, 26*(5), 767-774.

Zawirska, W., Renata, Siwulski, M., & Mildner-Szkudlarz, S. (2009). Studies on the aroma of different species and strains of Pleurotus measured by GC/MS, sensory analysis and electronic nose. *Acta Scientiarum Polonorum Technologia Alimentaria, 8*(1), 47-61.

Zeman, L., Simecek, K., Krasa, A., Simek, M., & Lossmann, J. (1995). *The tables for nutritional values of feeds (in Czech). VUVZ, Pohorelice, 465 pp*. Retrieved from

Zhan, X., Zhang, Y.-H., Chen, D.-F., & Simonsen, H. T. (2014). Metabolic engineering of the moss Physcomitrella patens to produce the sesquiterpenoids patchoulol and α/β-santalene. *Frontiers in plant science, 5*.

Zhang, Z., & Pawliszyn, J. (1993). Headspace solid-phase microextraction. *Analytical chemistry, 65*(14), 1843-1852.

Zhang, Z., Yang, M. J., & Pawliszyn, J. (1994). Solid-phase microextraction. A solvent-free alternative for sample preparation. *Analytical chemistry, 66*(17), 844A-853A.

Zheng, Z., & Shetty, K. (2000a). Enhancement of pea (Pisum sativum) seedling vigour and associated phenolic content by extracts of apple pomace fermented with Trichoderma spp. *Process Biochemistry, 36*(1), 79-84.

Zheng, Z., & Shetty, K. (2000b). Solid state production of polygalacturonase by Lentinus edodes using fruit processing wastes. *Process Biochemistry, 35*(8), 825-830.

Zygmunt, B., Zaborowska, A., & Namiesnik, J. (2007). Solid phase microextraction combined with gas chromatography-A powerful tool for the determination of chemical warfare agents and related compounds. *Current Organic Chemistry, 11*(3), 241-253.

APPENDIX A

LIST OF PUBLICATIONS

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 (ICOST2017), 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 (ICoRAST2017). 7 – 8 November 2017, Novotel Melaka, Malaysia