BIOTRANSFOR ID ISOEUGENOL TO VANILLIN BY *ASPERGILLUS NIGER*

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ABSTRACT

Eugenol and isoeugenol are main chemical components of clove oil. These both chemical compounds are phenylpropene, the class of phenylpropanoids chemical compounds. Clove oil extracted from the leaves, stem, and buds of clove tree *Syzygium aromaticum*. It has clear to pale yellow oily liquid and used widely as an herbal drug. Eugenol and isoeugenol can produce vanillin by biosynthesis process. Vanillin is flavoring agent used extensively in food and perfume industry. Since consumer request on vanillin increasing, the study aim on producing vanillin compound by biosynthesis using *Aspergillus niger*. The objectives of the study are to learn the techniques of preparing the culture for growth of *Aspergillus niger* and to carry out biotransformation of eugenol and isoeugenol using *Aspergillus niger.* Methodology involves were culture growth of fungus, cultivation, inoculum development and analysis of compounds. The biotransformation process using *Aspergillus niger* is to change the eugenol and isoeugenol into vanillin compounds. All the compounds formed characterized by chromatographic technique using Gas chromatography-Flame Ionization Detector (GC-FID) and Thin Layer Chromatography (TLC).

ABSTRAK

Eugenol dan Isoeugenol adalah komponen kimia utama minyak cengkih. Kedua-dua komponen kimia ini merupakan fenilpropena, iaitu kelas sebatian bagi fenilpropanoids. Minyak cengkih diekstrak daripada daun, batang dan tunas pokok cengkih yang dikenal sebagai *Syzygium aromaticum.* Minyak ini bewarna kuning pucat dan digunakan secara meluas sebagai ubat herba. Eugenol dan isoeugenol mampu menghasilkan vanilin melalui proses biosintesis. Vanilin merupakan ejen perisa yang digunakan secara meluas dalam industri makanan dan industri minyak wangi. Vanilin yang dihasilkan mendapat permintaan tinggi daripada pengguna. Oleh yang demikian, matlamat kajian ini adalah untuk meghasilkan sebatian vanilin secara biosintesis dengan menggunakan *Aspergillus niger*. Objektif kajian adalah untuk mempelajari teknik penyediaan *Aspergillus niger* dan menjalankan proses biotransformasi terhadap eugenol dan isoeugenol dengan menggunakan *Aspergillus niger*. Dalam kajian ini, beberapa kaedah telah digunakan antaranya pertumbuhan *Aspergillus niger*, inoculum dan analisis data. Proses biotransformasi yang menggunakan *Aspergillus niger* adalah untuk menukarkan eugenol and isoeugenol menjadi sebatian vanilin. Sebatian yang dibentuk dikaji dengan menggunakan teknik kromatografi seperti GC-FID dan TLC.

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LIST OF SYMBOLS

°C	Degree Celcius
$	Dollar
g	Gram
h	Hour
kg	Kilogram
<	Less than
lbs	Pound
L	Liter
mg	Milligram
ml	Milliliter
min	Minutes
ppm	Part per million
%	Percent
rpm	Revolutions Per Minutes
sec	Second
v/v	Volume per volume
µL	Microliter
µm	Micrometer

LIST OF ABBREVIATION

Z	Cis
EPA	Environmental Protection Agency
GC-FID	Gas Chromatography-Flame Ionization Detector
NFPA	National Fire Protection Association
PDA	Peptose Dextrose Agar
SDA	Sabouraud Dextrose Agar
E	Trans
UV	Ultraviolet
US	United State
USFDA	United State Food and Drug Administration
USA	United State of America

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CHAPTER 1

INTRODUCTION

1.1 BACKGROUND OF THE STUDY

Eugenol and its derivatives isoeugenol ($C_{10}H_{12}O_2$) are type of phenylpropene, the class of phenylpropanoids chemical compounds that was extracted particularly from clove oil. Eugenol and isoeugenol are the main composition found in the clove oil. These chemical compounds are responsible to give the fragrant-smelling in the essential oil. Since the compounds are major components that give aroma to the clove oil, it has been used in food stuffs, perfumeries, cosmetics and in medicine. About 72 to 90 percent of eugenol can be found in this oil. Isoeugenol can be synthesized from eugenol and further oxidation of these both compounds can form vanillin. Vanillin is an organic compound used widely in flavor industry, fragrance industry such as in perfumes and also as a chemical intermediate in the production of pharmaceuticals and agrochemicals. Eugenol and isoeugenol compounds undergo biotransformation process to produce vanillin. This process goes through chemical conversion of substrate by living organisms. In this method, *Aspergillus niger* is used to modify the structure of eugenol and isoeugenol to produce vanillin compound.

1.2 PROBLEM STATEMENT

The worldwide demand for vanilla flavoring is increasing per year due to the increasing popularity and price of vanillin. (Ashengroph et al., 2011). To fulfill consumer request, more vanillin is synthesized chemically. Vanillin that produces artificially has stronger odor than natural vanillin. In 1954, US Food and Drug Administration has banned vanillin that chemically synthesized from cheaper sources like waste sulphate liquor from paper mills. This is due to the carcinogenic properties and toxicity on liver of test animals. According to journal written by Achterholt et al. (2000), vanillin is used frequently for the production of flavors for foods. About 70% of natural food flavors were used in Germany since 1990. Thus, it increases the health and nutrition conscious lifestyle of the customer. Due to the price variation and consumer high demand, natural flavor have attracted attention towards production of vanillin from other natural sources using biotransformation. (Tillay et al., 2010). The legal definition for 'natural flavor' includes products obtained by fermentation and enzymatic processes. Therefore, US and European legislation legalize that the use of microbial transformation as a suitable alternative to generate some products such as vanillin and vanillic acid which are considered as 'natural'. As the customer request for natural vanillin is rising, the production of vanillin by bioconversion has brought more importance since natural eugenol and isoeugenol from essential oil are more resourceful and more economical. Therefore, the aim of this study is to produce natural vanillin from eugenol and isoeugenol by biotransformation process using *Aspergillus niger*.

1.3 OBJECTIVES

The objectives of this study are:

1.0 To learn the techniques of preparing the culture and growth of the *Aspergillus niger*.

2.0 To carry out the biotransformation of eugenol and isoeugenol using *Aspergillus niger*.

3.0 To produce natural vanillin from eugenol and isoeugenol by biontransformation process using *Aspergillus niger.*

4.0 To characterized the compounds by chromatographic technique using GC-FID

1.4 SCOPE OF STUDY

As a way to achieve the objectives of this research, the scope of this study has been identified and focuses on how to culture and growth the fungus *Aspergillus niger*. It also involves biotransformation of eugenol and isoeugenol by *Aspergillus niger* and at the end of experiments it focuses on the production of vanillin compound. The compound produced is characterized by chromatographic technique using GC-FID.

1.5 SIGNIFICANCE OF THE STUDY

The clove oil contains eugenol and its derivates isoeugenol as a main constituent. (Rabenhorst, 1996). These compounds are believed to give scent and pleasant aroma to the oil. As far as we concern, natural vanillin is expensive and has high demand compare to synthetic vanillin. Thus, there have been a number of studies on the biotransformation of eugenol and isoeugenol to synthesis vanillin compound. In this study, biotransformation process is done by using *Aspergillus niger* to chemically modify the structure of eugenol and isoeugenol and finally increases the yield of compound called vanillin. If this study can be achieved successfully then it may be a great use to the perfume, aromatherapy industries and food industries as it can produce more products with lower cost and increase their profit.

CHAPTER 2

LITERATURE REVIEW

2.1 INTRODUCTION

In this chapter, the detail of eugenol, isoeugenol, vanillin, *Aspergillus niger* and biotransformation process will be discussed.

2.2 EUGENOL AND USES

2.2.1 Source and Properties of Eugenol

Eugenol, 2-Methoxy-4-(2-Propenyl) phenol is a major chemical compound found in clove oil isolated from the clove tree S*yzigium aromaticum.* (Rabenhorst, 1996). Clove is an aromatic crop cultivated commercially in India, Madagascar, Sri Lanka, Indonesia and the South of China for the production of essential oil. (Bhuiyan et al., 2010). Eugenol oil or known as clove oil is made up from the extract of dried flower buds, leaves and stems of clove tree. The chemical compound of eugenol also can be found in nutmeg, cinnamon, basil and bay leaf. Eugenol with empirical formula $C_{10}H_{12}O_2$ is a clear to pale yellow oily liquid and has spicy odor together with taste of clove. The physical and chemical properties of eugenol are shown in Table 2.1. Figure 2.1 shows the picture of flower bud of clove tree while Figure 2.2 shows the dried flower buds and pure eugenol oil. Figure 2.3 illustrate the chemical structure of Eugenol.

Table 2.1: Physical and Chemical Properties of Eugenol

	Properties
Physical State	Clear to pale yellow oily liquid
Melting Point	-9°C
Boiling Point	254°C
Specific Gravity	1.066
Solubility in water	< 1mg/ml
Solvent solubility	Miscible in alcohol, ether, chloroform; Soluble in acetic acid, alkali hydroxide solutions
NFPA Rating	Health : 0; Flammability: 1; Reactivity : 0
Refractive Index	1.5410
Flash point	104°C
Stability	Stable under ordinary conditions. Light sensitive

Source: http://chemicalland21.com/specialtychem/perchem/EUGENOL.htm

Figure 2.1 Unopened Flower Buds of the Evergreen Clove Tree.

Sources: (Pati, 2010)

Figure 2.2: Dried Flower Buds and pure Eugenol oil

Source: Vikram Aromatic, 2005

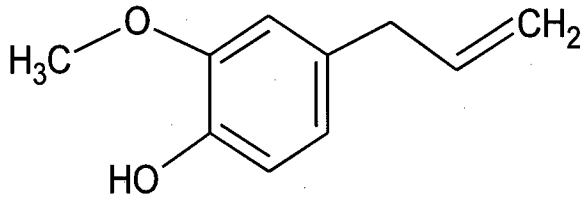

Figure 2.3 Chemical Structure of Eugenol

Source: Li et al. 2004

2.2.2 Uses of eugenol

Eugenol ($C_{10}H_{12}O_2$) causes the aromatic smell of clove oil and because of that it has been used widely in perfumeries and flavoring. Clove oil has been approved by the US Food and Drug Administration (USFDA) to be used in food as a flavoring agent and as a fragrance in personal care products. Eugenol used in the field of medicine such as application in dentistry for analgesic and antiseptic properties. It also used to make zinc-oxide eugenol paste in dental medicine for temporary fillings. Eugenol from clove oil can be act as antifungal, antibacterial and antimicrobial material in food. In Korea, the essential oil which has distinctive odor

that is pleasant used in the field of aromatherapy and successfully used for asthma and allergic disorder. The low concentration of eugenol can act as an antioxidant and anti-inflammatory agent while high concentration of eugenol act as a pro-oxidant ensuing from the enhanced generation of tissue damaging free radicals. Other than perfumeries, medicine, aromatherapy and flavoring, eugenol is found in formulating insects' attractants and UV absorbers. Eugenol are classified by Environmental Protection Agency (EPA) as minimum risk pesticides and therefore it being used more frequently to control Coleoptera (weevils and beetles), moth caterpillars and cockroaches. Furthermore, eugenol also used for the manufacture of vanillin using biotransformation techniques with fungus and bacteria such as *Aspergillus niger*, *Bacillus fusiformis* and *Pseudomonas resinovorans*.

2.2.3 Effects of Eugenol to Health

Eugenol oil is considered safe as a food additive if in a small quantities (<1,500 ppm). However, it is toxic to human cells if ingested in sufficient quantity and can cause life threatening complication and Central Nervous System Depression. Eugenol oil at 0.03 percent (v/v) was highly cytotoxic to human skin cells. Overdose of eugenol can be hepatotoxic where it might cause damage to the liver. Other symptoms are diarrhea, nausea unconsciousness, dizziness and rapid heartbeat. This essential oil has been used traditionally as treating burns, cuts and dental care for tooth infections and toothache. Several decades ago, there are a study shows that eugenol be a contact allergen when used in dentistry.

2.3 ISOEUGENOL AND USES

2.3.1 Source and Properties of Isoeugenol

Isoeugenol, 2-methoxy-4-propenylphenol ($C_{10}H_{12}O_2$) can be extracted from plant directly. It also can be synthesized from eugenol under strong basic condition or isomerization. (Ashengroph et al., 2008). This compound may occur either in the form of cis (Z) or trans (E) isomer. The trans (E) isoeugenol is crystalline while cis *(Z)* isoeugenol is a liquid. Isoeugenol is a colorless to light yellow-brown and transparent liquid. Isoeugenol is a cheap natural substrate that can be isolated from the essential oil *Syzygium aromaticum.* (Ashengroph et al., 2008). The physical and chemical properties of isoeugenol are shown in Table 2.2. Figure 2.4 shows the chemical structure of isoeugenol.

Table 2.2: Physical and Chemical Properties of Isoeugenol

	Properties
Physical State	Colorless to light yellow-brown, transparent liquid
Melting Point	-10°C
Boiling Point	266°C-268°C
Specific Gravity	1.077
Solubility in water	Slightly soluble
NFPA Rating	Health : 0; Flammability: 1; Reactivity : 0
Refractive Index	1.5760
Flash point	112°C
Stability	Stable under ordinary conditions.

Source: http://chemicalland21.com/specialtychem/perchem/ISOEUGENOL.htm

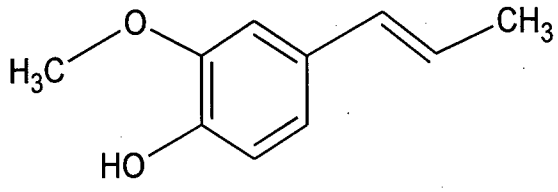

Figure 2.4 Chemical Structure of Isoeugenol

Source: Li et al. 2004

2.3.2 Uses of Isoeugenol

Isoeugenol is used as flavoring agent for non-alcoholic beverages, baked goods, and chewing gum. It also has been used in manufacturing perfumeries, essential oils and in medicine. This compound is essential for manufacture of vanillin compound since it can serve as a potential substrate for the production of valuable aromatic compounds. Many researchers have been reported on biotransformation isoeugenol to vanillin and vanillic acid for a variety of microbial species such as *Arthrobacter, Aspergillus niger, Bacillus, Corynebacterium, Pseudomonas, Rhodococcus* and with crude enzyme extract from soybean. (Seshadri et al., 2008). The bioconversion of isoeugenol using fungus and bacteria has always been a hot topic because it is a natural renewable resource and the conversion processes are environmentally friendly. (Ashengroph et al., 2008)

2.4 VANILLIN AND USES

2.4.1 Source and Properties of Vanillin

Vanillin, (4-hydroxy-3-methoxybenzaldehyde) is a phenolic aldehyde. The functional group for this organic compound includes aldehyde, ether and phenol. *Vanilla planifolia* is the main species harvested for vanillin. It is a climbing terrestrial orchid grown in warm humid tropics. Madagascar is the world's largest producer of Vanilla. This plant also cultivated in Indonesia, Comoros, Uganda and India.

Vanillin in the extraction of vanilla is primarily responsible for the characteristic flavor and smell of vanilla. Gobley in 1858, was the first person isolated vanillin from vanilla pods. Vanillin can be extracted naturally and by chemically synthesized. (Zhao et al., 2006). Natural vanillin cost's ranges between US$ 2000 to US$ 3000 a kg. Although it is expensive, it has high demand in world's wide marketing. Artificial vanillin is produced due to demand for vanilla flavoring has long exceeded the supply of vanilla beans. In 2001, the annual demand for vanillin was 12,000 tons, but the natural vanillin produced was only 1800 tons. The remainder was produced by chemically synthesis. (Zheng et al., 2007). According to the US and European legislation, chemically synthesized flavor could not be used for natural flavors.

Vanillin can be prepared by oxidation of eugenol and isoeugenol using microorganism. (Jurgen and Rudolf, 1991). Further oxidization produce vanillic acid with molecular formula 4-hydroxy-3-methoxybenzoic acid. Vanillin is biodegradable and not classified as dangerous to the environment but GPS Safety Summary Vanillin, 2011, state that vanillin considered to be unsafe and harmful to fish, algae and invertebrates. Table 2.3 shows the physical properties of vanillin.

Table 2.3 Physical and Chemical Properties of Vanillin

Properties	
Molecular Formula	$C_8H_8O_3$
Molar Mass	152.15 g mol^{-1}
Appearance	White crystals
Physical State	Solid at 20°C
Odor	Floral pleasant
Melting Point	81°C – 83°C
Boiling Point	285°C
Solubility in water	10 g dm^{-3}
NFPA Rating	Health : 1
	Flammability : 1
	Reactivity : 0
Flash point	147°C

Source: http://cira.ornl.gov/documents/vanillin.pdf

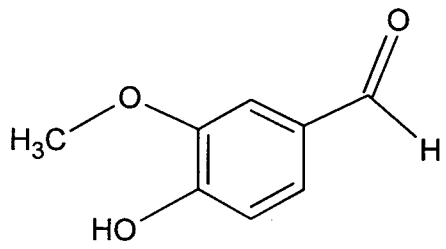

Figure 2.5 Chemical Structure for Vanillin

Source: Burger et al. 1993

2.4.2 Uses of Vanillin

Vanilla aroma is one of the most valuable flavors. There are more than 100 aroma components such as esters, acids, aldehyde, phenols and alcohols found in the composition of vanilla. The entire components that present in vanilla flavor gives characteristic to vanilla taste. However, vanillin the aldehyde compound is the major principle for the aroma of vanilla. It mainly produced by chemical synthesis and used widely as flavors and fragrances. The aroma used for foodstuffs such as in sweet foods like ice cream and chocolate, dairy products, coffee, olive oil, butter and beverages. (Buddo, 2003; Kasana, 2007; Ryu et al., 2010.). About 75 % of the market used vanillin as flavoring and the remainder used for confections and baked goods. Vanillin used in the fragrance industry, in perfumes, cosmetics, personal care products, cleaning products, detergents and to cover unpleasant odor or taste in medicines. Besides that, vanillin is also used also as a synthesis intermediate in agrochemicals and pharmaceuticals. (GPS Safety Summary Vanillin, 2011).