BIOTRANSFORMATION OF EUGENOL AND ISOEUGENOL TO VANILLIN BY *ASPERGILLUS NIGER*

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Report submitted in partial fulfillment of the requirements
for the award of
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SUPERVISOR'S DECLARATION

I hereby declare that I have checked this project report and in my opinion this project is satisfactory in terms of scope and quality for the award of the degree of Bachelor of Applied Science (HONOR) in Industrial Chemistry.

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Date : 29 DECEMBER 2011

STUDENT'S DECLARATION

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

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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.

TABLE OF CONTENTS

CHAPTER 5 CONCLUSION & RECOMMENDATIONS

LIST OF TABLES

LIST OF FIGURES

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

LIST OF APPENDICES

APPENDIX C

APPENDIX D

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 ℃
Boiling Point	254 ℃
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 ℃
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

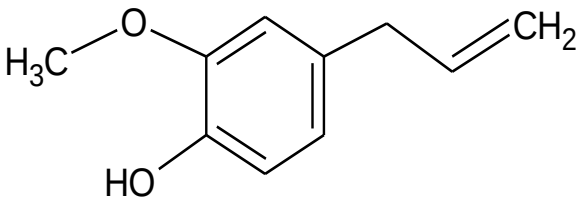


Figure 2.3 Chemical Structure of Eugenol

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 ℃
Boiling Point	266 ℃-268 ℃
Specific Gravity	1.077
Solubility in water	Slightly soluble
NFPA Rating	Health : 0; Flammability: 1; Reactivity : 0
Refractive Index	1.5760
Flash point	112 ℃
Stability	Stable under ordinary conditions.

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

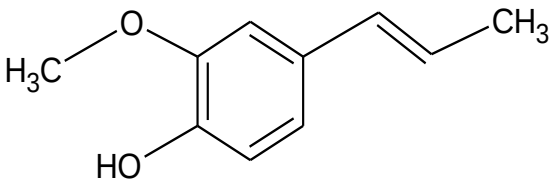


Figure 2.4 Chemical Structure of Isoeugenol

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 ℃
Odor	Floral pleasant
Melting Point	81 ℃ – 83 ℃
Boiling Point	285 ℃
Solubility in water	10 g dm^{-3}
NFPA Rating	Health : 1
	Flammability : 1
	Reactivity : 0
Flash point	147 ℃

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

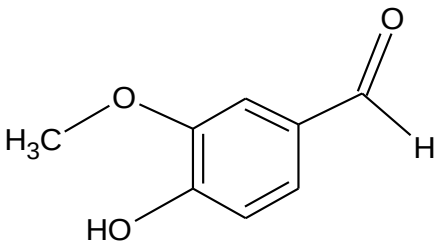


Figure 2.5 Chemical Structure for Vanillin

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).

2.5 *ASPERGILLUS NIGER*

Fungus includes microorganisms such as yeast and molds. Fungus is responsible for spoilage, production of mycotoxins and also for bioconversion. Fungus needs enough nutrients, water, temperature, oxygen and other factors to growth. *Aspergillus niger* is one type of common fungus found on dead leaves, stored grain, compost, foods, and decaying vegetation. (Hoffmann, 2010) It contaminates foods and formed black mold on certain fruits and also vegetables such as onions, grapes, and peanuts. This fungus is morphologically black with white margin and has yellow surface mycelium. It grows better at the temperature 33 ℃ to 35 ℃. *Aspergillus niger* is used for citric acid production in the food industry. (Kubicek and Rohr, 1986). The spore of *Aspergillus niger* is present in the air but normally it does not cause illness. However, those people have low immune system and weak might be risk to *Aspergillus* infection. *Aspergillus niger* has been exploited in the biotransformation of various substrates. Many researchers have been done using this fungus to study whether these fungi functionalize other part of molecule. For that reason, in this study, *Aspergillus niger* is used to study whether this fungi can change or modify the structure of eugenol and isoeugenol into vanillin. Table 2.3 below shows the scientific classification for *Aspergillus niger*. Table 2.4 present classification of *Aspergillus niger* and Figure 2.6 shows growth of *Aspergillus niger* on SDA media.

Table 2.4 Scientific Classification for *Aspergillus niger*

Scientific Classification	
Kingdom	Fungi
Phylum	Ascomycota
Class	Eurotiomycetes
Order	Eurotiales
Family	Trichocomaceae
Genus	*Aspergillus*
Species	*Aspergillus niger*

Source: http://epa.gov/biotech_rule/pubs/fra/fra006.htm

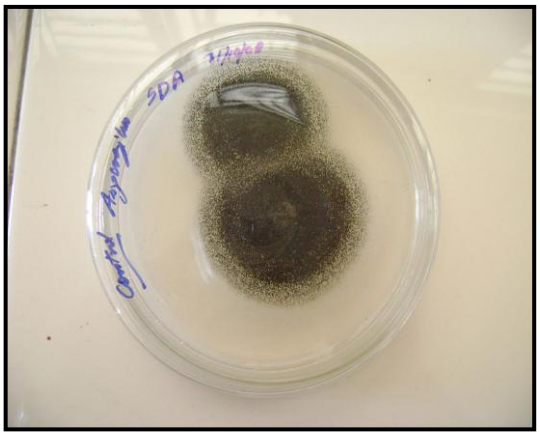


Figure 2.6 *Aspergillus niger* on SDA

Source: http://en.wikipedia.org/wiki/Aspergillus_niger

2.6 BIOTRANSFORMATION

Biotransformation is a process of metabolism that involves the use of microbes, living organism and enzyme preparation to create specific molecules from precursor molecules. Nowadays, this process is widely use by the synthetic chemist as it has many commercial applications and also environmental friendly. The recent biotransformation technologies have become more sophisticated, as the production of new compounds is used for the pharmaceuticals and chemical industries. This alternative process is deemed economically and also ecologically as the development of methods use in the process reduces the environmental pollution. Biotransformation reactions in stereoselective is the process of hydroxylation involves the direct oxidation of a C–H bond to produce an alcohol. These reactions may take place at various points on the molecule, especially hydroxylations of non-activated centers that are difficult to be achieved using classical chemical methods. Biotransformation of organic compounds by cultured plant cells includes oxidation, reduction, hydroxylation, esterification, methylation, isomerization, hydrolysis and glycosylation. (Shimoda et al., 2006)

Eugenol and its derivatives isoeugenol obtained from the clove tree *Syzygium aromaticum.* Since it is cheap and valuable, the raw material of eugenol and isoeugenol commercially available for biotransformation processes. (Priefert et al., 2001; Shimoni et al., 2000). Therefore, in this study, biotransformation process is applied using the *Aspergillus niger* to change the structure of eugenol and isoeugenol to form vanillin compound. Figure 2.7 and Figure 2.8 below describes how eugenol and isoeugenol transforms into vanillin by using bioconversion process.

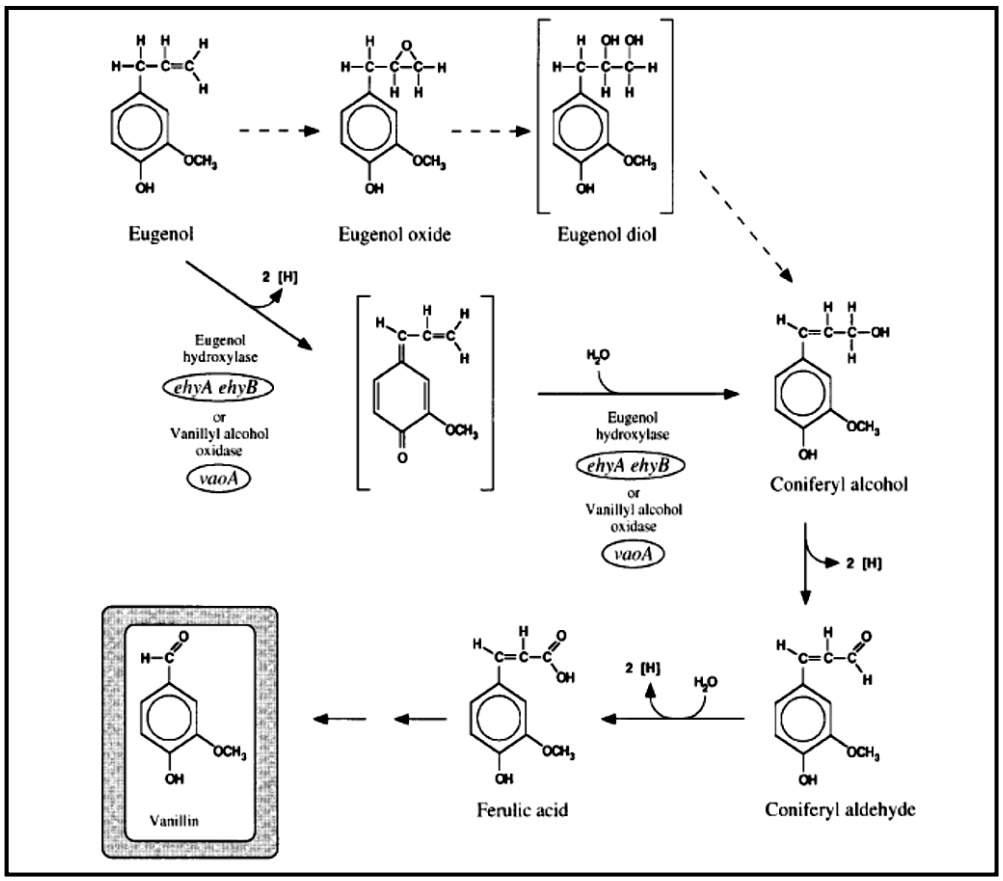
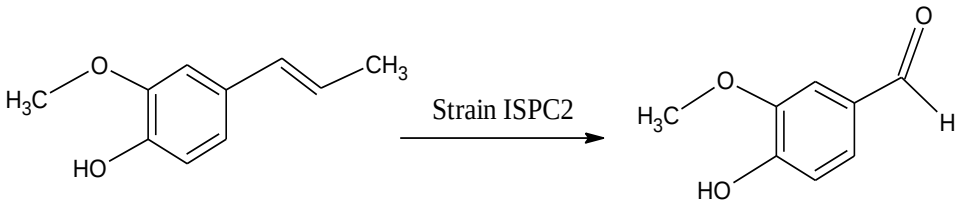


Figure 2.7 Biotransformation of Eugenol to Vanillin that Involves Enzymes and Genes.

Source: Priefert et al. (2001)



Isoeugenol **Vanillin**

Figure 2.8 Representation of the Bioconversion of Isoeugenol into Vanillin Catalyzed by *Pseudomonas* sp., labeled as ISPC2

Source: Ashengroph et al. (2008)

2.7 STERILIZATION

Sterilization is the process that eliminates or removes all forms of microbial life such as fungi, bacteria, and viruses that present in a compound of biological culture media. Sterilization can be achieved by autoclaving where the items are sterilized at 121 ℃ and 15 lbs of pressure for 20 min. At these conditions, microorganisms cannot survive longer than 12 min to 13 min. It has been designed to ensure that all air has expelled and only steam is present in autoclave chamber. (Prescott, 2002). The modern autoclave can sterilized almost all media, wastes and glassware such as pipette and petri dishes based on the mode settings in autoclave. These items are generally dry-heat sterilized and set between 160 ℃ and 170 ℃ for 2 h longer.

CHAPTER 3

MATERIALS AND METHODS

3.1 INTRODUCTION

This chapter discussed about the study of the materials, apparatus, instruments and the methodology of research carried. The methodology of the research is sub divided into five parts which include preparation of culture media for growth of *Aspergillus niger*, cultivation of *Aspergillus niger*, *Aspergillus niger* growth measurement, inoculums development in liquid media and media preparation for vanillin production.

3.2 MATERIALS

Eugenol and isoeugenol was purchased from Merck Schuchardt OHG while vanillin ($C_8H_8O_3$) is a product of USA. Vanillin purchased from SIGMA-ALDRICH. *Aspergillus niger* was procured from Fisher Scientific Co. Distilled water and deionized water used were from laboratory resources. Ethanol, Methanol, and Chloroform were purchased from Merck Germany. Sabouraud Dextrose Agar, Glucose, Ammonium Tartarate, Potassium Dihydrogen Phosphate (KH_2PO_4), Calcium Chloride ($CaCl_2.H_2O$), Magnesium Sulfate ($MgSO_4.7H_2O$), Yeast Extract, and Thiamin Hydrochloride.

3.3 APPARATUS

Schott bottles, glass rod, spatula, separation funnel, dropper, 5 ml and 10 ml of measuring cylinder, magnetic stir bar, petri dishes, universal bottles, plastic weighing boats, Bunsen burner, marker pen, parafilm, 500 ml and 250 ml of conical flask, 100 ml, 500 ml and 1 L of beakers, heat-proof gloves, aluminium foil, hot plate, paper towel, inoculating needle, 100-1000 µl micropipette and 20-200 µl micropipette with tips, syringe, needle, syringe filter membrane, glass vial, membrane filter and vacuum filter.

3.4 INSTRUMENTS & EQUIPMENTS

Ultrasonic bath, Incubator, Laminar Flow, Autoclave, Weighing Scale, eppendorf, GC-FID Agilent Technologies 7890 A GC system.

3.5 METHODOLOGY

3.5.1 Preparation of Culture Media for Growth of *Aspergillus niger*

Sabouraud Dextrose Agar (SDA) was used as a medium for growing the *Aspergillus niger*. 65 g of SDA was weighed and mixed into 1 L of distilled water. The mixture in the Schott bottle was stirred using a glass rod and then boiled using a stirring hot plate. The media prepared was then sterilized in an autoclave for 20 min at 121 ℃ (15 lbs pressure). After sterilization, Schott bottle was removed from the autoclave while the agar was still melted. About 12 ml of SDA media was poured into standard size (85 mm) plastic petri dishes. The remainder SDA was then poured into universal bottles (5 ml). The medium in the petri dishes were kept undisturbed until it cooled while the medium in the universal bottles, were allowed to harden in a slanted position. After the agar plates were cooled, petri dishes are inverted to prevent condensing moisture from accumulating on the agar surfaces. All the steps were done inside the Laminar Flow. Ten petri dishes and 15 universal bottles were used for cultivation while the remainders were placed in the refrigerator in order to prevent drying of the agar.

3.5.2 Cultivation of *Aspergillus niger*

Two months old cultures grown on Peptose Dextrose Agar (PDA) was used for cultivation. Cultures were grown on each medium of petri dishes and universal bottles. Inocula were prepared in the laminar flow. A*spergillus niger* was transformed to the prepared petri dishes and universal bottles that contained agar using the inoculating needle. The needle was sterilized first by passing the end of wire to the flame from a Bunsen burner. The wire was then turned to the red with the heat. The needle was used before it contaminated. Then, carefully, a drop of suspension of each fungus was inoculated, separately, on the centre of each culture medium. Once cultivated, the inoculating needle was sterilized again by heating it using Bunsen burner. All the agar plates prepared were inverted and sealed using parafilm. The plates were incubated in the incubator at temperatures 30 ℃ for 5 days.

3.5.3 Growth Measurements of *Aspergillus niger*

The growth of *Aspergillus niger* on petri dishes was observed each day to analyze the growth measurement. The observation was done for 5 days of incubation. *Aspergillus niger* that growth successfully in agar plates were kept inside the refrigerator to use for next step.

3.5.4 Preparation of Standard Solution for Eugenol, Isoeugenol and Vanillin

Stock solution for eugenol was prepared at the concentration 1000 ppm. The calculation showed the preparation of 1000 ppm of stock solutions.

1000 ppm of stock solution in 0.1 L of methanol,

$$\frac{X \text{ mg}}{0.1 \text{ L}} = 1000 \text{ ppm}$$

$$X \text{ mg} = 100 \text{ mg}$$

$$= 0.1 \text{ g}$$

0.1 g of eugenol was weighed inside 100 ml of volumetric flask. Then, 100 ml of methanol was added in the volumetric flask. Therefore, 1000 ppm of stock solution for eugenol was prepared. The same procedure has been followed for 1000 ppm stock solution for isoeugenol and vanillin.

3.5.5 Inoculum Development in Liquid Media

20 g of Glucose, 1.8 g of Ammonium Tartarate, 0.5 g of Yeast Extract, 0.5 g of $MgSO_4.7H_2O$, 0.2 g of K_2HPO_4, 0.0013 g of $CaCl_2$ and 0.0025 g of Thiamin Hydrochloride were weighed on analytical balance. (Zheng et at., 2007). All the chemicals prepared were dissolved in 1 L of distilled water in Schott bottle. The liquid media was then sterilized for 20 min in autoclave. After sterilization, 250 ml of the stock media was transferred into 500 ml of conical flask. The remains media was kept inside refrigerator.

The conical flask contained liquid media was placed in laminar flow and left it for cool. 10 mycelium disks of *Aspergillus niger* at 4 mm in diameter from the agar plate that was prepared earlier were transferred into the conical flask using inoculating needle and subsequently used as an inoculum. (Tillay et al., 2010). The cultivation of *Aspergillus niger* was undertaken at 30 ℃ and 180 rpm for 48 h inside the incubator shaker.

3.5.6 Media Preparation for Vanillin Production

Another liquid media was prepared using the same chemicals as above but different amount of yeast extract. 3.0 g of yeast extract was used for this liquid media. (Zheng et al., 2007). 60 ml from the liquid media was poured into 14 conical flasks. The conical flask was covered with aluminum foil before put in autoclave. After sterilization, 5 ml from the inoculum development that was prepared earlier was used and transferred into the fourteen conical flasks. All the conical flasks were placed in incubator shaker at 30 ℃ and 180 rpm for 48 h.

After two days, 7 conical flasks were added with 120 µL of eugenol and another seven conical flasks were added with 120 µL of isoeugenol. All the conical flasks used were shaked for 7 days continuously. Each day, one conical flask was taken out and the white spore of *Aspergillus niger* in the sample separated using vacuum filter. Then, the sample solution was added with chloroform solvent inside the separative funnel. Two layers of the sample solution were formed and the bottom layer was removed and kept inside 20 mL vials. The sample solution was then filtered using syringe filter and placed into glass vial by using syringe. The sample was prepared to run under GC-FID. Appendix C shows the formation of spore from *Aspergillus niger* after transferred in liquid media.

3.6 GAS CHROMATOGRAPHY-FLAME IONIZATION DETECTOR

Qualitative GC analysis was performed on Agilent Technologies 7890A GC-FID System. Instrumental conditions used for the analysis of the sample were shown in Table 3.1 while Figure 3.1 shows the flow chart for the biotransformation process of eugenol and isoeugenol to vanillin using *Aspergillus niger*.

Table 3.1: GC Conditions

PARAMETER	SETTING DESCRIPTION	
Column	Hp-5, 30 m X 320 μm X 0.25 μm	
Injection	Injection Volume : 1 μL	
	Split Ratio : 50:1	
	Heater for front inlet : 250 ℃	
	Flow : 1mL/min	
Oven	Initial : 80 ℃	Hold Time : 1 min
	Ramp 1 : 200 ℃	Hold Time : 1 min
	Ramp 2 : 280 ℃	Hold Time : 1 min
	Run time : 13 min	
	Post run : 2 min	
Detector	Flame Ionization Detector (FID)	
Gas flow rates	FID heater : 240 ℃	
	Air flow at 350 mL/min	
	H_2 flow at 30 mL/min	
	Make up flow/N_2 at 25 mL/min	

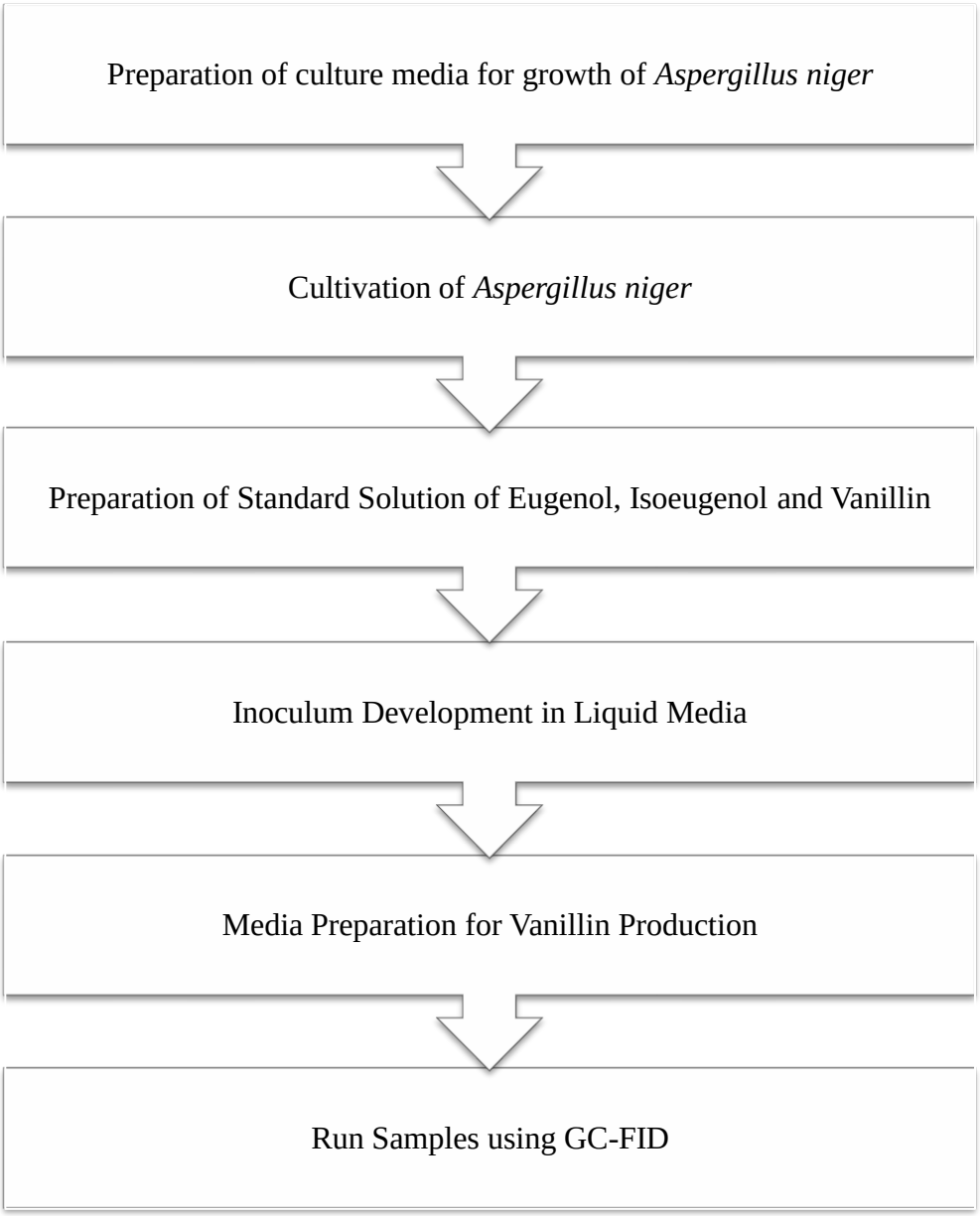


Figure 3.1: Flow Chart for Biotransformation of Eugenol and Isoeugenol to Vanillin using *Aspergillus Niger*

CHAPTER 4

RESULTS AND DISCUSSION

4.1 INTRODUCTION

In this section of this report, findings and data on the experiment conducted based on the general methodology in Chapter 3 is discussed. This section comprises data findings and discussion on the chromatogram of eugenol and isoeugenol obtained from GC-FID, the yield percentage obtained from the biotransformation of eugenol and isoeugenol to vanillin using *Aspergillus niger*, mechanism that shows how eugenol and isoeugenol converted to vanillin product.

4.2 GROWTH MEASUREMENT OF ASPERGILLUS NIGER

The cell culture for *Aspergillus niger* was done by following the steps as stated in the methodology part. The growths are measured for each day in an incubator. *Aspergillus niger* takes about four to six days to growth at suitable temperature in a petri dishes.

For inoculum development, once the mycelium was transferred into the liquid media, the observation of *Aspergillus niger* was noted. It was found out that, *Aspergillus niger* form white spore in the liquid media. Appendix A shows pictures taken during observation on growth of *Aspergillus niger*.

4.3 STANDARD CHROMATOGRAM

Internal Standard for Eugenol, Isoeugenol and Vanillin were shown in the Appendix D. From the chromatogram obtained, the peaks identification based on retention time were listed in Table 4.1.

Table 4.1: Peaks Identification for Standards

COMPOUND	RETENTION TIME(min)
Eugenol	7.006
Cis-Isoeugenol	7.322
Trans-Isoeugenol	7.609
Vanillin	7.284

All the chromatograms obtained for eugenol and isoeugeol from day 1 to day 7 were analyzed. Then, the vanillin yields obtained for each days were discussed in next section.

4.4 YIELD PERCENTAGE OF VANILLIN IN BOTH EUGENOL AND ISOEUGENOL

In this experiment the yield percentage of vanillin produced were tabulated in the Table 4.2 and Table 4.3. The GC-FID chromatograms for eugenol shown in Appendix A1-A14 while chromatogram for isoeugenol shown in Appendix B1-B14.

Table 4.2: Yield Percentage of Vanillin Obtained from Biotransformation of Eugenol.

Day	Yield (%)
1	-
2	-
3	0.14
4	0.16
5	0.22
6	-
7	-

Table 4.3: Yield Percentage of Vanillin Obtained from Biotransformation of Isoeugenol.

Day	Yield (%)
1	3.29
2	6.96
3	12.57
4	8.85
5	8.57
6	6.41
7	4.41

Based on the graph from Figure 4.1, no vanillin was produce in day 1, 2, 6, and 7 from substrate eugenol. The highest yield of vanillin was produced in day 5 which is 0.22 % and followed by day 4 where 0.16 % of vanillin obtained. Day 3 has the least yield of vanillin at 0.14 %. From the graph obtained, it can be summarized that the pure eugenol compound has been converted to vanillin compound on day 3, 4, and 5. While for day 1, 2, 6, and 7, there were no biotransformation process take place and the pure eugenol used does not transformed into any compound.

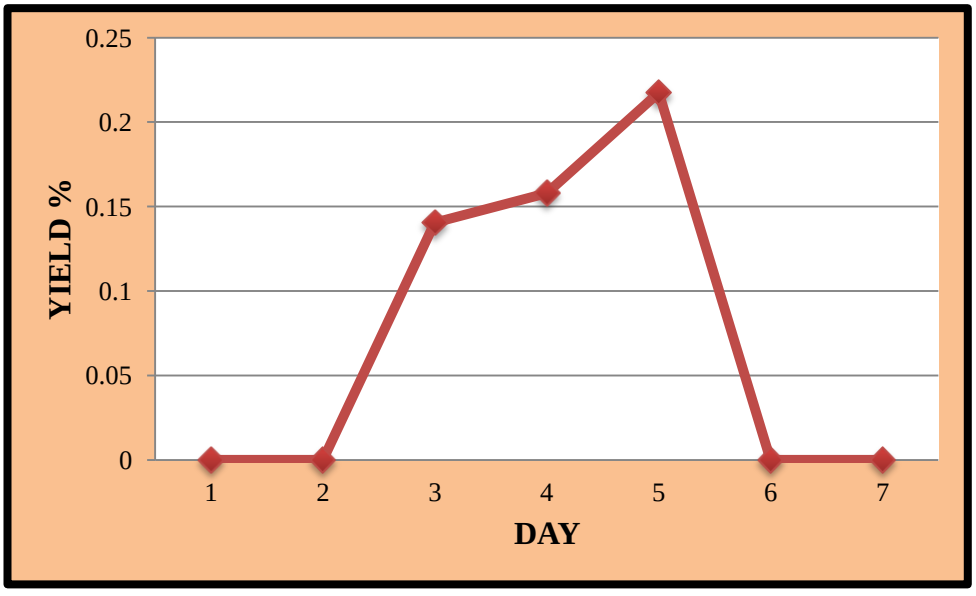


Figure 4.1: Graph Shows Yield Percentage of Vanillin Obtained after Biotransformation of Eugenol.

According to the few journals, the metabolic pathway of eugenol in *Aspergillus niger* or other fungus and bacteria generally will take a few step where in the initial reaction, it converted to coniferyl alcohol. The degradation of eugenol which proceeds via ferullic acid and vanillin has been studied in detail in *Pseudomonas*. (Overhage et al., 1999). Based on the journal written by Rabenhorst (1996), the double bond of side chain of eugenol was oxidized to coniferyl alcohol. Then, it converted to coniferyl aldehyde in small amounts. From coniferyl alcohol, it further oxidized to ferulic acid and then the final product produce was vanillin. Figure 4.2 shows the metabolic pathway proposed for the bioconversion of eugenol to vanillin in *Aspergillus niger.*

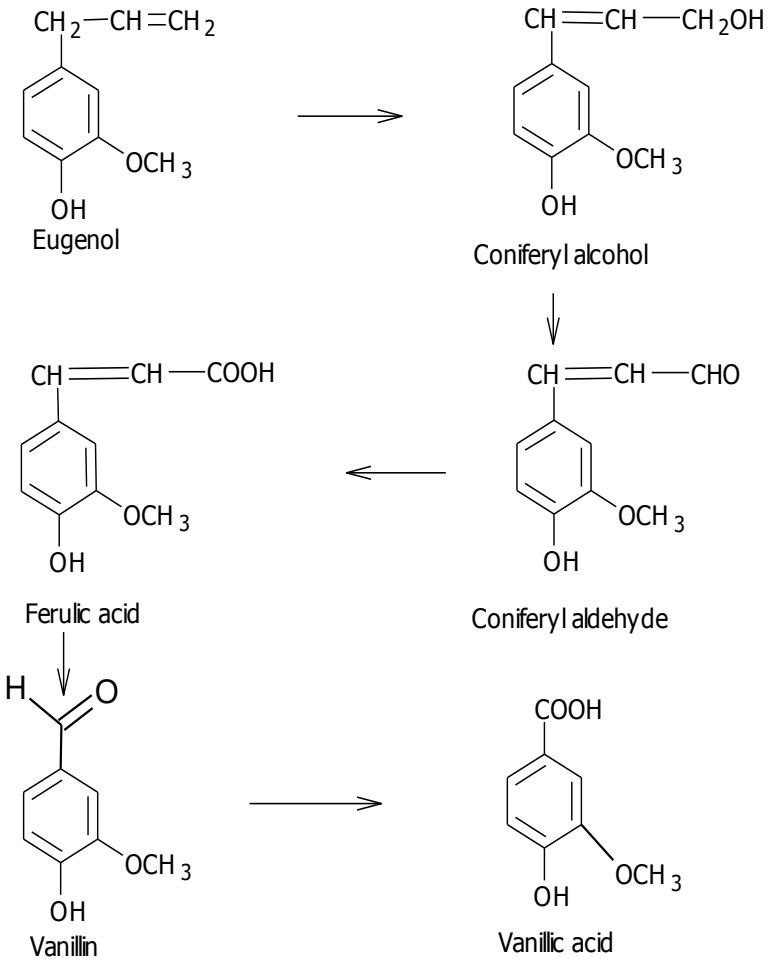


Figure 4.2: Metabolic Pathway Proposed for the Bioconversion of Eugenol to Vanillin in *Aspergillus niger*

Source: Rabenhorst, 1996; Furukawa et al. 2003.

The graph obtained in Figure 4.3 shows that highest vanillin was produced on day 3 which is 12.57 %. Start from the day 1 to the day 3, the yield percentage of vanillin has been increasing rapidly. Then, the following day from day 4 to day 7, the product of vanillin was decreasing. This shows that, the isoeugenol compound has been converted to vanillin by *Aspergillus niger* in day 1 to day 7.

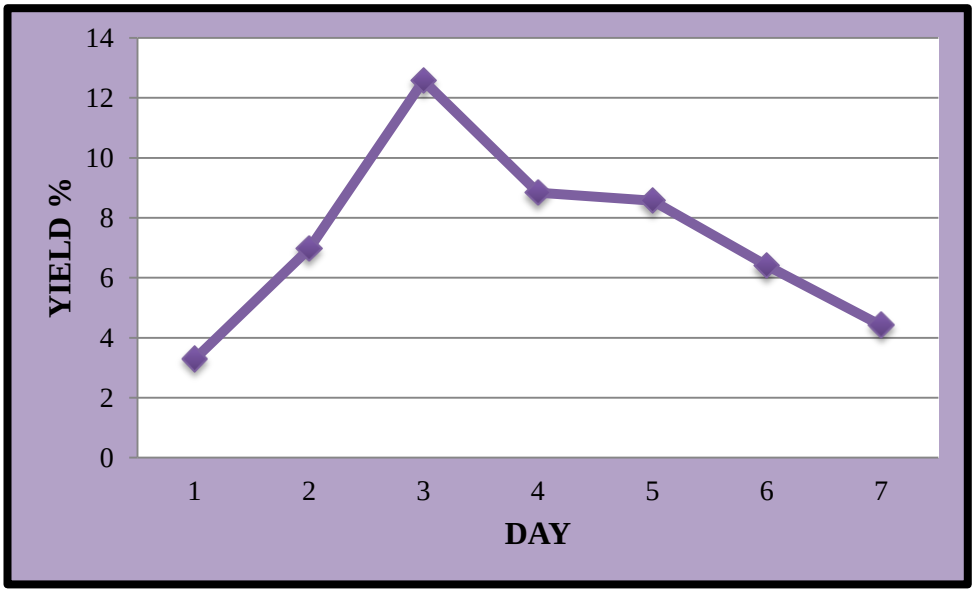


Figure 4.3: Graph Shows the Yield Percentage of Vanillin Obtained after Biotransformation of Isoeugenol

The both graphs are compared and it was found out that biotransformation of isoeugenol give the better yield of vanillin compared to eugenol. The maximum vanillin yield of 12.57 % from isoeugenol at day 3 was achieved after 72 h in 60.12 ml of reaction mixture. This showed that both substrates used were oxidized by *Aspergillus niger*. *Aspergillus niger* able to functionalize other part of molecule and since of that, it can changed and modified the structure of the eugenol and isoeugenol into vanillin.

Initially, the biotransformation of isoeugenol substrate with *Aspergillus niger* modified the structure of isoeugenol to isoeugenol epoxide. Then, the epoxide ring breaks down by the hydroxyl, nucleophilic group and formed isoeugenol diol. This hydroxyl was from the water molecules that present in the surrounding or can be from the impurities of isoeugenol. The isoeugenol diol compound modified the

structure and formed vanillin. Figure 4.4 shows metabolic pathway proposed for the bioconversion of isoeugenol to vanillin in *Aspergillus niger*. The dashed arrow represents the unidentified step.

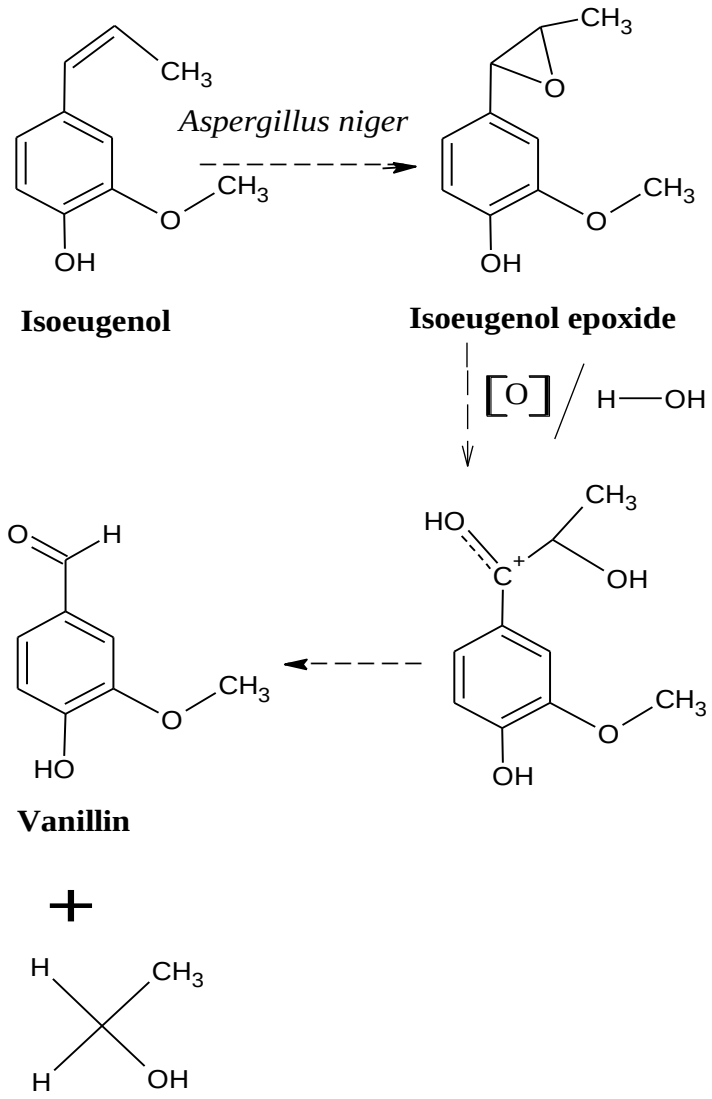


Figure 4.4: Metabolic Pathway Proposed for the Bioconversion of Isoeugenol to Vanillin in *Aspergillus niger*

Source: Hua et al. (2007)

Vanillin ($C_8H_8O_3$) is responsible for the characteristic aroma and flavor. It is the highest volume aroma chemical and ranks amongst the top aromas in terms of value. The usage of vanillin has been increasing at the beginning of the sixteenth century. Since it has been used widely especially in the industry of food and perfume, synthetic vanillin was produce to replace the natural vanillin. The shortage of vanilla beans in 1979/1980 resulted in many users switching to synthetic vanillin. The main source of synthetic vanillin was from the lignin. Lignin is chemical compound commonly derived from wood. Vanillin obtained in this way has the disadvantages that it is not a natural substance within the meaning of foodstuffs legislation. The demand of natural vanillin in the market is always higher than synthetic vanillin. As results, many researchers have been done to increase the natural vanillin and fulfill the customer requirement on the vanillin products.

According to the journal written by Abraham et al. (1988), it state that the first biotransformation of isoeugenol to vanillin was achieved with *Aspergillus niger* ATCC 9142. The efficiency of this transformation was only 10 % and gave relatively low yields. This is due to the further degradation of vanillin to vanillyl alcohol and vanillic acid. In general, it stated that, the percentage of vanillin is quite low because the expected compound undergoes further oxidation and forming vanillic acid. (Li and Rosazza, 2000). Therefore, it can be say that, isoeugenol at day 3 produces high yield and the day after 72 h the percentage of vanillin reduced because the fungus may further oxidized the vanillin compound to form vanillin acid.

Based on journal written by Hua et al. (2007), adding the substrate isoeugenol directly to the immature culture is not recommended since isoeugenol is toxic to bacterial cells. In this project, vanillin production was performed by adding isoeugenol to stationary phase culture. After, 48 h cultivation, the substrate isoeugenol was added into the culture. Isoeugenol was converted to vanillin on the first day itself. At first, vanillin was gradually accumulated at high until day 3 and after reached at maximal point, the concentration of vanillin immediately declined. The yield percentage of vanillin reduced on fourth day onwards until day 7.

Based on this experiment, it was identified that the yield of vanillin produce were low and the time consuming for the biotransformation process are long. This might be due to the metabolic pathway of the eugenol and isoeugenol to convert to vanillin take a long step. According to the journal written by Rabenhorst and Hopp (1977), they have done research producing natural vanillin using ferulic acid and found out only bioconversion of ferulic acid has been developed to an economically feasible process. However, the use of ferulic acid as the substrate of the bioconversion is expensive. (Ashengroph et al., 2008). Therefore, a much cheaper substrate such as eugenol and isoeugenol was used. (Overhage et al., 2006).

For this project, the parameter such as the duration of eugenol and isoeugenol taken to convert to vanillin product, initial concentration of substrate and pH condition of the media were optimized. During this biotransformation, the highest percentage of vanillin produce from the substrate eugenol was 0.22 % and for isoeugenol was 12.57 %.

CHAPTER 5

CONCLUSION

5.1 INTRODUCTION

In this chapter, the conclusion of the study has been discussed where all the overall research done is summarized. It includes the general significance of the research to the industry and the approach on whether research carried out met the objectives of the study.

5.2 CONCLUSION

The objectives of this study have successfully achieved. At the end of the project I able to carry out the biotransformation process and produce natural vanillin from eugenol and isoeugenol using *Aspergillus niger*, learned techniques of preparing the culture and growth the *Aspergillus niger*. Besides, from this project I finally characterized the compounds produce by chromatographic technique using GC-FID.

Based on this research, it was proved that natural vanillin can be produce throughout bioconversion method even though the yield produces was relatively low. Since the source of eugenol and isoeugenol is low price, it is encourage to do more research on producing vanillin and give high yield of percentage. The product of vanillin will be more demand if it is a natural vanillin. *Aspergillus niger* is useful biocatalyst for producing vanillin from eugenol and isoeugenol. It provides very important solution for chemical industry going green. Therefore, all researches that

based on the producing vanillin will absolutely be useful for industries such as food industry, perfume industry and pharmaceutical industry.

5.3 RECOMMENDATIONS FOR FUTURE RESEARCH

Aspergillus niger has converted eugenol and isoeugenol substrate to vanillin compound. For the better results, it is recommended to do further investigation on biotransformation process to increase the yield of vanillin. The calculation of Kovats Index based on standard carbon is also recommended to use to identify the presence of other compounds in the sample. Besides that, another suggestion can be given by running the biotransformation process using other microorganisms such as fungus or bacteria. This is because, different microorganism will have different bioconversion reaction of eugenol and isoeugenol thus it may produce different yield of vanillin compound.

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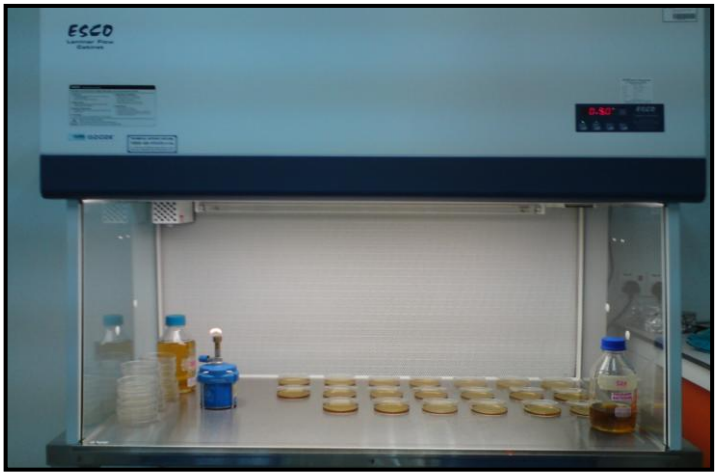
APPENDIX A

Appendix A1: SDA Media Preparation for *Aspergillus niger* inside Laminar Flow

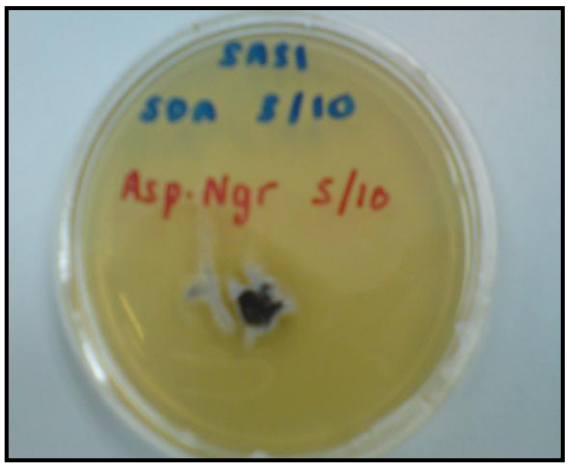


Appendix A2: SDA Media in Petri Dishes

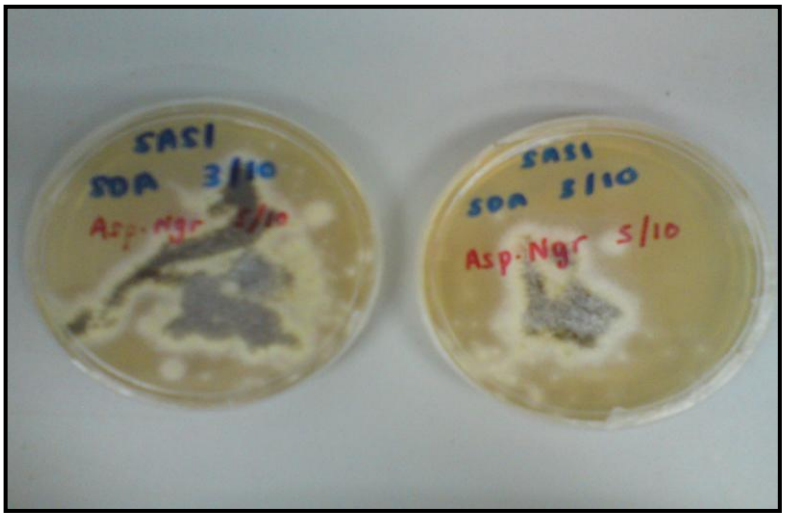
Appendix A3: Cell Culture of *Aspergillus niger* in Universal Bottle

Appendix A4: Cell Culture of *Aspergillus niger* kept inside Incubator

Appendix A5: Cell Culture of *Aspergillus niger* in Petri Dish



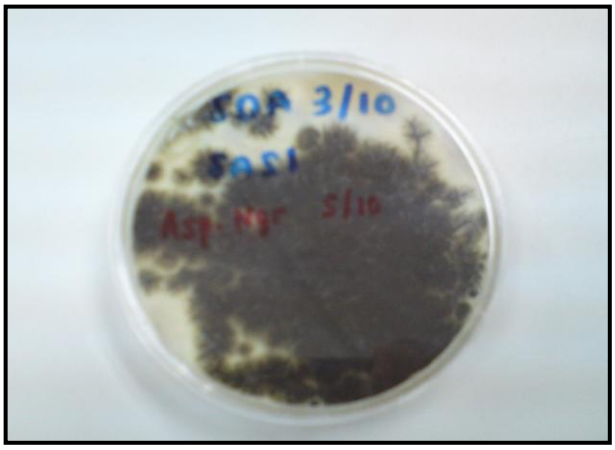
Appendix A6: Cell Culture of *Aspergillus niger* at Day 3

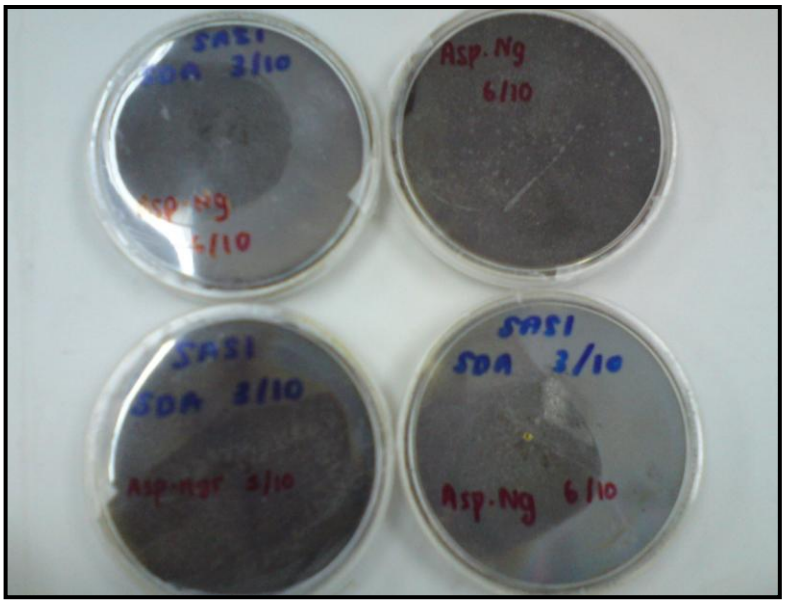


Appendix A7**:** Cell Culture of *Aspergillus niger* at Day 4

Appendix A8**:** Cell Culture of *Aspergillus niger* at Day 5

Appendix A9: Cell Culture of *Aspergillus niger* at Day 6

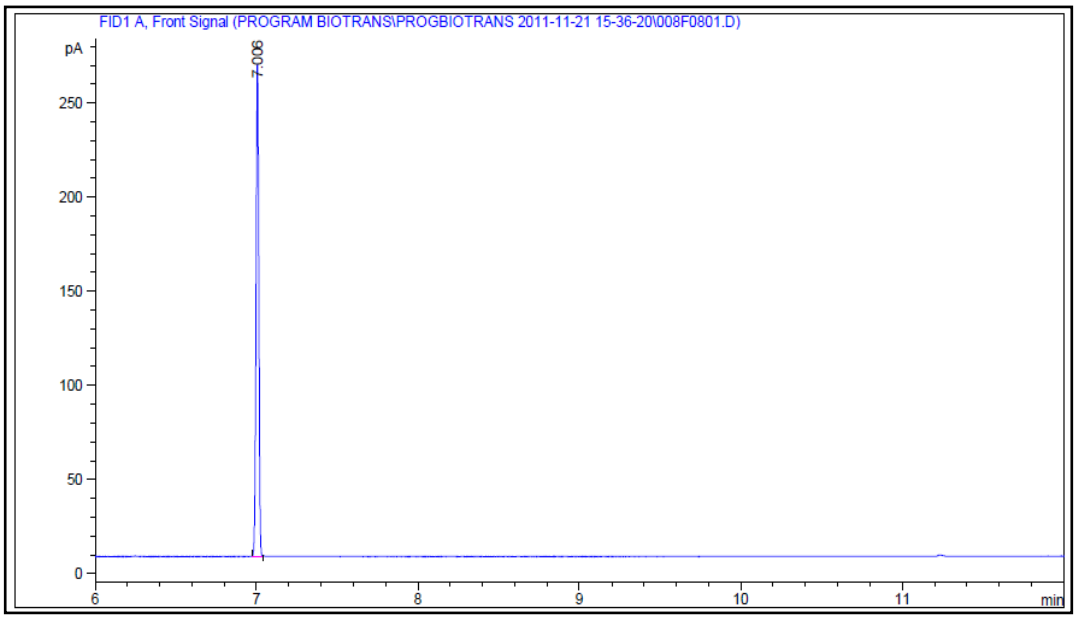




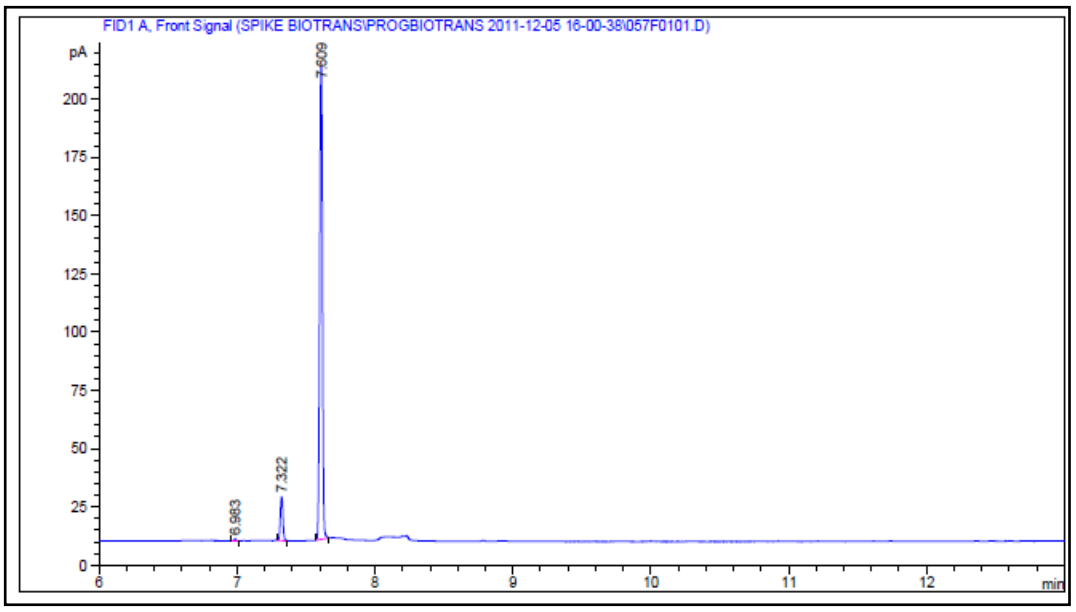
Appendix A10 : *Aspergillus niger* Spore in Inoculum Development of Liquid Media

APPENDIX B

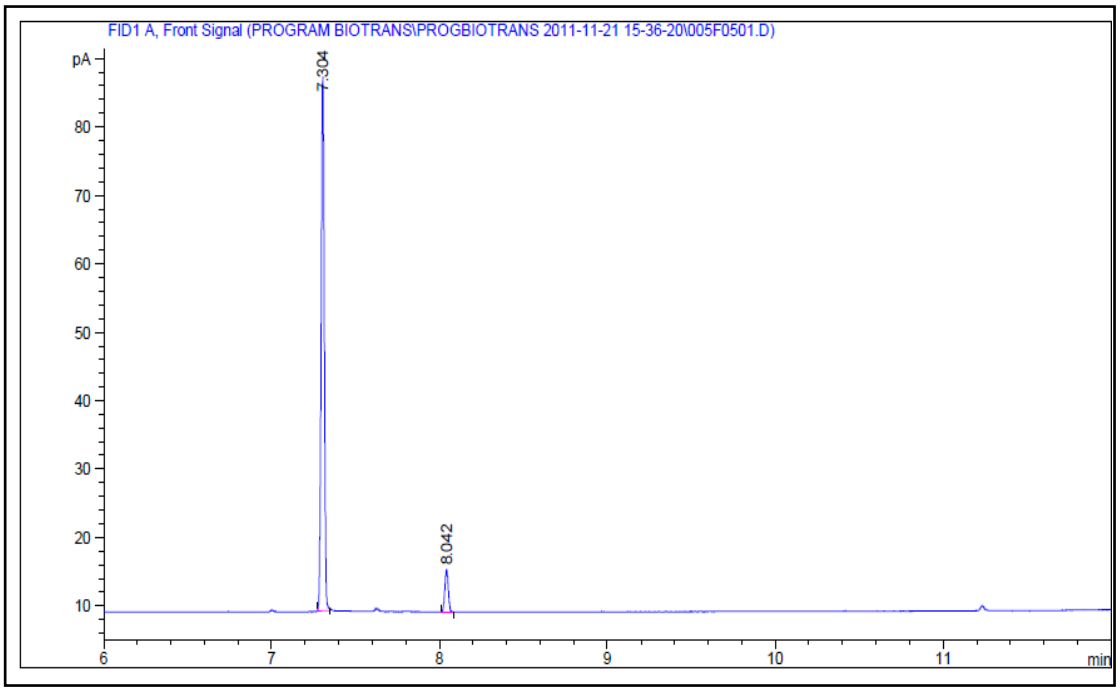
Appendix B1: Standard Eugenol



Appendix B2: Standard Isoeugenol

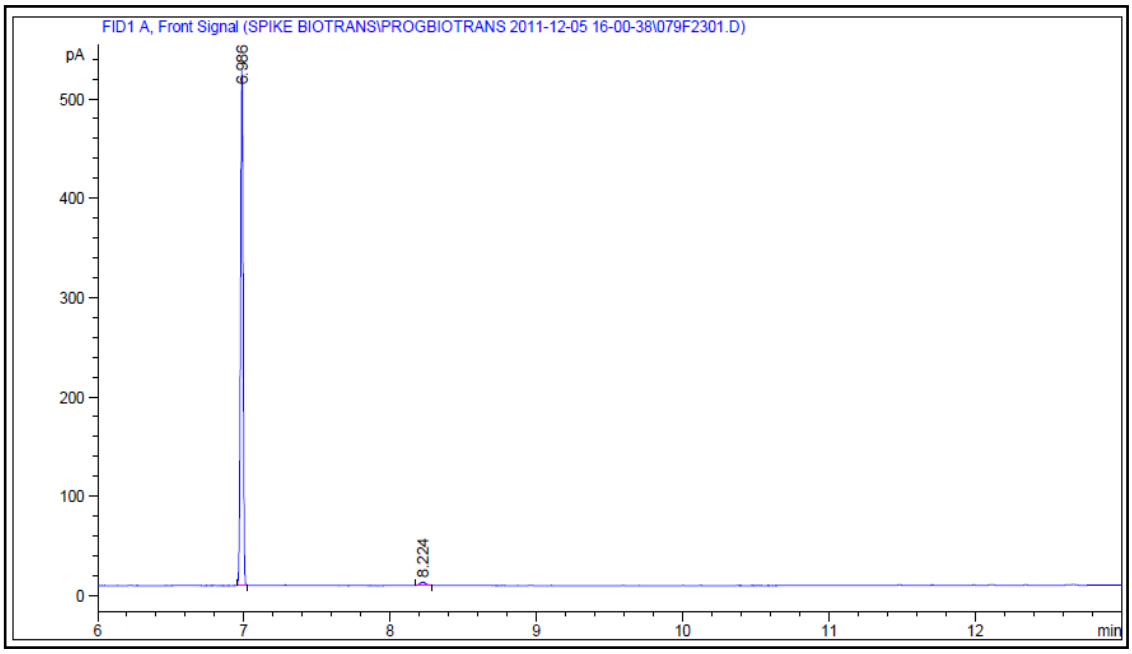


Appendix B3: Standard Vanillin

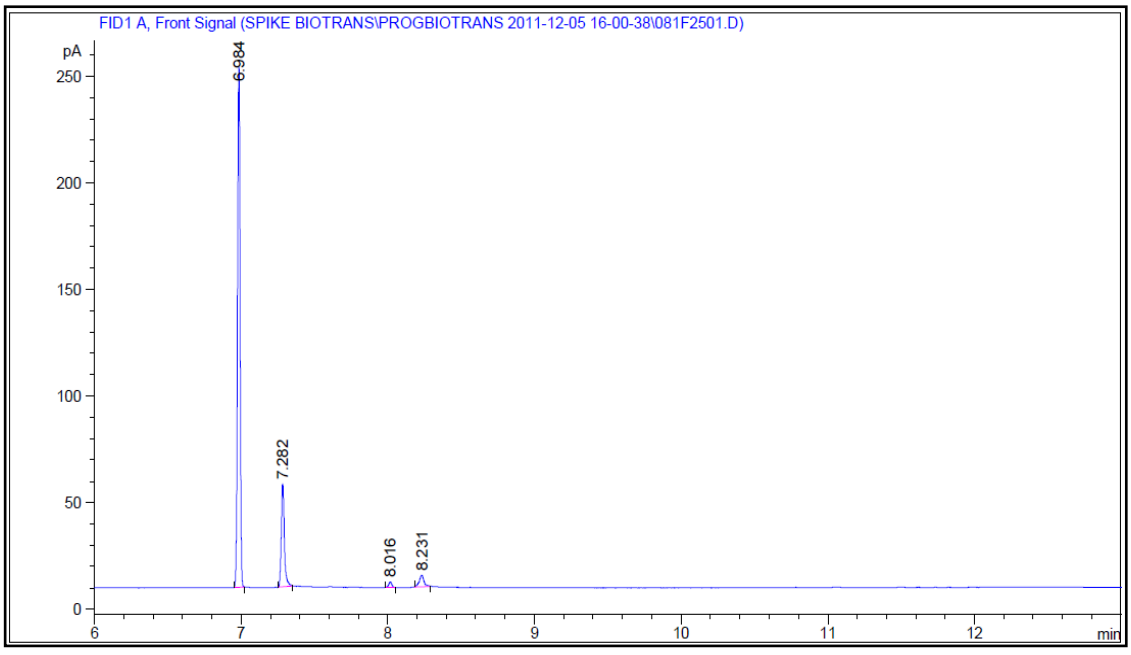


APPENDIX C

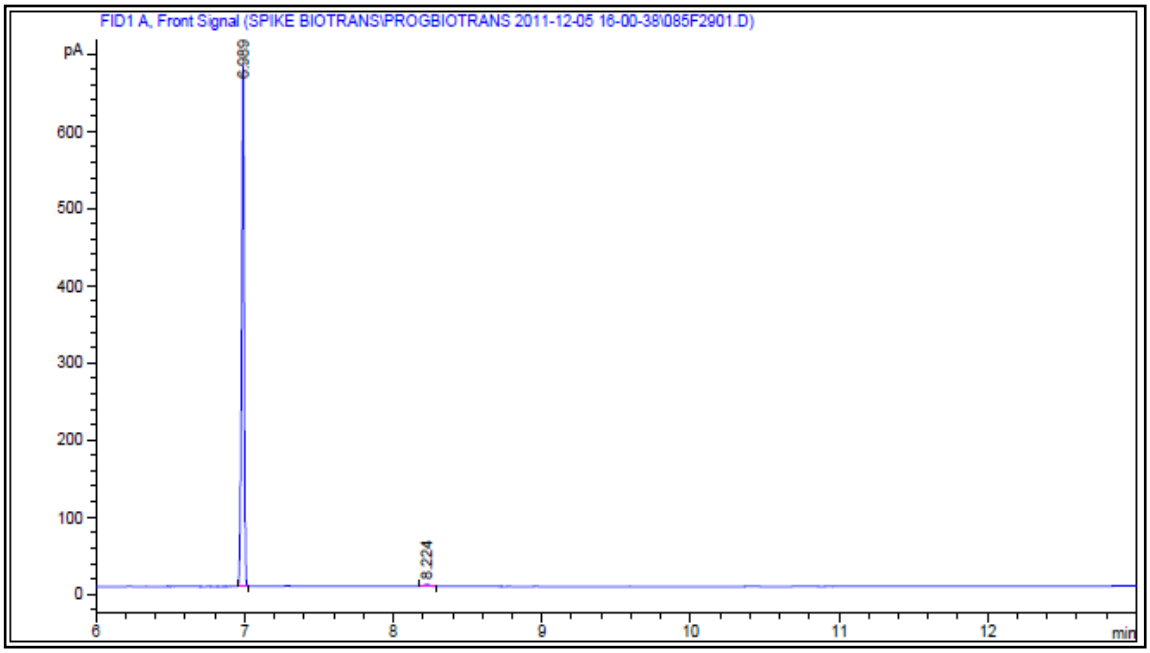
Appendix C1: Eugenol Day 1



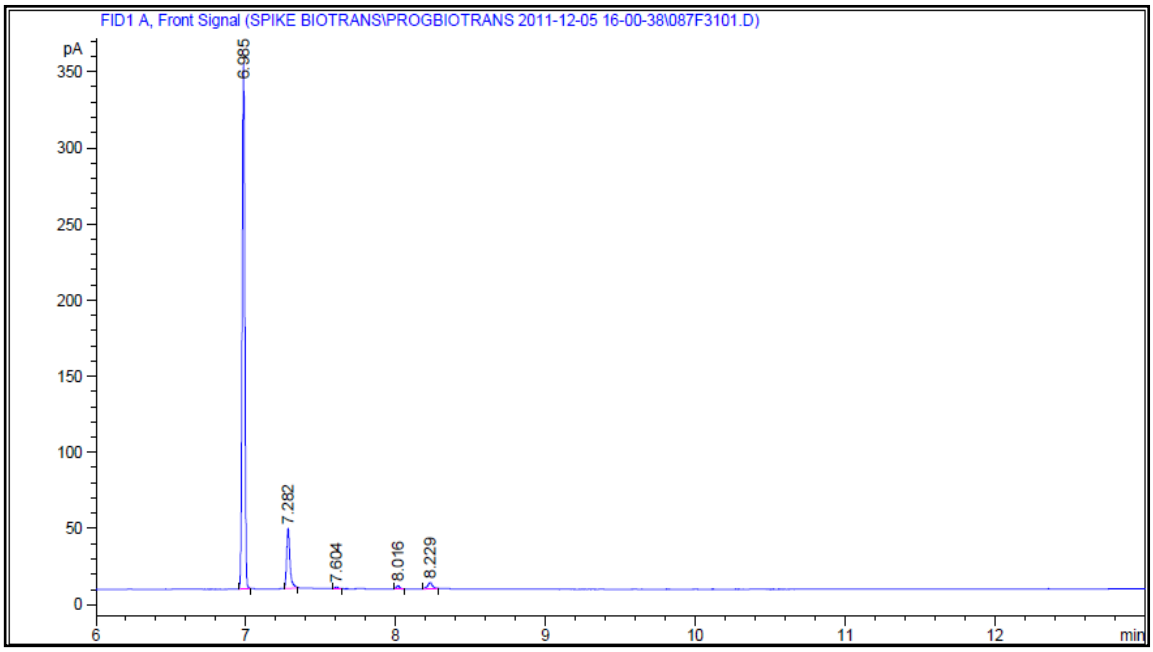
Appendix C2: 50 % Spike Vanillin in Eugenol Day 1



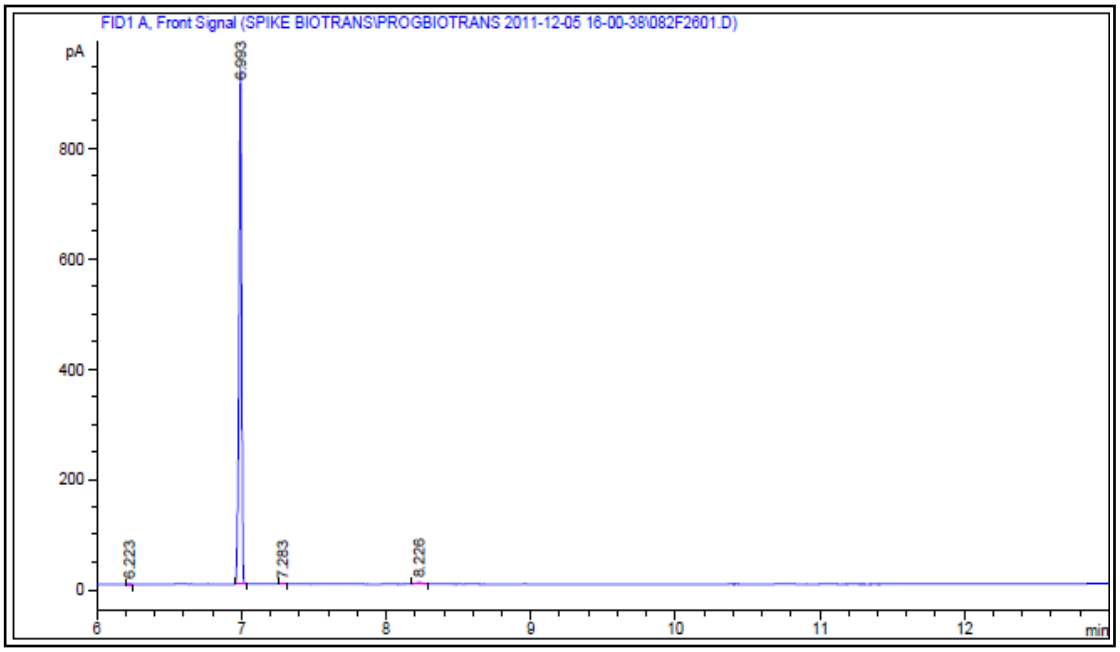
Appendix C3: Eugenol Day 2



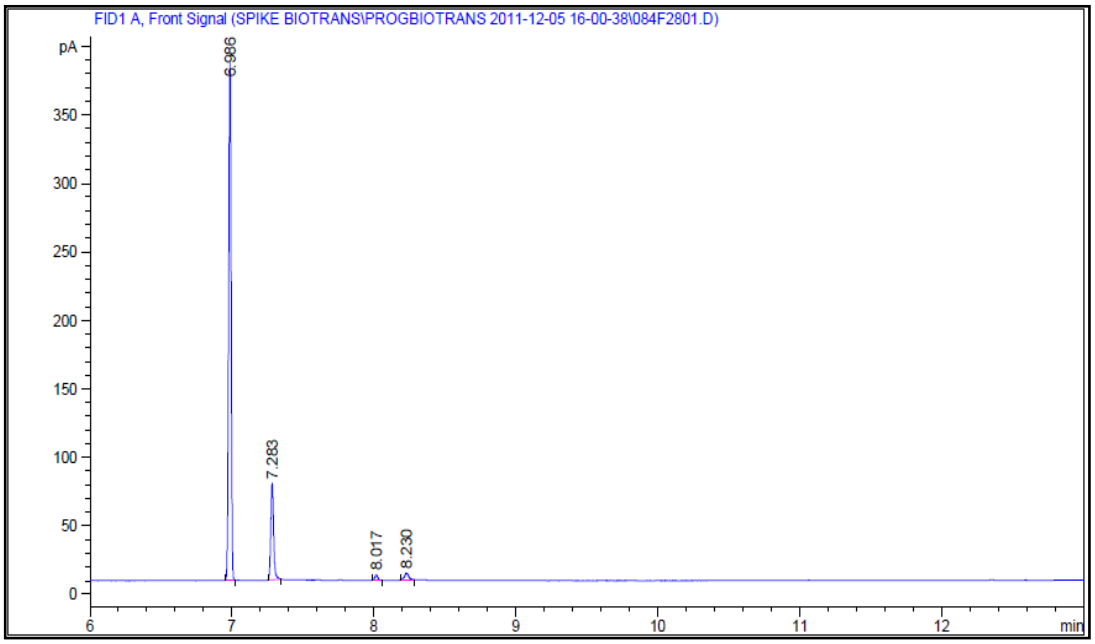
Appendix C4: 50 % Spike Vanillin in Eugenol Day 2



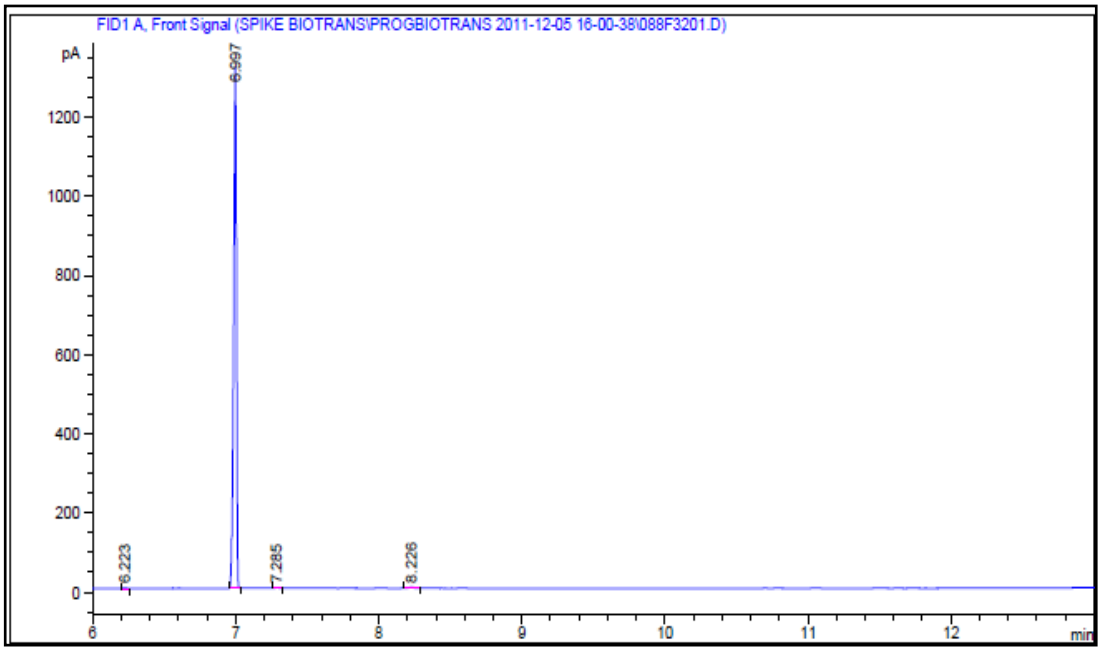
Appendix C5: Eugenol Day 3



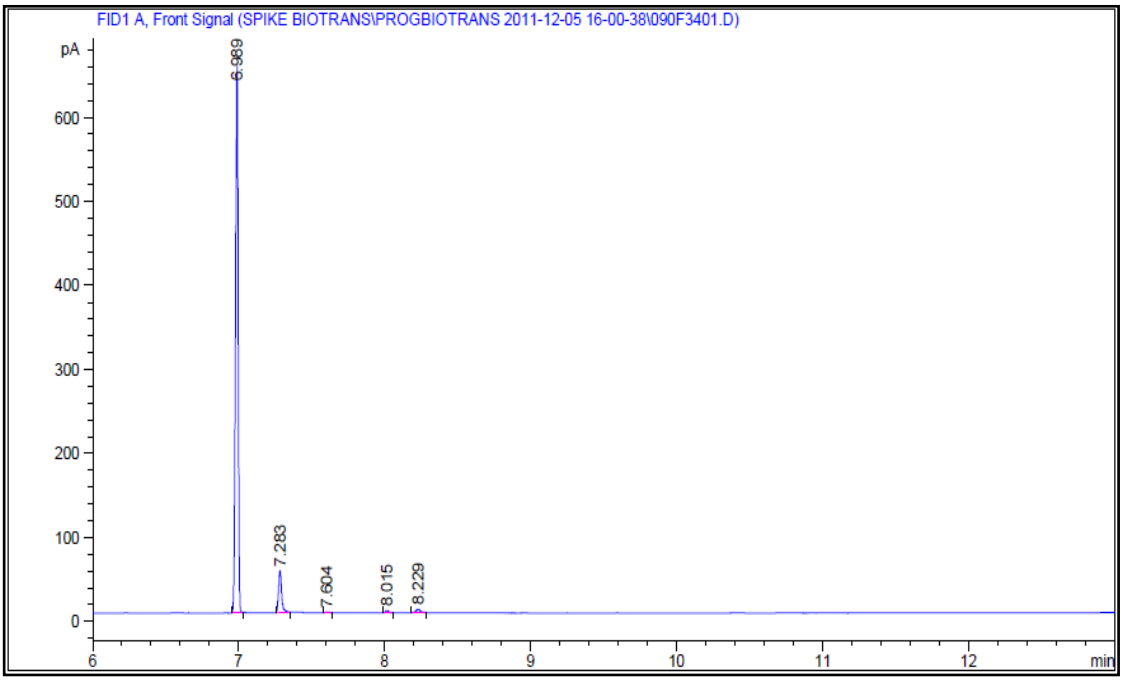
Appendix C6: 50 % Spike Vanillin in Eugenol Day 3



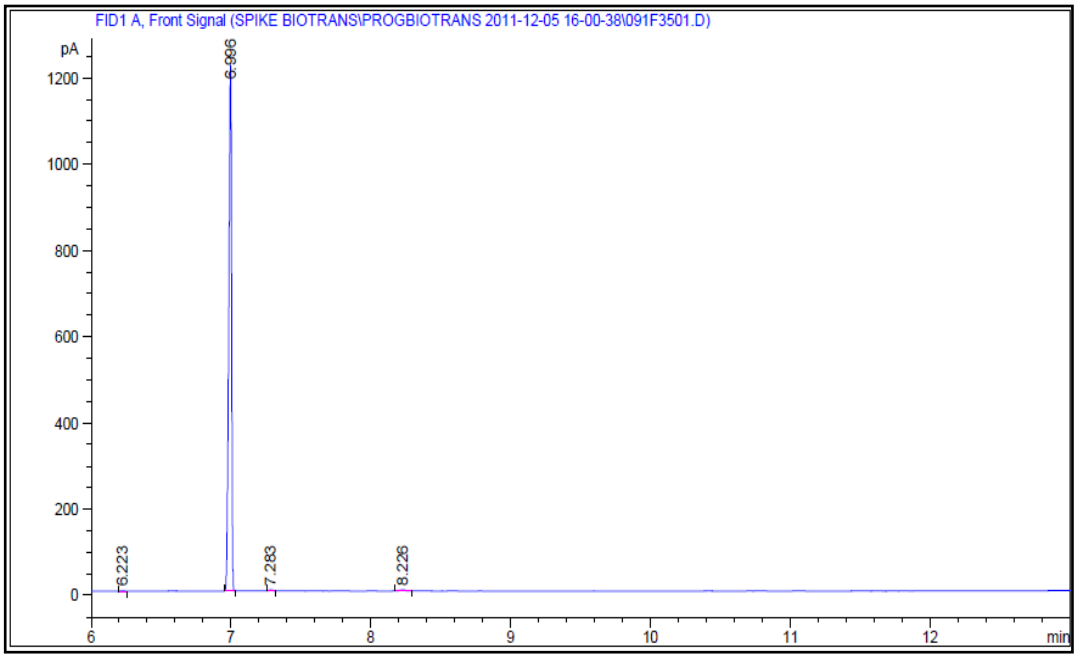
Appendix C7: Eugenol Day 4



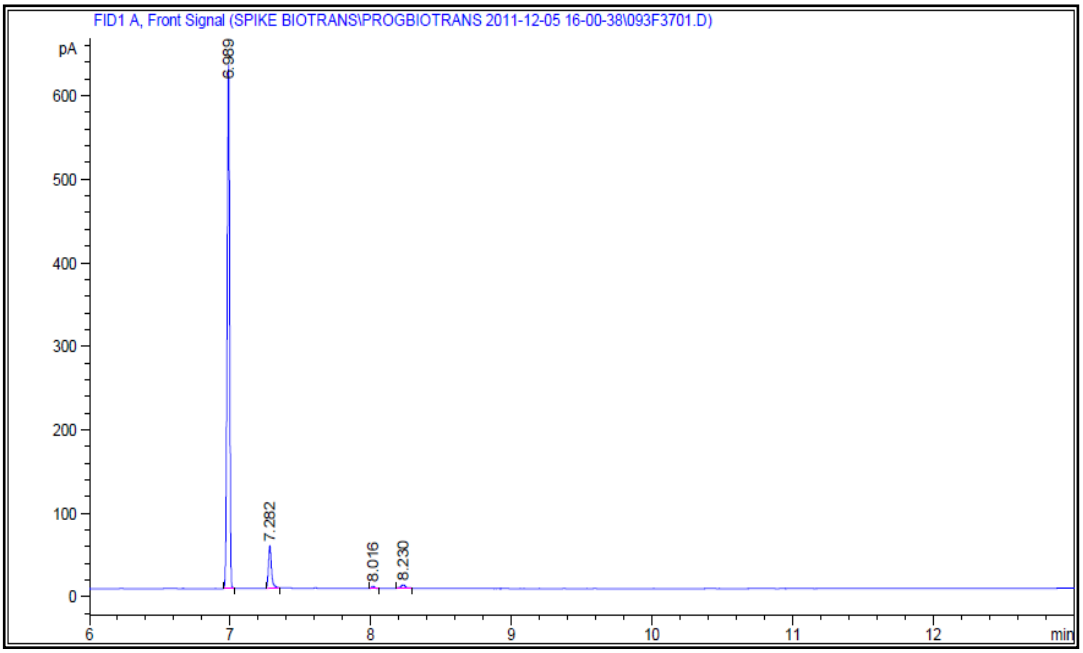
Appendix C8: 50 % Spike Vanillin in Eugenol Day 4



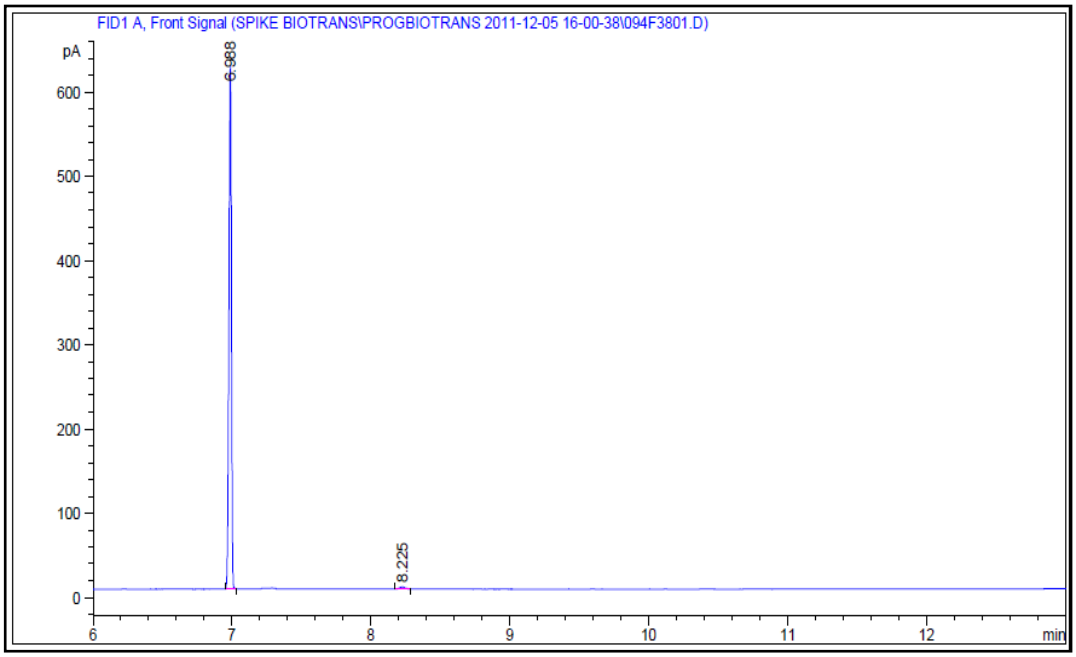
Appendix C9: Eugenol Day 5



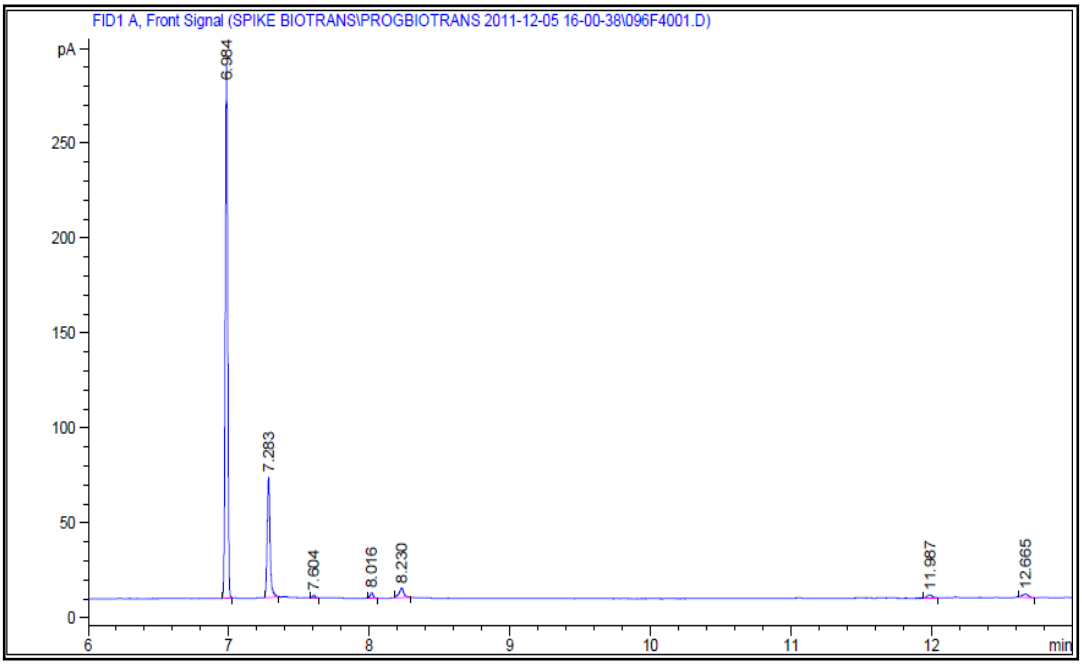
Appendix C10: 50 % Spike Vanillin in Eugenol Day 5



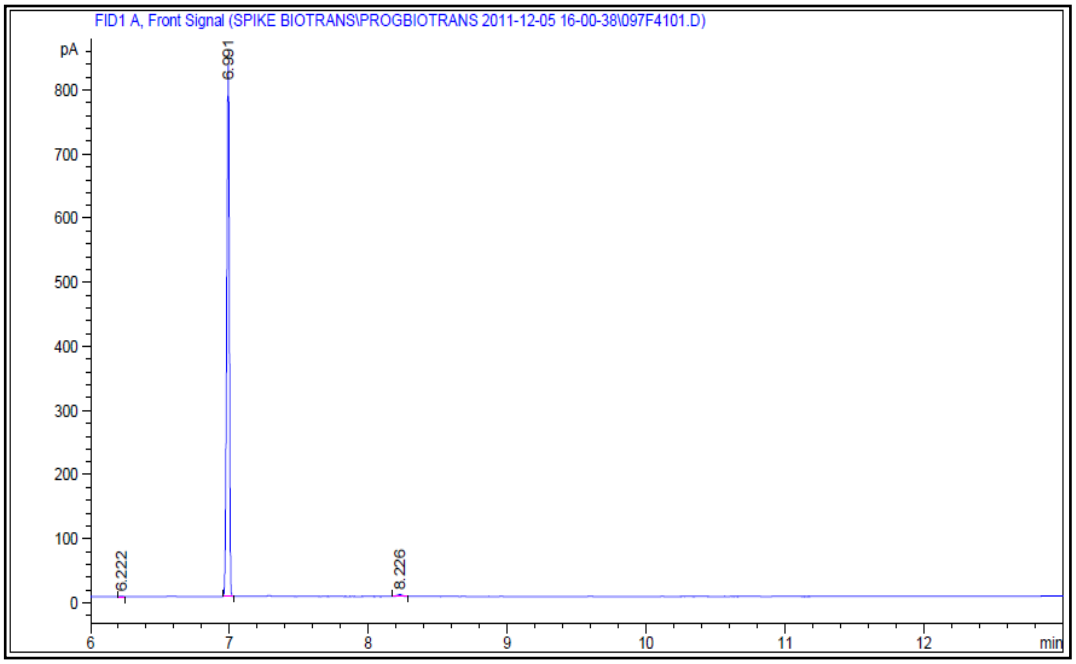
Appendix C11: Eugenol Day 6



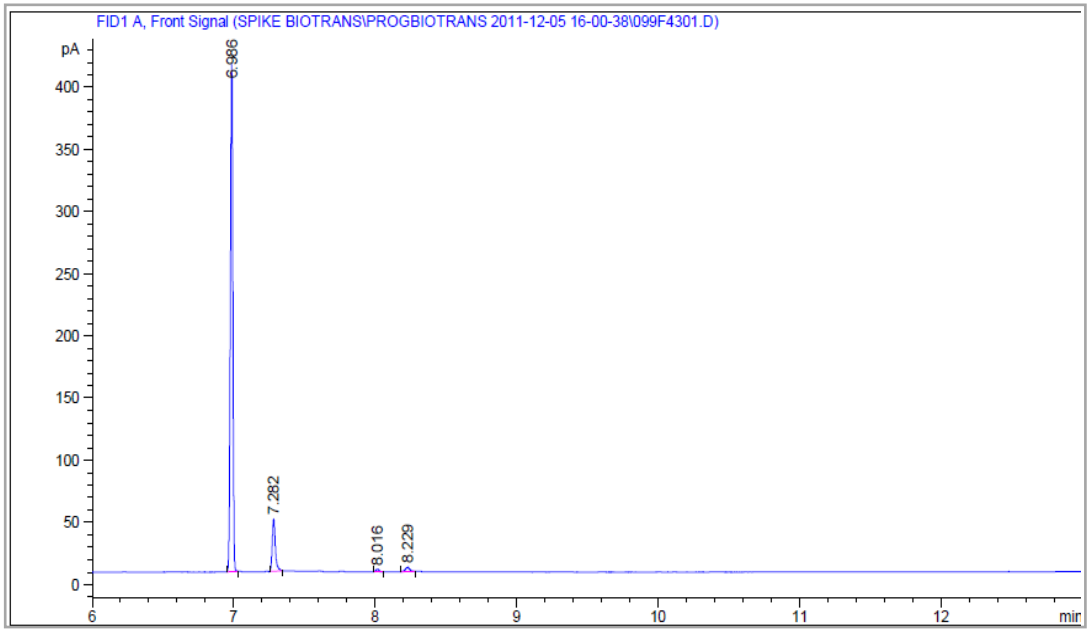
Appendix C12: 50 % Spike Vanillin in Eugenol Day 6



Appendix C13: Eugenol Day 7

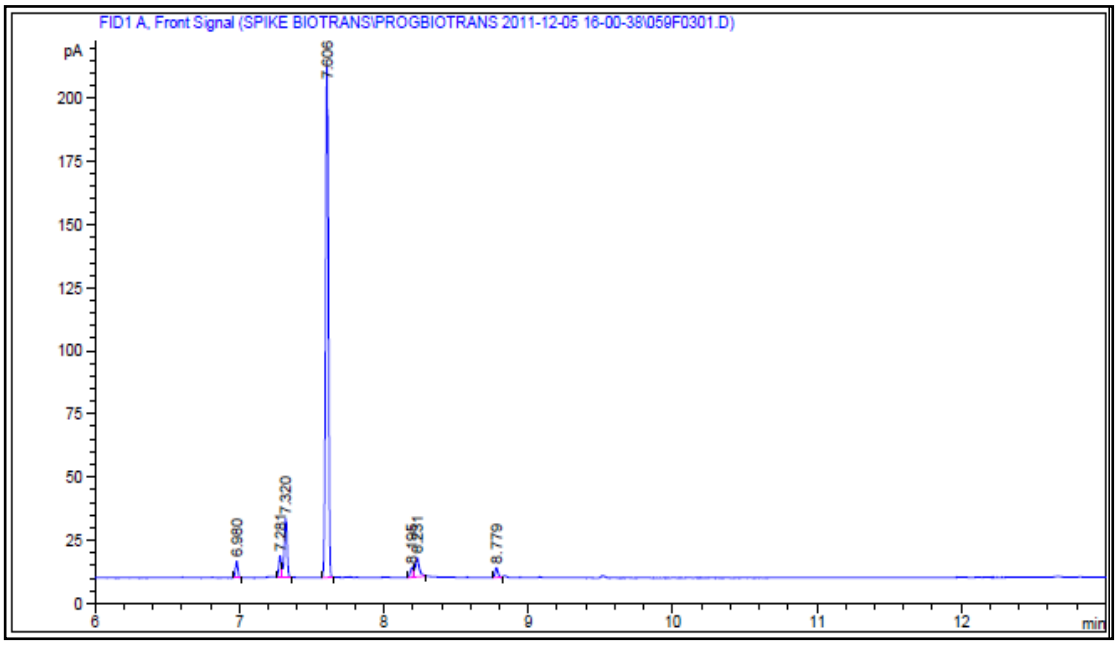


Appendix C14: 50 % Spike Vanillin in Eugenol Day 7

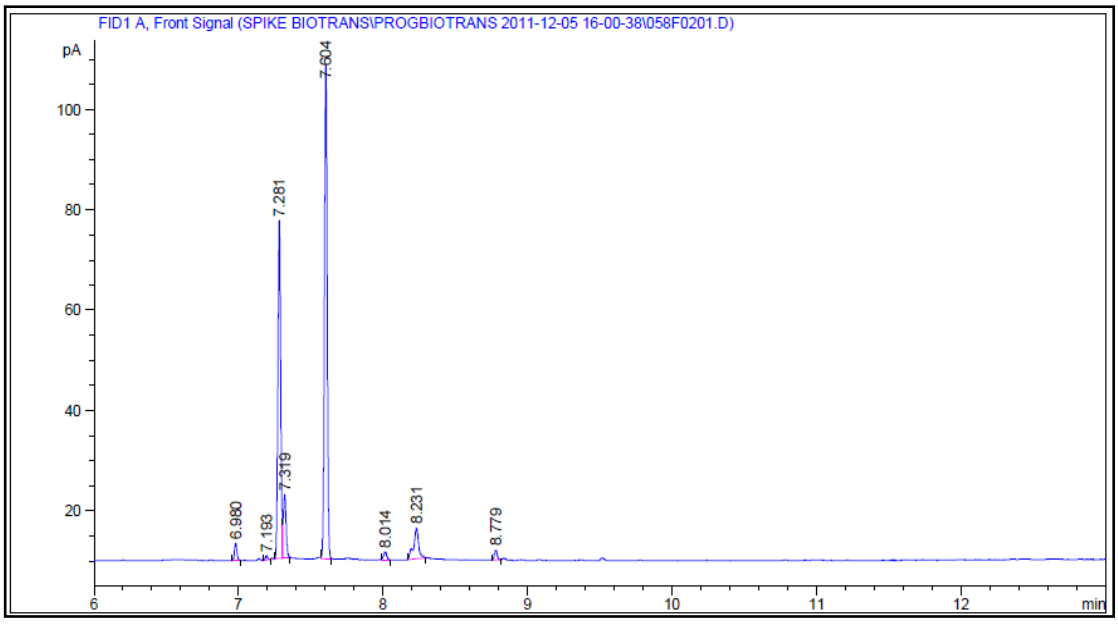


APPENDIX D

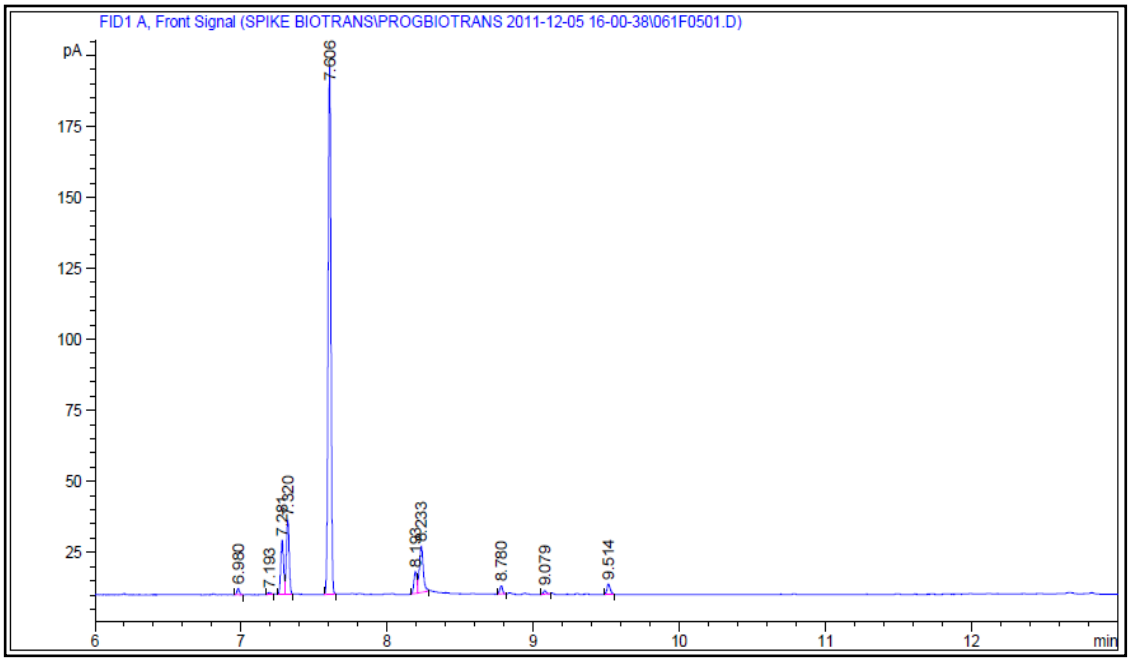
Appendix D1: Isoeugenol Day 1



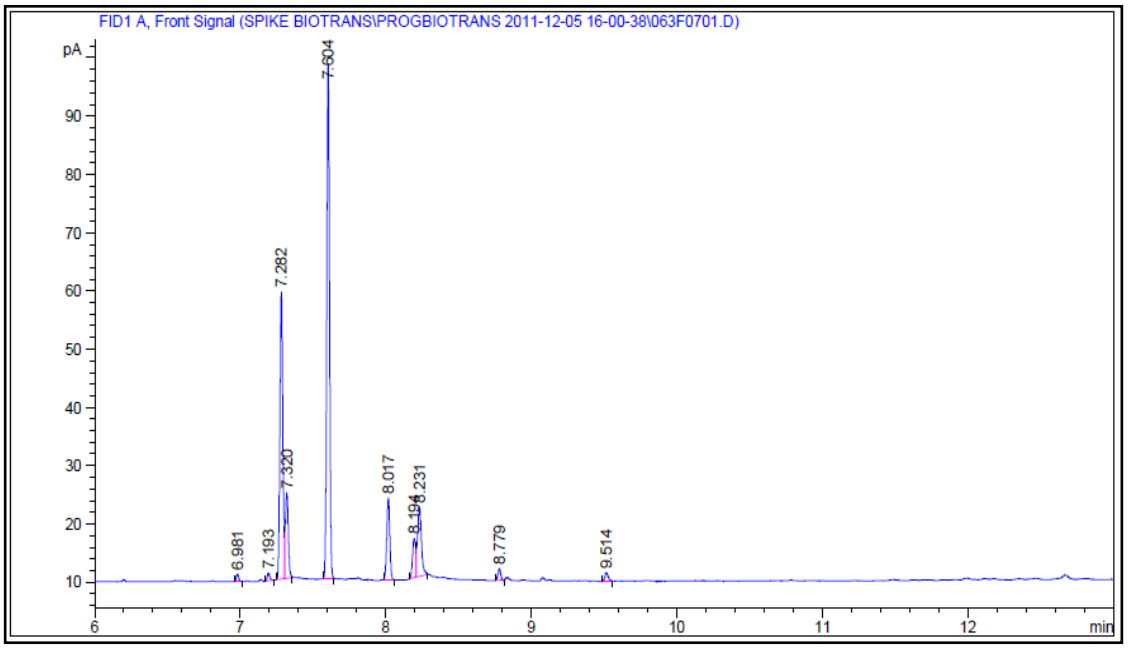
Appendix D2: 50 % Spike Vanillin in Isoeugenol Day 1



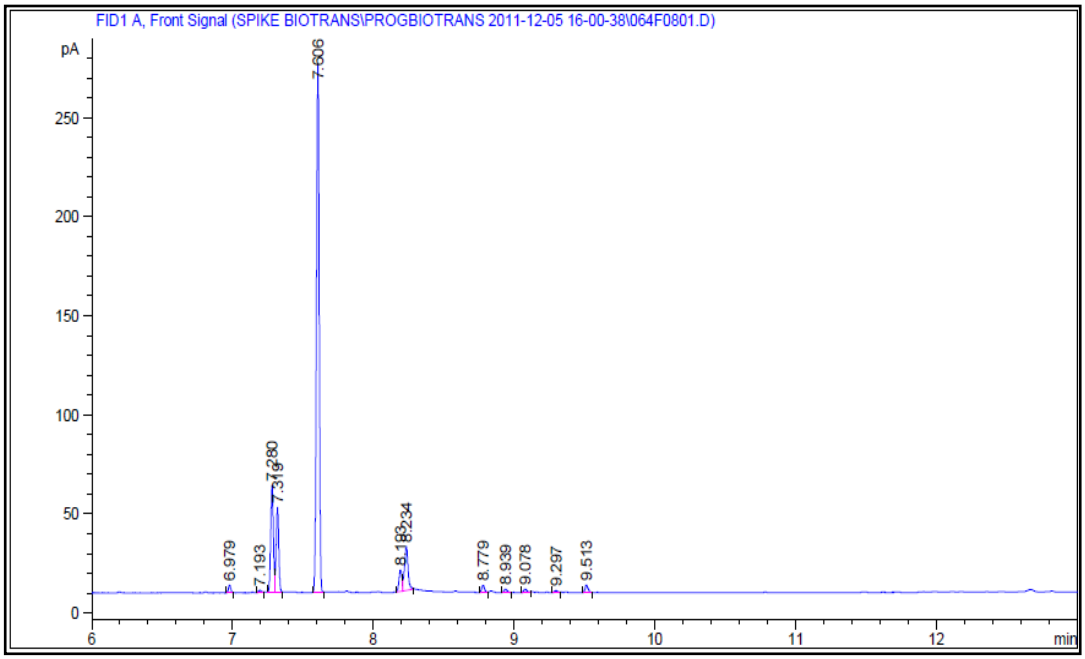
Appendix D3: Isoeugenol Day 2



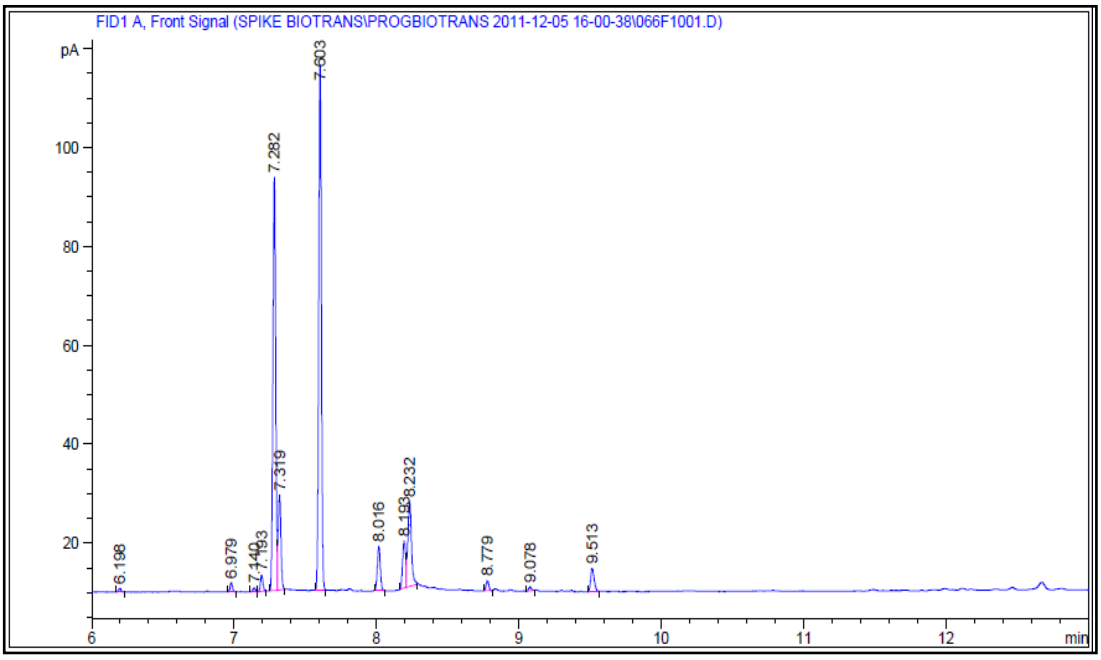
Appendix D4: 50 % Spike Vanillin in Isoeugenol Day 2



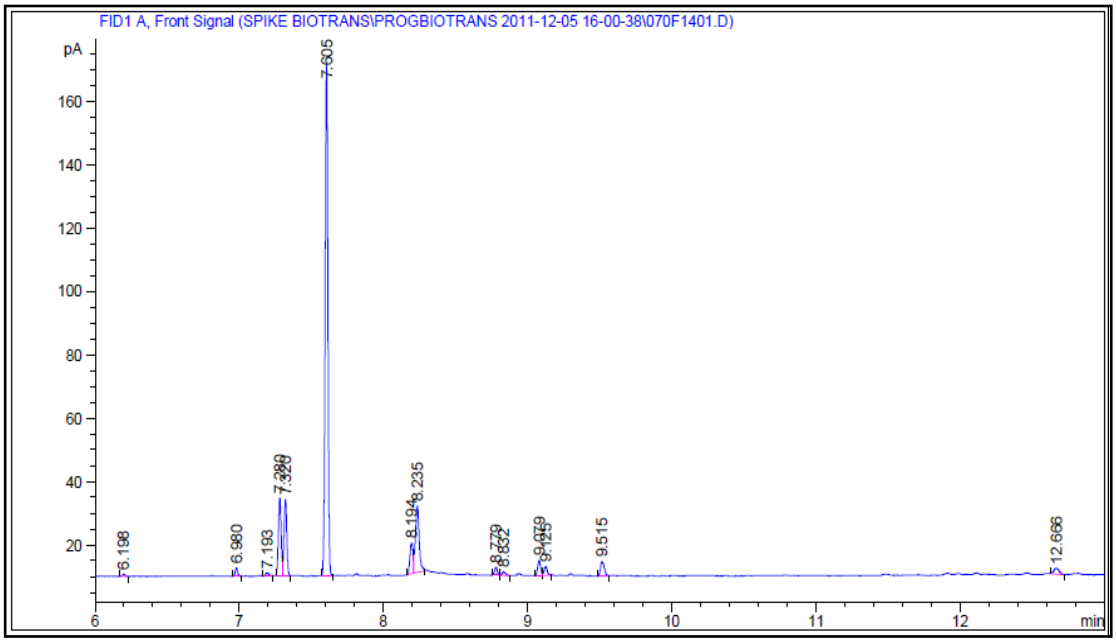
Appendix D5: Isoeugenol Day 3



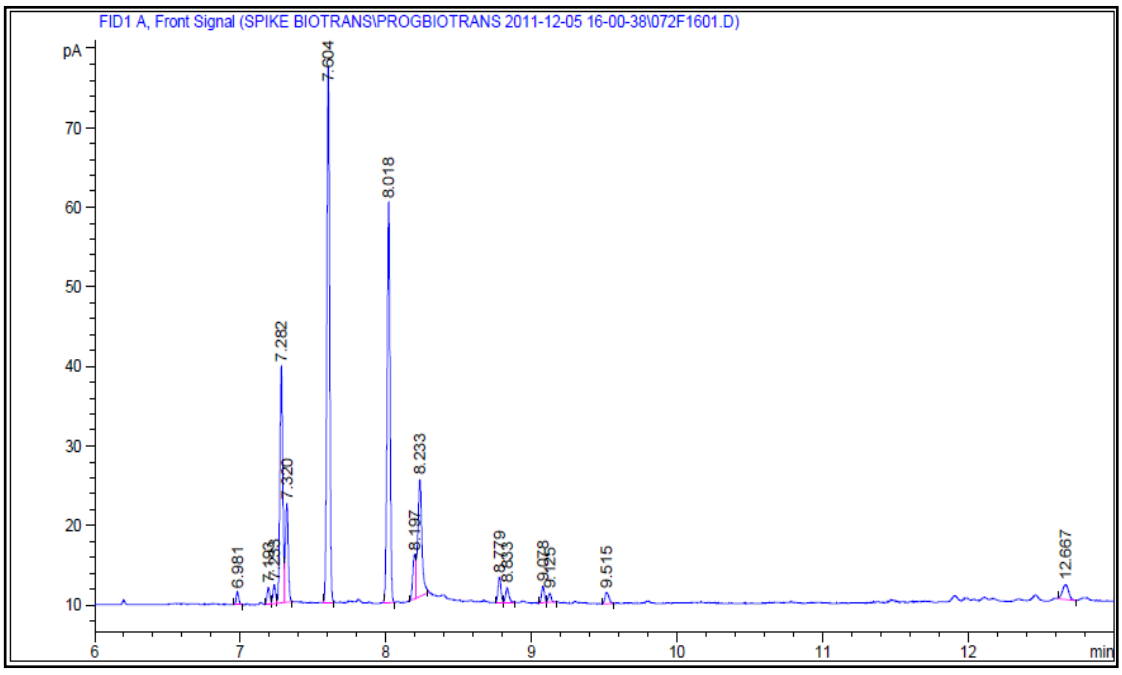
Appendix D6: 50 % Spike Vanillin in Isoeugenol Day 3



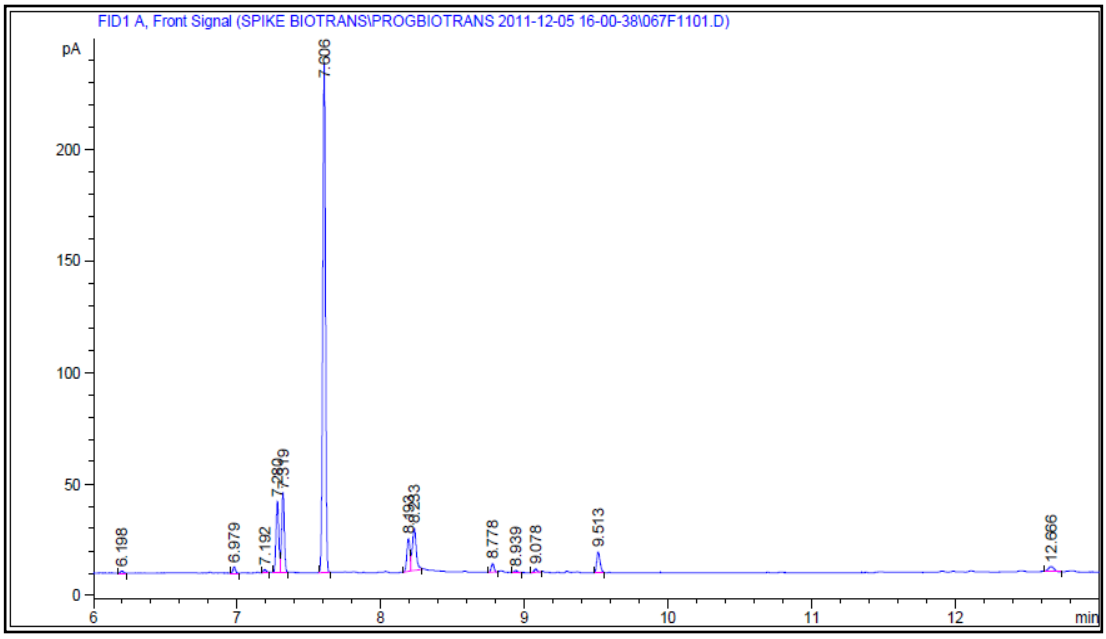
Appendix D7: Isoeugenol Day 4



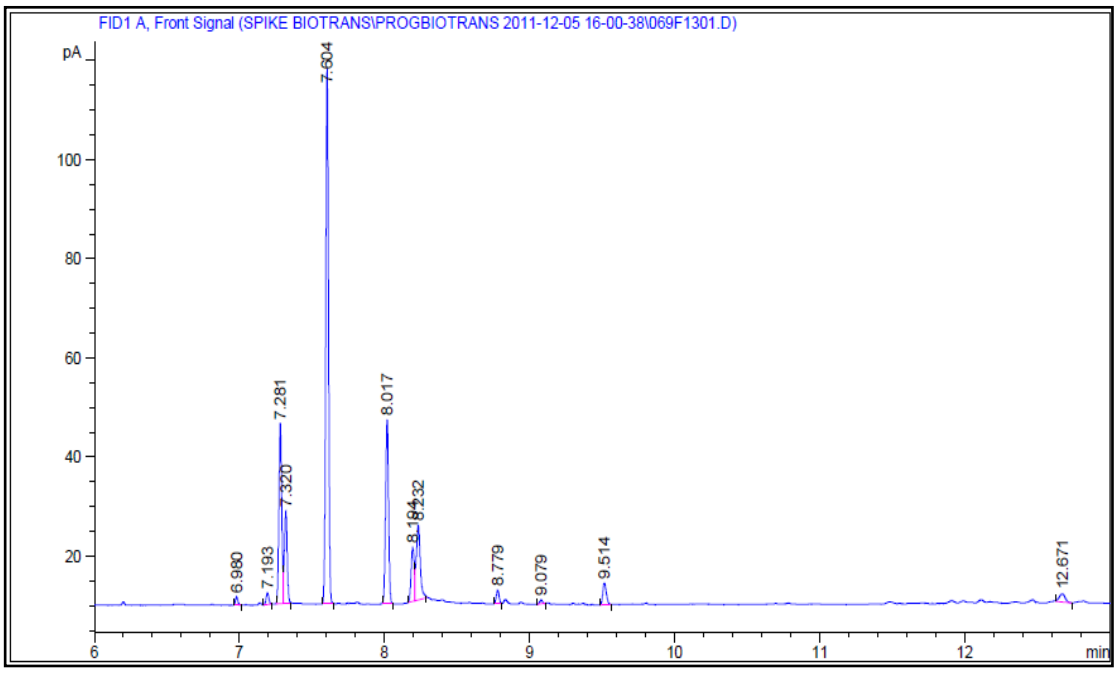
Appendix D8: 50 % Spike Vanillin in Isoeugenol Day 4



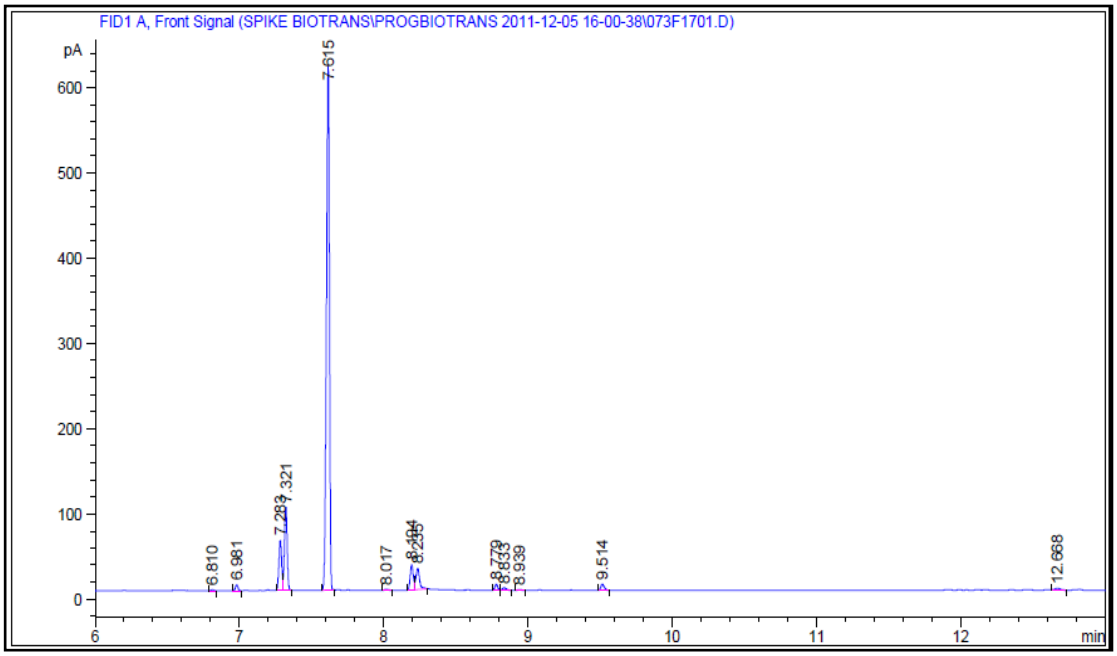
Appendix D9: Isoeugenol Day 5



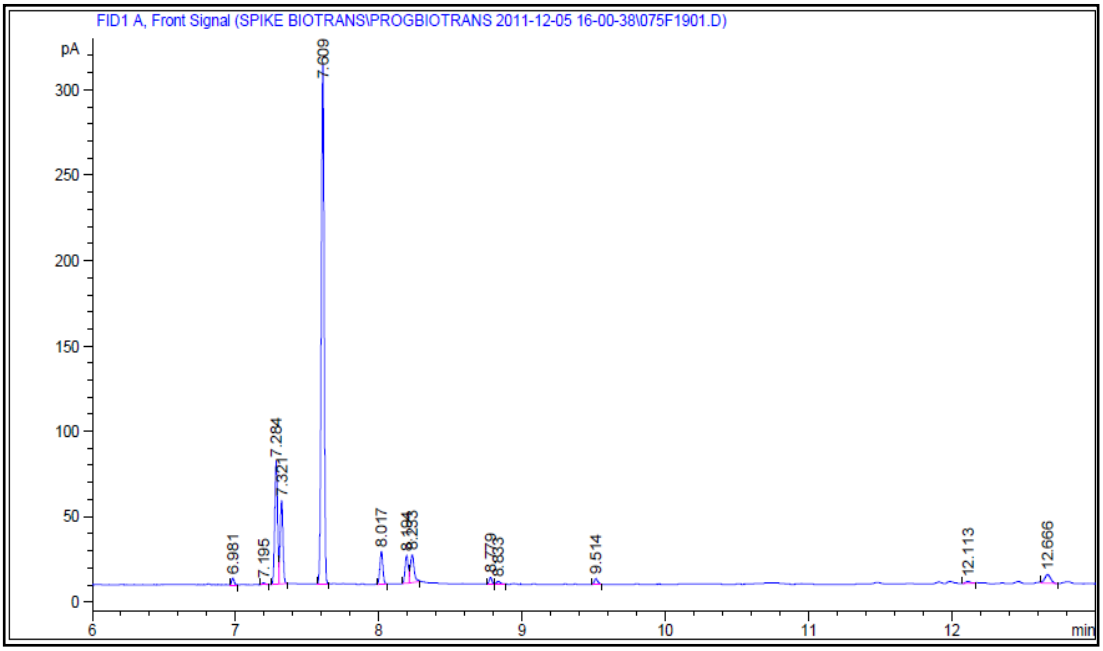
Appendix D10: 50 % Spike Vanillin in Isoeugenol Day 5



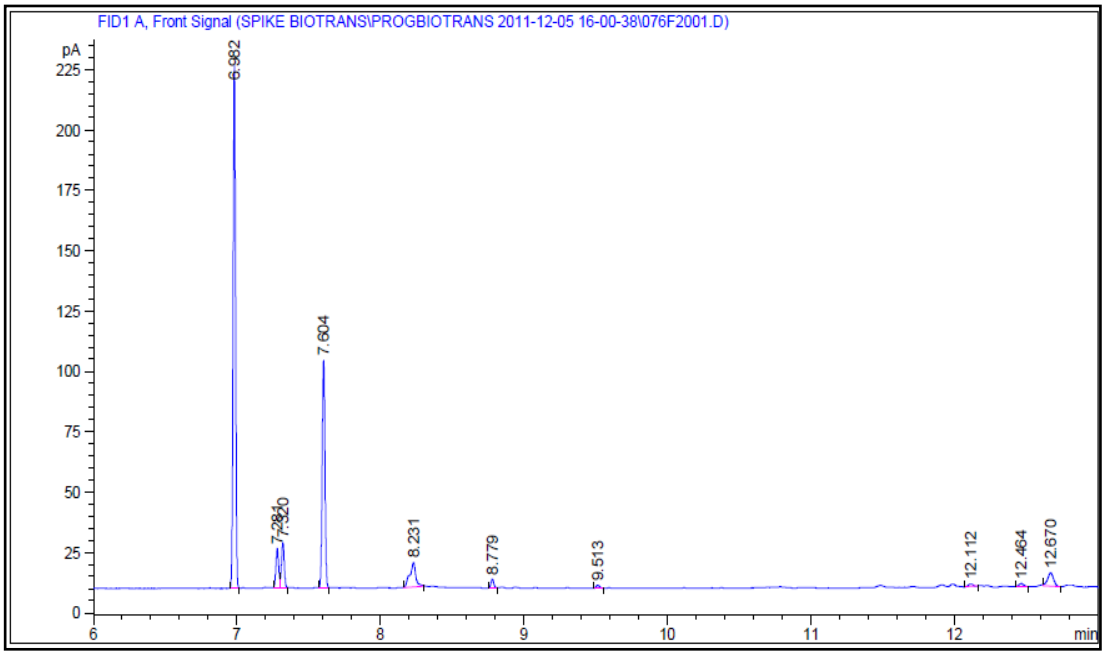
Appendix D11: Isoeugenol Day 6



Appendix D12: 50 % Spike Vanillin in Isoeugenol Day 6



Appendix D13: Isoeugenol Day 7



Appendia D14: 50 % Spike Vanillin in Isoeugenol Day 7