BIOCONVERSION OF ISOEUGENOL TO VANILLIN WITH DIFFERENT STRAINS OF *PSEUDOMONAS AERUGINOSA*

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ABSTRACT

The experiment was conducted at the Faculty of Industrial Sciences and Technology lab at Universiti Malaysia Pahang to investigate the bioconversion of isoeugenol to vanillin. Vanillin is a simple monoterpenoid which is considered as one of the world's principal flavouring compound used extensively in the food, beverage, perfumery, and pharmaceutical industries. Vanillin can be produced using bioconversion of isoeugenol via microorganism and it could be used to substitute synthetic vanillin with a natural vanillin flavor at an affordable price. This study was conducted to screen the *Pseudomonas aeruginosa* strains for the bioconversion of isoeugenol to vanillin. Initially isoeugenol was obtained from extraction of crude clove bud oil. Two different methods of extraction were done to extract the crude clove bud oil which were microwave extraction and steam distillation. Through microwave extraction of clove bud oil, eugenol can be extracted at minimum time of 75 minutes with an optimum yield of 9.09% as compared to the steam distillation technique where it took time to achieve higher yield of eugenol. Purified eugenol (purity ≥99%) was obtained using 1.2 moles of sodium hydroxide with recycle water. Ruthenium acetylacetonate was used as catalyst to produce isoeugenol by synthesis. The conversion was almost 99% but the method is very expensive and cannot be further use as a substrate in biotransformation process. API-20E test was selected as a biochemical test to identify the characteristics of *Pseudomonas aeruginosa* strains P178, U641, S376, B932 and ETT187. In fact, all *Pseudomonas aeruginosa* strains were also confirmed using 16S rRNA gene sequencing and obtained that all the strains were *Pseudomonas aeruginosa*. In this study, the subculture of different strains of *Pseudomonas aeruginosa* was used to convert isoeugenol to vanillin by oxidation. Vanillin formation was analyzed directly by gas chromatography mass spectrometry (GCMS). All the strains exhibited good potential as whole-cell bio-catalysts for direct bioconversion of isoeugenol to vanillin. During biotransformation screening by whole cell culture of *P. aeruginosa strains*, *P. aeruginosa* ETT187 showing a good vanillin produced which is 2.312±0.006 g/l at only 1% (v/v) isoeugenol added for 24 hours incubation at 200 rpm agitation. Furthermore, the effect of vanillin production versus time with 1% induction of isoeugenol was observed at 12, 24, 36, 48, 60, 72, 84, and 96 hours. *P. aeruginosa* P178 demonstrated consist the highest production of vanillin which was 2.97g/l at 72 hours of incubation while the isoeugenol decreased over time. Meanwhile, *P. aeruginosa* ETT 187 presented the highest amount of vanillin produced in only 24 hours with 2.31 g/l. Furthermore, strains U641, S376 and B932 produced the highest amount of vanillin at maximum of 96 hours with 2.62 g/l, 3.56 g/l and 2.49 g/l respectively. The reaction also produced the following by-products, namely, isovanillic acid and isovanillin, ethyl vanillate and also vanillyl methyl ketone. As a conclusion, the *P. aeruginosa* strains which were *P. aeruginosa* P178, *P. aeruginosa* U641, *P. aeruginosa* S376, *P. aeruginosa* B932 and *P. aeruginosa* ETT187 can be proposed to pilot scale as bicatalytic to convert isoeugenol to vanillin at a reasonable price.

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LIST OF SYMBOLS

α	alpha
β	beta
°C	degree Celsius
°F	degree Fahrenheit
γ	gamma
λ	lambda
g	gram
mg	milligram
kg	kilogram
k	kilo
ml	milliliter
ml/min	milliliter per minute
eV	electron volt
L	liter
g/L^{-1}	gram per liter
µ	micro
µL	microliter
mmol	millimole
mM	millimolar
M	molar
≥	greater and equal
=	equal
W	Watt
%	percentage

v/v	volume per volume
w/v	weight per volume
K_a	acid dissociation constant
CFU/ml	colony-forming units per milliliter
rpm	revolution per minute
OD_{600}	optical density at wavelength, 600 nm
RM	Ringgit Malaysia
USD	United States Dollar

LIST OF ABREVIATIONS

GCMS	Gas Chromatography-Mass Spectrometry
GCFID	Gas Chromatography-Flame Ionization Detector
M^+	molecular ion
MS	mass spectrometry
m/z	mass to charge ratio
ICU	intensive care unit
$CHCl_3$	chloroform
HCl	hydrochloric acid
H_2SO_4	sulfuric acid
NA	nutrient agar
NB	nutrient broth
NaCl	sodium chloride
NaOH	sodium hydroxide
KOH	potassium hydroxide
$(NH_4)_2SO_4$	ammonium sulfate
$CaCl_2.6H_2O$	calcium chloride hexahydrate
$MgSO_4.7H_2O$	magnesium sulfate heptahydrate
KH_2PO_4	potassium dihydrogen phosphate
$Na_2HPO_4.12H_2O$	disodium phosphate
TAE	tris acetate ethylenediaminetetraacetic acid
NIST	National Institute of Standards and Technology
pH	power of hydrogen
TIC	total ion current chromatogram
PCR	polymerase chain reaction
DNA	deoxyribonucleic acid

16S rRNA	16S ribosomal ribonucleic acid
BLAST	Basic Local Alignment Search Tool

CHAPTER 1

INTRODUCTION

1.1 BACKGROUND

The term biotransformation which is also known as bioconversion refers to the process of a substance which is changed from one chemical to another and transformed by a chemical reaction within the body (Havkin-Frenkel and Belanger, 2011). The process involves the use of living organisms to modify substances that are not normally used for growth. An alternative route for flavour synthesis is based on microbial biosynthesis or bioconversion (Yamada et al., 2007). The most popular approaches involve the use of microbial cultures or enzyme preparations, although plant cell cultures have also been reported as suitable production systems.

4-Hydroxy-3-methoxy-benzaldehyde or vanillin is the world's principal flavouring compound which is used extensively in the food industry, perfumery, and beverage besides being applied in the pharmaceutical industry (Rabenhorst and Hopp, 1999). Vanillin can be produced synthetically or naturally from vanilla beans (Havkin-Frenkel and Belanger, 2011). Owing to the increasing demand for healthy and natural food, there is a growing interest in producing vanillin from natural raw materials by bioconversion (Priefert and Rabenhorst 2001). According to Seshadri et al. (2008), the price of vanillin varies from USD 15 per kilogram for synthetic vanillin to USD 1200 and sometimes even as high as USD 4000 per kilogram for natural vanillin. Of the total vanillin produced annually, less than 1% is from the vanilla plant and the remainder is prepared mostly by chemical process. Vanillin produced by such a route could then be regarded as a natural aroma chemical (Ashengroph et al., 2008).

Biotechnologically produced vanillin is not intended as a replacement for vanilla extract but it could be used to substitute synthetic vanillin with a natural vanillin flavour at an affordable price (Overhage et al., 2002). Production of vanillin by microbial cultures has been widely used for biotechnological production of vanillin using a wide array of substrate as precursors which include linens, Stevens, ferulic acid, vanillic acid, eugenol and isoeugenol (Seshadri et al., 2008).

Microorganisms can also be adapted for the formation of other vanillin related flavourings where they present either economic advantage or distinctive end products (Priefert and Rabenhorst 2001). In general, biological processes are performed under gentle processing conditions and tend to have lower yields than chemical reactions (Havkin-Frenkel and Belanger, 2011). In order to attain high yields and economic feasibility, the engineering of the process must be coupled with a detailed understanding of metabolic pathways. Alternative classifications could be established as a function of the chemical family by the precursor used for their production by bioconversion. The ability of *Pseudomonas aeruginosa* strains to oxidize a variety of aromatic compounds has led to its use in the study of vanillin production (Ashengroph et al., 2008). In this research, the conversion ability of a subculture of *Pseudomonas aeruginosa* for terpenic compounds was examined. This species was preselected because of its high resistance to toxic monoterpenic substrates and is hereby reported for the first time for the biocatalytic conversion of isoeugenol to vanillin (Ashengroph et al., 2011). This could be attributed to the high reactivity of vanillin that forces the applied microorganism to detoxify this compound by either oxidation or reduction.

Production of vanillin by microbial or enzymatic conversion of natural precursors such as ferulic acid, vanillic acid, glucose and eugenol has been investigated (Havkin-Frenkel and Belanger, 2011). Most of the bioconversion processes studied so far resulted in low product concentrations below 1 g/l. One cheap alternative feedstock for biotechnological production of natural vanillin type aromatic compound is the isoeugenol, which is the main component of the essential oil of the clove tree via extraction. It is also often prepared from eugenol via a chemical route involving isomerization. Isomerization of eugenol to the corresponding thermodynamically stable isomer is an industrially important olefin isomerization reaction wherein the products

find applications in the fragrance and pharmaceutical industries (Kishore dan Kannan 2002). Isomerization of eugenol to isoeugenol is catalyzed by metal ions at high temperature between 200^{o}C to 300^{o}C which resulting in high production cost (Givaudan et al. 1977). Isoeugenol can serve as a potential substrate for the production of valuable aromatic compounds (Yamada et al., 2007). Isoeugenol can serve as a potential substrate in a bioconversion process to produce vanillin. Nowadays, bioconversion of isoeugenol has high demand investigate because it is a natural renewable resource besides the conversion processes are environmentally friendly (Ashengroph et al., 2008).

1.2 PROBLEM STATEMENT

Vanillin is widely used in foods, beverages, perfumes, pharmaceuticals and in various medical industries. Natural vanillin extracted from botanical sources represents approximately only 0.2 % of the global market and costs 4000 USD/kg, whereas chemically synthesized vanillin costs about 12 USD to 1200 USD/kg. An opportunity for biotechnology therefore lies in producing a replacement for synthetic vanillin that is produced non-chemically from sources other than the vanilla bean. The demand for natural flavors is growing and the production of vanillin from natural raw materials by biotransformation processes is becoming attractive because the product can be regarded as a natural aromatic chemical. One of the methods to produce natural vanillin economically is by carrying out biotransformation of isoeugenol. Isoeugenol is thought to be derived from lignin precursors and are major constituents of essential oil from clove buds and they are available relatively cheap.

1.3 RESEARCH OBJECTIVES

The objectives of the research were to perform:

(a) to purify eugenol from clove bud oil obtained by steam distillation and microwave extraction

(b) to isomerizes eugenol to isoeugenol using ruthenium acetylacetonate catalyst with vacuum distillation.

(c) to isolate and screen different strains of *Pseudomonas aeruginosa* for bioconversion of isoeugenol to vanillin.

1.4 SCOPE OF RESEARCH

In order to accomplish the objectives of this study, the scope of the research are as follows:

- different methods of crude clove oil preparation and consideration of process parameters which were used in method of extraction.

- purification of clove oil such as eugenol contents of more than 98%.

- determination of the synthesis reaction of eugenol derivatives by using metal ruthenium acetylacetonate catalyst.

- identification of microbial strain as a biocatalyst.

- bioconversion of eugenol to isoeugenol using different strains of *P. aeruginosa.*

- calculation of the cost of vanillin production using microbial technique.

CHAPTER 2

LITERATURE REVIEW

2.1 VANILLIN

The major aroma component of vanilla is 4-hydroxy-3-methoxy-benzaldehyde, also known as vanillin (Figure 2.1). It is the only one of 250 or so components that contribute to vanilla's characteristic and complex aroma. Vanillin is present in trace amounts in potato parings, Siam benzoin and tobacco but the main source for natural vanillin is the *vanilla* orchid. Zheng et al., (2007) has reported that vanillin is the second largest aroma chemical in the world with an output of 15000 tonnes per year. According to Priefert (2001), isolated vanillin is present in white, needle-like crystalline powder with sweet and vanilla-like odour (Table 2.1).

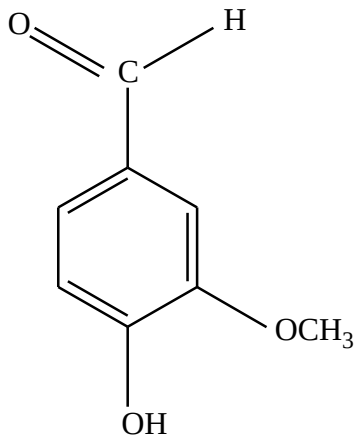


Figure 2.1: Molecular structure of vanillin [4-hydroxy-3-methoxy-benzaldehyde]

Table 2.1: Physical properties of vanillin (Source: Ravendra Kumar et al. (2012)

Characteristic	Identify
Molecular formula	$C_8H_8O_3$
Common synonyms	4-hydroxy-3-methoxybenzaldehyde; vanillic aldehydes; 3-methoxy-4-hydroxybenzaldehyde
Physical state	White or slightly yellow needles
Melting point	178-181^oF
Specific gravity	1.056 at 68^oF
Boiling point	545 ^oF at 760mm Hg
Molar mass	152.15 g/mol
Odour	Floral pleasant
Acidity	(pK_a) 7.781
Basicity	(pK_b) 6.216
Crystal structure	Monoclinic
Water solubility	1/100 g/ml
Density	1.056 g/ml
Vapor density	(air-1)5.2
Vapor pressure	2.2 x 10^{-3} mm Hg
Reactivity	Can react violently with bromine, potassium tert-butoxide,
Solvent solubility	chloroform, acetic acid

Vanillin is used extensively in the food industry, perfumery, beverage and pharmaceutical industry. It is essential in confectionery, chocolates, baked goods, beverages and many other foods, as well as in perfumes, cosmetics, personal care products and detergents (Havkin-Frenkel and Belanger, 2011). Vanillin is also used as a synthetic intermediate in agrochemicals and pharmaceutical production.

According to Sesahdri et al. (2008), the price of vanillin varies from USD 15 per kilogram for synthetic vanillin to USD 1200 and sometimes even as high as USD 4000 per kilogram for natural vanillin. Of the total vanillin produced annually, less than 1% is from vanilla plant while the remainder is produced chemically or by biotechnologically routes.

2.2 PRODUCTION OF VANILLIN

Presently, there are several common ways to produce vanillin. These include natural vanillin extract from vanilla pods, synthetic vanillin production and biotechnological vanillin production (Rabernhorst et al., 1991; Shrader et al., 2004).

2.2.1 NATURAL VANILLIN

Vanillin is the primary chemical component of the extract of vanilla beans. Natural vanilla extract is a mixture of several hundred different compounds in addition to vanillin (Kumar et al., 2012). Natural vanillin is obtained from the cured pods or fruits of the vanilla plant, *Vanilla planifolia*. Vanilla is a perennial climbing orchid with sessile leaves and succulent green stems, producing aerial roots at the nodes (Seshadri et al., 2005). There are three important cultivated species of vanilla namely, *Vanilla planifolia* (Mexican vanilla), *Vanilla pompon* (West Indian vanilla), and *Vanilla tahitensis*. *Vanilla planifolia* is predominantly cultivated for the production of vanillin. *Vanilla tahitensis* and *V. pompon* also yield vanillin but they are of inferior quality (Frenky et al. 2011).

Havkin-Frenkel and Belanger (2011) reported that the vanilla orchid is cultivated in tropical areas by vegetative propagation. The orchid starts flowering 2 to 3 years after planting and the flowers have a tightly closed structure which makes self-pollination very difficult. Artificial pollination is done manually with a bamboo stick. The flowers remain in bloom for less than 24 hours. Hence, artificial pollination needs to be done within a very tight time period for fertilization to occur. Once fertilization occurs, the vanilla beans start to mature in a process that takes 10 to 12 months. The fresh beans have to be cured before the characteristic aroma is obtained from the vanilla beans. The curing of the vanilla beans consists of four steps, namely killing, sweating, drying and conditioning.

Harvested vanilla beans can be killed by anyone of the following: hot water scalding, sun drying, oven wilting, ethylene gas treatment or freezing (Yamada et al., 2007). The most commonly used methods are sun drying and hot water scalding. The killing step helps to disrupt the cell membrane. Distruption of the cells structure helps enzymes to come into contact with the substrate, vanillin glucosides. Frenky et al. (2011)

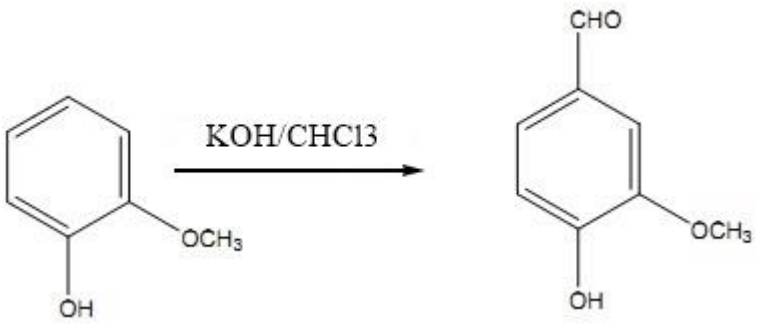
studied the sweating process whereby moisture is allowed to escape rapidly until it reaches a level where microbial spoilage is minimized. Curing enzymes are most active and it takes 7 to 10 days to complete during this step. Vanillin and other related components like vanillic acid, *p*-hydroxybenzaldehyde, *p*-hydroxybenzylmethyl ether and sugars are released from their glucosylated states during this step.

The beans contain 600 to 700 g/kg of moisture at the end of sweating. They are further dried to avoid microbial spoilage and other unwanted enzyme reactions. The beans lose more than half of their moisture content during the drying step. The beans are finally conditioned by placing them in closed boxes and allowing the various chemical and biochemical reactions like esterification, etherification and oxidative degradation to occur (Kumar, 2012). These processes require between 40 days to 6 months depending on the method used to condition them. Vanilla flavour is extracted from the beans by the percolation method or the oleoresin method where the beans are first pulverized before treatment with ethanol.

Production of vanillin from natural vanilla suffers from many disadvantages. It is a laborious, time consuming and expensive process. This leads to the high cost of natural vanillin.

2.2.2 SYNTHETIC VANILLIN

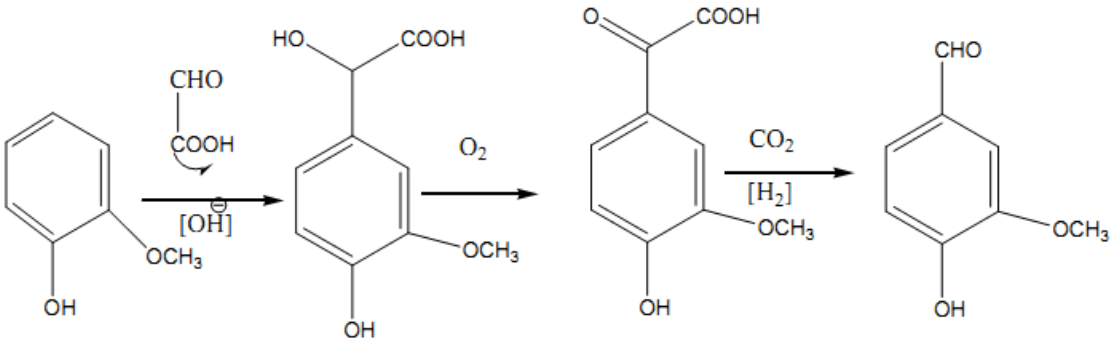
According to Reimer et al. (1876), vanillin is synthesized from guaiacol. Guaicol is obtained from the reaction of eugenol with potassium hydroxide. When distilled with alkaline chloroform, the final product obtained is vanillin (Figure 2.2).



Guaiacol Vanillin

Figure 2.2: Reaction of guaiacol to form vanillin

The most common method involves reacting guaiacol, obtained from catechol, with glyoxylic acid. The more significant of this is the two-step process practiced by Rhoda (1970) in which guaiacol is reacted with glyoxylic acid by electrophilic aromatic substitution. The resulting vanillylmandelic acid is then converted to 4-hydroxy-3-methoxyphenylglyoxylic acid to vanillin by oxidative decarboxylation (Figure 2.3).



Guaiacol Vanillin

Figure 2.3: The two-step vanillin production

Seshadri (2005) had stated that another convenient two-step synthesis of vanillin is by using electrophilic aromatic substitution followed by an organometallic methoxylation procedure based on copper (I) bromide and sodium methoxide (Figure 2.4).

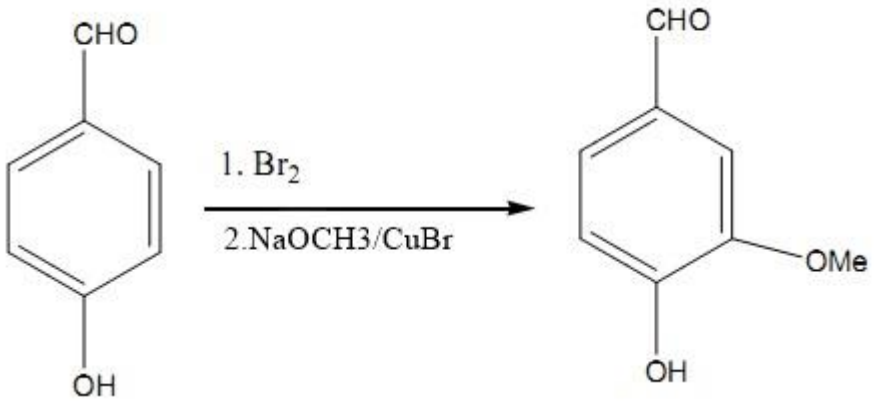
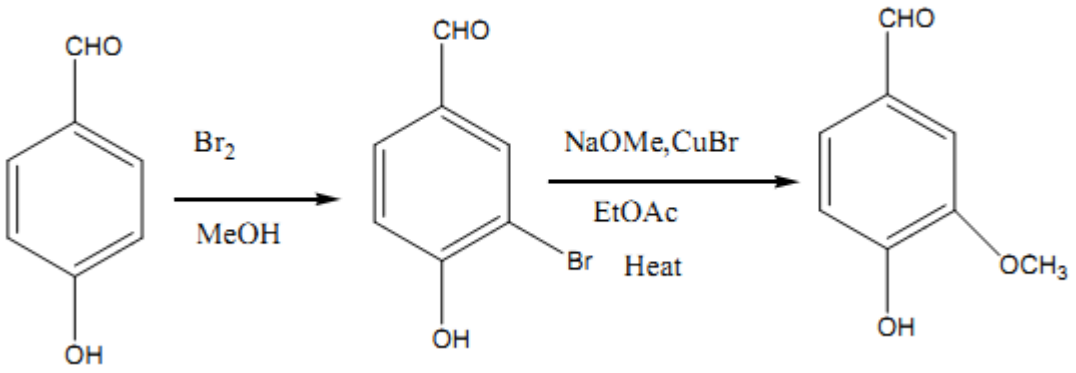


Figure 2.4: Synthesis of vanillin according to Seshadri (2005)

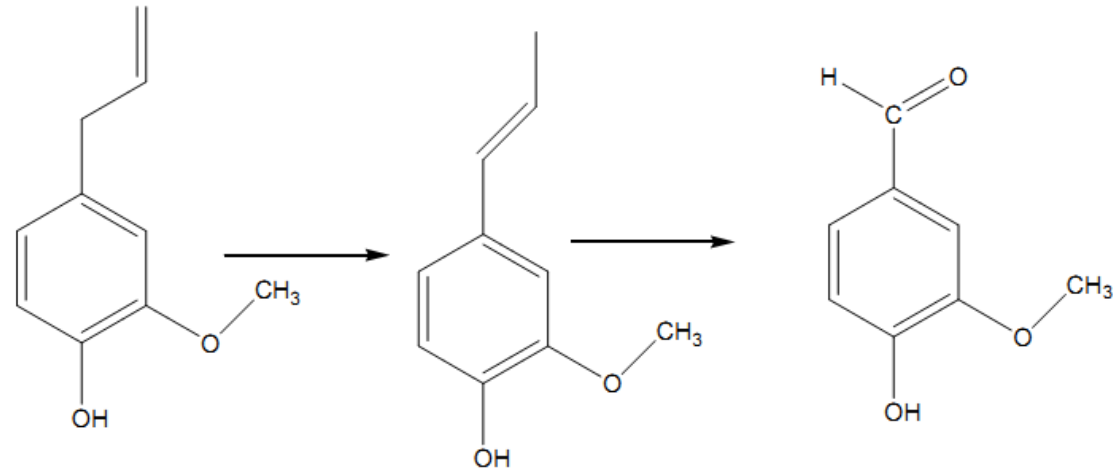
Seshadri (2005) reported a synthesis that involved an electrophilic bromination of 4-hydroxybenzaldehyde and copper-catalyzed methoxylation to yield vanilla fragrance (Figure 2.5). Copper-mediated coupling with methoxide results in regioselectivity of the reaction. The initial monobromo product disproportionates easily to the starting material and 3,5-dibromo-4 hydroxybenzaldehyde. Hence, bromination is completed within 30 seconds and the reaction mixture is then brought directly to the next step where bromide is replaced with methoxide in the presence the copper catalyst in the pathway that probably involves oxidative addition and reductive elimination.



4-hydroxybenzaldehyde Vanillin

Figure 2.5: Synthesis of vanillin according to Seshadri (2005)

As far as large-scale industrial syntheses are concerned, an early classic method involves cloves-derived eugenol as precursor, from nutmeg and cinnamon. It is isomerized to isoeugenol in alkaline solution, and this in turn can be oxidized (by nitrobenzene) to vanillin (Figure 2.6). Other oxidizing agents like acidified potassium dichromate can also be used and this will involve protection of the OH group by acetylation prior to oxidation. The double bond will undergo isomerizations, and then oxidized and cleaved to form vanillin.



Eugenol Isoeugenol Vanillin

Figure 2.6: Synthesis of vanillin from isoeugenol

2.2.3 BIOTECHNOLOGICAL VANILLIN PRODUCTION

Nicholes (2000) had stated that biotechnologically produced vanillin has been developed by *in vitro* production using plant tissue culture, metabolic engineering and microbial cultures. The production of vanillin from plant cell or tissue culture has been effective at the laboratory scale. The main problems encountered in scaling-up procedures for commercial levels are the low levels of vanillin formed and also the competent growth of plant cultures that makes the maintenance of sterile environments difficult.

Meanwhile, metabolic engineering affords an attractive path for producing vanillin. Currently, two biological systems have been developed for the biosynthesis of vanillin (Onozali et al., 1988). The first system involves expression of cloned vanillin biosynthetic genes in plants while the second uses microorganism. The advantage of biosynthesis is the use of cheap precursor like glucose, while the main disadvantages are a separate step for the reduction of vanillic acid is involved and the high cost of cofactor recycling (Figure 2.7).

The production of vanillin with microbial cultures is a largely popular biotechnological method utilizing wide array of substrates as precursors (Koeduka et al., 2006). This includes lignin, phenolic stilbenes, ferulic acid, vanillic acid, eugenol and