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Laporan Akhir Projek Penyelidikan Jangka Pendek

Enhancing The Biocatalytic Performance of Stereospecific Biotransformation of Geraniol into Citronellol using Genetically Engineered Baker's Yeast Type-II, Saccharomyces Cerevisiae

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TECHNICAL REPORT

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GENETIC ENGINEERING OF *ESCHERICHIA COLI* OVER-EXPRESSING YEAST ALCOHOL DEHYDROGENASE (YADH) FOR BIOCATALYTIC PRODUCTION OF CITRONELLOL

By

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INTRODUCTION

1.1 Flavours and Fragrances as Food Additives

Food additives are any compounds or substances added into food which can either form the food components or change the characteristics. These substances can perform a number of functions, which are generally aimed at maintaining freshness, prevention of deteriorations, and improvement of appearances or flavour and fragrance enhancement. The functions of various food additives can be grouped into several categories as summarised in Table 1.1. In food making industries, these substances can be added or used in the production, processing, treatment, packaging, transportation or storage of foods.

Table 1.1: Different categories of food additives and their functions.

Categories	Functions	Examples
Sweeteners	Increase the sweetness of foods	Sucrose, aspartame
Stabilisers/Emulsifiers	Maintain a desirable texture of foods	Carboxymethyl cellulose, glycerides
Preservatives	Prevent decomposition by microbial growth or by undesirable chemical changes	Calcium propionate, Sodium nitrate, Formaldehyde
Nutrition enhancers	Improve the nutritious contents	Vitamins, amino acids, beta-carotene
Colouring agents	Enhance the colour and appearance of fresh and processed foods	Tartrazine, beta-carotene
Flavouring agents	Enhance the tastes or improve the fragrances	Aromatic, Esters, Turpenes, Benzene

In today's food industries, flavouring agents have been one of the most critical ingredients required for the purpose of taste improvement or flavour enhancements such as rose flavour for syrup,

vanilla flavour for ice creams, fruity like tastes for desserts and mint flavour for lollies and so on. Most of the flavouring substances used for these purposes are derived from different sorts of aroma compounds such as monoterpenes, higher terpenes, vanillin and benzaldehyde (Krings & Berger, 1998). They are either extracted from natural source or artificially synthesised (Schrader, 2007, Serra *et al.*, 2005, Patett *et al.*, 2006).

1.2 Biocatalysis in Production of Chemical Compounds

1.2.1 Introduction to Biocatalysis

Biocatalysis is defined as the employment of natural catalyst, such as enzymes, to perform chemical transformation on fine organic compounds. This transformation process relies on the utilization of enzymes or whole cells as the biocatalyst. In the history of the synthesis of organic compounds, biocatalysis has been long employed for more than one hundred years ago. The sole purpose was mainly for the chemical transformations of natural compounds. Over the last 30 years, there has been a substantial increase in the employment of biocatalysis, widely applied for the synthesis of a large numbers of fine chemicals (i.e chemicals with low molecular mass and with specific conformation). This is particularly true in the pharmaceutical and agrochemical industries including food where optically pure molecules are particularly essential (Lye *et al.*, 2002).

Today, the production of highly diversified and structurally complex natural products has been made possible through advancement in transformation process catalysed by large numbers of enzymes which are either isolated from or residing inside living cells. For instance, yeast is one of the most popular organisms used in the catalytic fermentation for the production of flavouring compounds such as geraniol. Yeast producing YADH enzyme catalysed the bioconversion of its geraniol precursor into active citronellol (Gramatica *et al.*, 1982).

1.2.2 Advantages of biocatalysts over chemical catalysts

Many industrial processes have been depending on the use of chemical catalysts for the performance of a chemical reaction. Undeniably, chemical catalyst has been proven to speed up the rate of a chemical conversion by lowering its activation energy. However, the reliance on the use of chemical catalyst possesses several drawbacks and limitations. Apart from the involvement of unenvironmental-friendly substances, the chemical-catalytic reactions also yield low productivity, which is represented by low value of enantiomeric excess (e.e) of the yield.

In order to overcome this issue, many chemical and biotechnological companies have turned their attentions into enzyme-catalysed reactions. In fact, the biocatalysis-involving processes have been experiencing a significant growth over the last few decades (Schmid *et al.*, 2001). In contrast to chemical catalysis, enzyme-catalysed reactions produce yields of higher enantiomeric excess. The key to the achievement of the enantiomerically pure compounds lies in the properties of enzymes with respect to chemoselectivity, regioselectivity and stereoselectivity. The advantages of biocatalytic reactions over the chemical catalytic reactions are summarised in table 1.2.

Table 1.2: Advantages of an enzyme-catalysed reaction over a chemical-catalysed reaction.

Enzyme-catalysis	Chemical-catalysis
Highly stereo-, regio- and chemoselective	Low product specificity
Mild reaction conditions	Extreme conditions
Unique and varied chemistry	Unwanted reaction with impure preparation
Environmental friendly	Chemical waste
Lower cost	Higher cost

Furthermore, the enzyme used for a biocatalysis reaction is mostly active at mild, near ambient conditions of temperature (30 to 40°C), pressure (atmospheric) and neutral pH, and preferentially in aqueous media (Bommarius and Riebel, 2004). This can consequently eliminate many of the operational difficulties of high pressures and temperature which often associated with chemical catalytic process, while at the same time avoiding costly and time consuming product purifications (Etschmann *et al.*, 2002).

In addition, limited availability of enzymes which has been the major issue in biocatalysis is now no longer a problem. Today, biocatalysis can catalyse an increasing number of reactions. The expansion on the number of enzymes exploited for this purpose has contributed to the wide application of biocatalysis on industrial scale (Bommarius & Riebel, 2004). Thanks to the genetic engineering and recombinant DNA technology which allow large pools of enzymes to be produced more efficiently, highly pure and less expensive.

1.3 Genetic Engineering

The terms genetic engineering, recombinant DNA technology and genetic modification (GM) refer to the direct manipulation of the genetic material of an organism. Unlike traditional breeding, where genes from an organism are manipulated indirectly, genetic engineering exploits the techniques of molecular cloning and genomic transformation to directly alter the structure and characteristics of genes. To date, genetic engineering techniques have been reported to be highly successful for many applications, ranging from agriculture, health and medicine, food making industries as well as research and development.

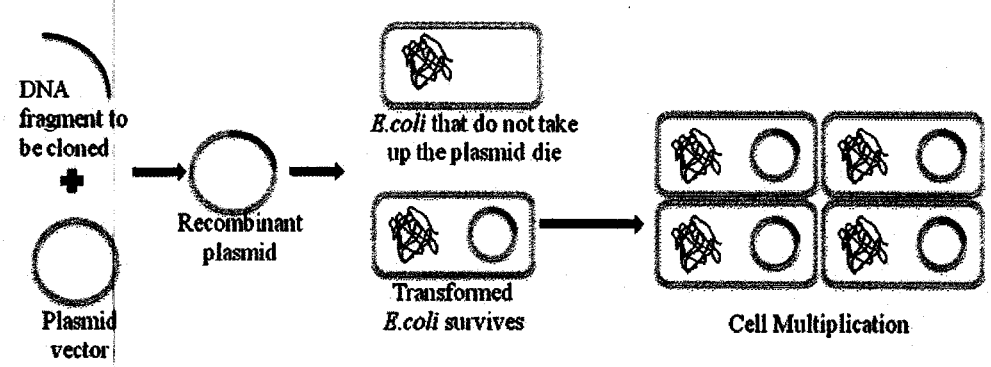


Figure 1.1: Basic steps in gene cloning and modification.

A general procedure of genetic modification comprises a few steps as summarised in figure 1.3. Prior to the genetic modification procedure, the gene of interest to be cloned, whose functions are usually known must be isolated and amplified using polymerase chain reaction (PCR) technique. As depicted in figure 1.3, the DNA fragment (gene) will be enzymatically inserted into a vector which is a plasmid. Restriction enzymes and ligases are of great use in this crucial step. This step yields a recombinant plasmid carrying the gene of interest. Next, the recombinant plasmid is then used to

transform a bacterial host which is *Escherichia coli* via several available protocols such as electrophoration, chemical catalysed reaction or direct gene transfer using DNA guns.

Following transformation, the transformed bacterial hosts, those which are able to take up the plasmids will be selected by using certain antibiotic supplemented in the growth nutrient. Only the transformed cells possessing the recombinant plasmid which carries the gene of interest and an additional selectable marker gene (a gene that confers resistance to the antibiotic) will grow on the media while the non-transformed cells will die. Eventually, only the transformed cells are allowed to multiply and grow as genetically modified organisms that can express their own proteins concurrently with the foreign protein. In subsequent steps, the foreign protein will be isolated and purified from the host proteins.

LITERATURE REVIEW

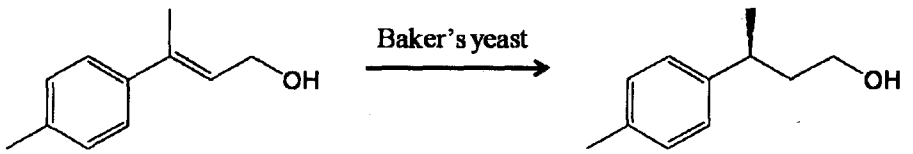
2.1 Biocatalysis in Flavour Synthesis

2.1.1 Baker's yeast for biocatalytic production of flavouring compounds

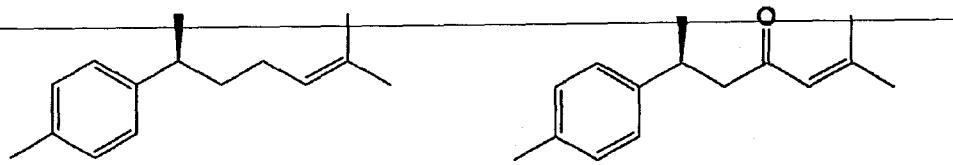
In the synthesis of flavours, a specific synthetic pathway is crucial since different enantiomers contribute to different sensorial properties (Brenna *et al.*, 2001). Therefore, the biocatalytic production of flavourants has been widely applied in food industries since this technique promises the production of product of interest in high enantiomeric purity. For this purpose, various types of biocatalysts are currently available and commonly employed for the production of various sorts of artificial flavouring compounds. They are ranging from bacteria, fungi, plants cells and even to genetically-engineered microorganisms (Priefert *et al.*, 2001). Among them, baker's yeast (*Saccharomyces cerevisiae*) which is a fungus is the most commonly used biocatalyst due to its properties such as easily available, very convenient, inexpensive, and versatile.

The use of baker's yeast for general interests, especially for the preparation of optically active compounds has long been applied (Ema et al, 1998;(Serra *et al.*, 2005). In food manufacturing industries, baker's yeast has been proven to catalyse the production of high enantiomerically pure (*S*)-(+)-3-(*p*-tolyl)-butanol (Figure 2.1 (a)), a chiral building block used for the synthesis of bisabolane sesquiterpenes (*S*)-(+)-curcumene, (*S*)-(+)-turmerone, (*S*)-(+)-dehydrocurcumene and (*S*)-(+)-nuciferal (Figure 2.1 (b)) (Fuganti *et al.*, 1999). These are flavour components of many essential oils. Besides, Brenna and co-workers (Brenna *et al.*, 2001) reported the first enantioselective synthesis of enantiomerically pure natural compounds that constitute the main odour component of the flowers of orchid *Aerangis confusa* (Kaiser, 1993), *cis*-Aerangis lactone (cis-4-methyl-5decanolide) and the (+)-*trans*–diasteroisomer. They demonstrated that baker's yeast can catalyse the enantio-specific and 100% diastereoselective reduction of 1,4-ketoacid into a compound used as the starting material for the preparation of these flavourants.

(a)

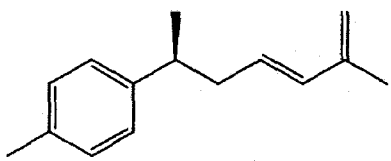


(b)

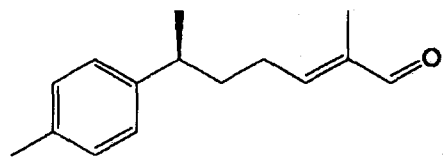


(S)-(+)-Curcumene

(S)-(+)-Turmerone



(S)-(+)-Dehydrocurcumene



(S)-(+)-Nuciferal

Figure 2.1: (a) Baker's yeast- mediated reduction of allylic alcohol forming enantiopure (*S*)-(+)-3-(*p*-tolyl)-butan-1-ol, a compound used for the preparation of **(b) monocyclic aromatic sesquiterpenes of bisabolane;** (*S*)-(+)-curcumene, (*S*)-(+)-turmerone, (*S*)-(+)-dehydrocurcumene and (*S*)-(+)-nuciferal.

Apart from producing high quality products, baker's yeast is also able to increase the product quantity while at the same time maintaining or even decreasing the cost of production. This is particularly true for the biological formation of methionine-derived flavours (odour reminiscent of soup, meat, onions, and potatoes) which is extensively studied in yeast (Hazelwood *et al.*, 2008, Etschmann *et al.*, 2008). The transformation of the amino acid precursor, L-methionine (Figure 2.2) in *Saccharomyces cereviciae* yielded significantly higher product concentration (Etschmann *et al.*, 2008, Etschmann and Schrader, 2006) with a lower cost of production in comparison with its previous chemical process (Schreier et al., 1976).

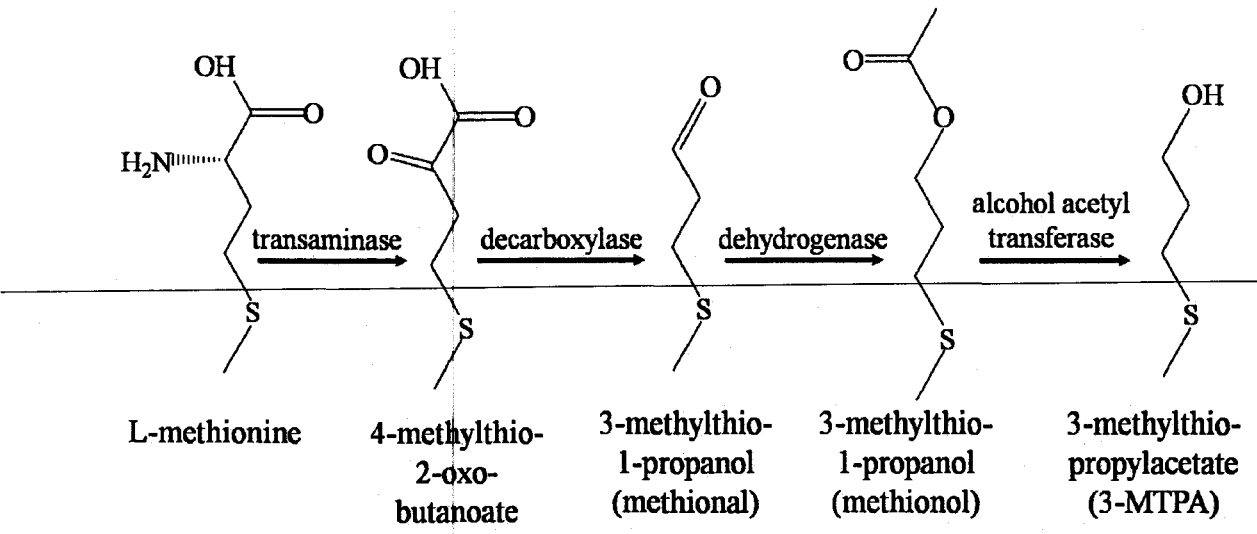


Figure 2.2: Biological formation of methionine-derived flavours using L-methionine amino acid as the substrate. The amino acid is transaminated and decarboxylated to aldehyde (methional), which is then reduced to higher alcohol (methionol). In yeast, where the activity of alcohol acetyl transferase is usually present, methionol will be transesterified into 3-MTPA.

2.1.2 Stereo- and Regio-specific Biotransformation of Geraniol into Citronellol using YADH-producing *S. cerevisiae*

Bakers' yeast has long been used for the asymmetric bioreduction of carbonyl compound (Ema *et al.*, 1998). For instance, this organism has been used to catalyse the stereospecific, asymmetric reduction of geraniol forming citronellol (a compound that constitutes the odour of rose). Gramatica and co-workers (1982) demonstrated the conversion of *trans*-3,7-dimethyl-2,6-octadiene-1-ol (geraniol) into 3,7-dimethyloct-6-en-1-ol ((R)-(+)-citronellol)) (Figure 8.3). The product formed was reported to be of high optical purity which is more than 98% enantiomeric excesses and is significantly higher than that available from natural pool (70- 80% e.e). Hence, this method promises a greater commercial value for the production of (*R*)-citronellol.

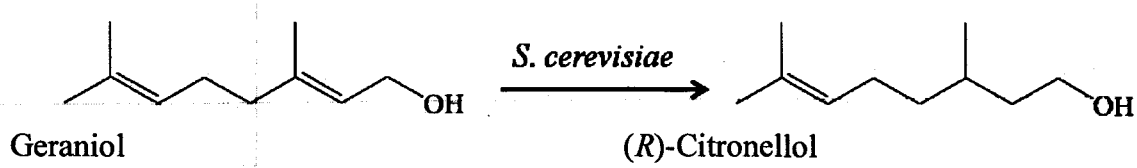


Figure 2.3: Biotransformation of geraniol into citronellol using *S. cerevisiae*.

The ability of bakers's yeast to catalyse a remarkable degree of regiospecific and stereospecific bioreduction of geraniol forming citronellol lies in the ability of this organism to produce yeast alcohol dehydrogenase (YADH) (Reynolds and Holland, 1997). The stereospecificity of the reaction catalysed by YADH has been reported by Fisher and co-workers (Fisher *et al.*, 1953). Their experiment demonstrated the transfer of a hydrogen from the *pro*-4R position of NADH (co-factor) to the *Re* face of acetaldehyde (substrate) (Figure 2.4) forming NAD^+ and ethanol as the product. In this reaction, the enzyme is shown to be able to discriminate between the diastereotopic hydrogens attached at a C4 of the co-factor.

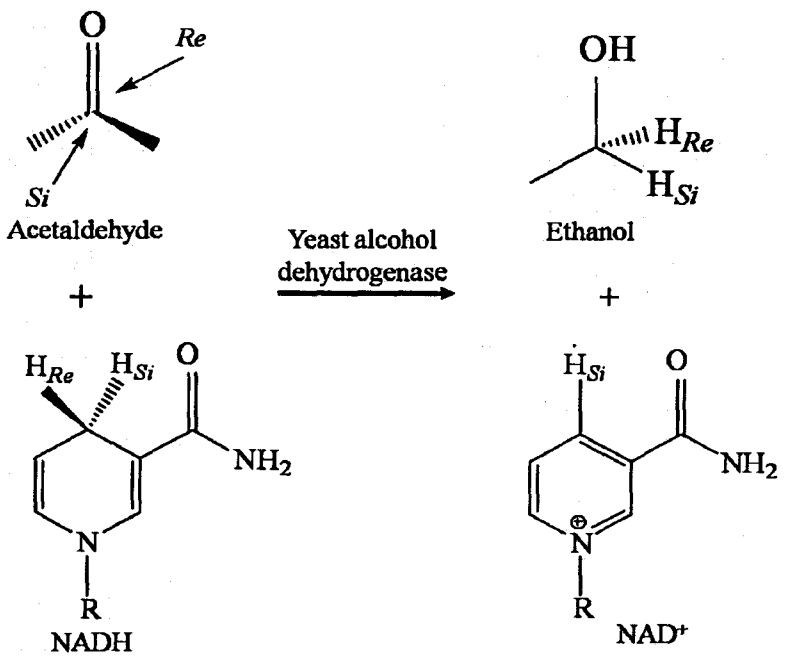


Figure 2.4: Stereochemical course of the reduction of acetaldehyde into ethanol catalysed by YADH enzyme. Adapted from Reynolds and Hollands (1997).

Furthermore, the specificity of the YADH is also given by the particular arrangement of substrate and co-factor at the active site. This was proven by the X-ray crystal structure of YADH with ethanol and NAD^+ at the active site which was clearly consistent with the observed stereospecific course of the enzyme-catalysed reaction (Weinhold *et al.*, 1991). In the context of geraniol as the substrate, the active site of YADH is positioned in a way that the hydrogen atom from NADH can be transferred to the *Si* faces of the central carbon of the geraniol (Figure 2.4). As a result, the methyl group attached to the central carbon is protruded to the *Re* face, forming (*R*)-citronellol instead of the (*S*)-isomer.

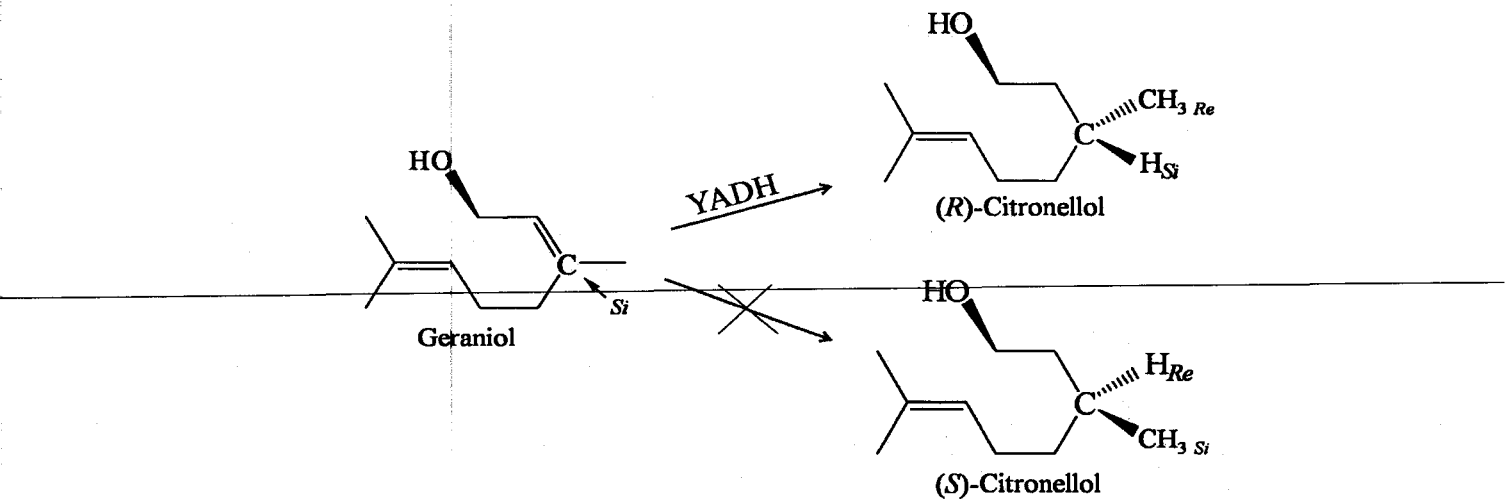


Figure 2.5: Stereospecific reduction of geraniol into (*R*)-citronellol catalysing by yeast alcohol dehydrogenase (YADH) enzyme.

Even though the reduction of geraniol forming citronellol using baker's yeast is described as stereospecific, however the percentage yield is still considerably low. In the previous work, only 0.02 g/l citronellol was obtained from 2 g/l geraniol, implying that the percentage yield was only 1%. Furthermore, at the optimum pH and temperature, the maximum rate of reaction achieved was only approximately 0.0004 g/(L.min) (Arifin, 2007). The reason behind this phenomenon was that the yeast concentration formed during the biotransformation was very limited, which consequently reduced the amount of yield that can be possibly produced (H'Ng, 2008). Besides, the level of YADH enzyme produced per unit biomass has never been reported in literature. Hence, more studies are still required since this information is highly essential in determining the relationship between the amount of enzyme and the percentage of product yield.

8.2 Genetic Engineering Approaches for an Optimised Enzyme Production in Flavour Synthesis

2.2.1 Gene cloning and recombinant DNA technique in enhancing enzyme production and biocatalytic performance

The use of a wildtype organism producing a particular enzyme catalysing vast number of biocatalysis reactions was found to have certain limitations (Kawagoe *et al.*, 1997, Arifin, 2007). Novel approaches for this problem could employ genetic engineering strategy, with the aim of achieving a rapid and inexpensive procedure for the production of aroma compounds in organisms such as yeast and bacteria that are easily cultivated and extracted. This includes several feasible innovative ways such as genetic knockout, overexpression and mutagenesis that have been proven to maintain both enantioselectivity and multi-stress resistance within the strains (Nir *et al.*, 2008). Among these strategies, an optimised production of recombinant protein or enzymes used as the biocatalyst in an engineered organism has become a popular method of choice.

For instance, the metabolic engineering production of the phenylpropanoid methyl benzoate, a type of plant odorants used as food flavourings and perfume ingredients (Boatright *et al.*, 2004) has been synthesised using genetically-modified *S. cerevisiae*. This employed heterologous expression of *Antirrhinum majus* (snapdragon flower) benzoic acid methyl transferase, a biocatalyst required for the transformation of benzoic acid forming methyl benzoate in *S. cerevisiae*. Even though this method is described as cost-effective and environmental friendly alternative to chemical synthesis, the direct cost of production is similar in magnitude to the price of naturally derived benzoate (~$1000/kg). This is due to the low efficiency (~1%) of biotransformation, which produces only 50 μg of methyl benzoate per litre of culture per hour (Farhi *et al.*, 2006). However, considering its huge potential in food industries such as for the fermentation of benzoic acid-rich fruit juices (Visti *et al.*, 2003), much efforts are still needed to optimize the production of this flavoring compounds using this genetic engineering approach.

Recently, a technique in genetic engineering that can significantly increase the yield of production and hence, reducing cost has been reported. For instance, the production of the acetate ester of methionol is greatly enhanced by overexpressing *ATF1 gene* coding for alcohol acetate transferase 1 enzyme in *S. cerevisiae*. It is an enzyme which catalyses the final step of transesterification of methional intermediates into final 3-methylthio-propylacetate (3-MTPA) product using L-methionine as the

substrate (Figure 2.2). The authors claimed that they have successfully produced the highest concentration of this methional-type flavour which has never been reported using this mutant yeast strain. A maximum concentration of 2.2 gL^{-1} 3-MTPA are produced via this metabolic engineering approach in comparison with only a small trace of the same compound produced by using the wildtype *S. cerevisiae strain* (Etschmann, 2008).

Moreover, some researchers have looked into the possibility of more than one foreign genes encoding for different enzymes being concomitantly expressed within one single host, aimed at optimisation of synthesis of a compound (Schewe *et al*, 2007; Waché *et al.*, 2006). For instance, Hausler *et al.* (2001) described in their patent for the production of green note compound such as hexen-1-ol (Figure 2.6), an alcoholic compound widely used in food industry to reconstitute the fresh green odour of food or vegetables lost during processing. The genes encoding hydroperoxide lyase isolated from banana and the gene coding alcohol dehydrogenase from baker's yeast were cloned into a strain of *S. cerevisiae.* Respectively, these enzymes perform their functions within a single pathway of hexen-1-ol synthesis as depicted in Figure 2.6. The production of the final product from this method is yet to be published.

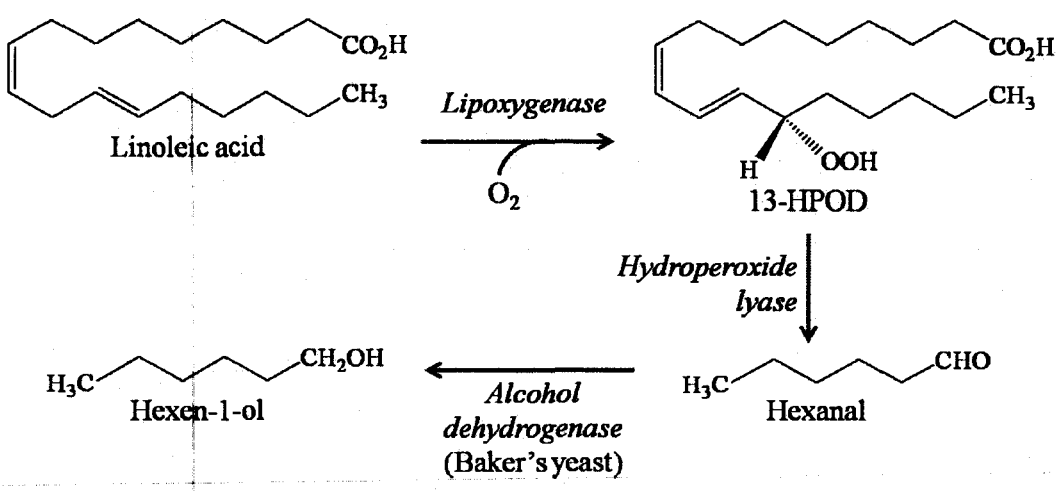


Figure 2.6: The synthesis of green notes compound via lipoxygenase and hydroperoxide pathway utilising genetically-engineered Baker's yeast as the main biocatalyst. The starting material, Linoleic acid is first deoxygenated by lipoxygenase enzyme forming 13-hydroperoxy-linoleic acids (13-HPOD). The hydroperoxide lyase enzyme then cleaves 13-HPOD to produce Hexanal, an aldehyde compound which is then enzymatically reduced by alcohol dehydrogenase to form hexen-1-ol alcohol. According to Wache and co-workers (2006) with modifications.

In addition to the metabolic engineering of yeast, Schewe and co-workers (2008) have also experimented the use of bacterial cell in order to optimise the whole-cell biocatalysis. On top of that, they have successfully integrated a recombinant intracellular NADPH regeneration system through co-expression of glucose facilitator (GLF) from *Zymomonas mobilis* for uptake of unphosphorylated glucose and a $NADP^+$-dependent glucose dehydrogenase (GlcDH) from *Bacillus megaterium* that oxidises glucose to gluconolactone in *E. coli* (Figure 2.7). The engineered bacterial strain was proven for its ability to take up glucose more efficiently and to carry out a straightforward intracellular co-factor generation. Consequently, the initial formation of α-pinene oxide as the final product was shown to be significantly increased by nine-fold via this improved system.

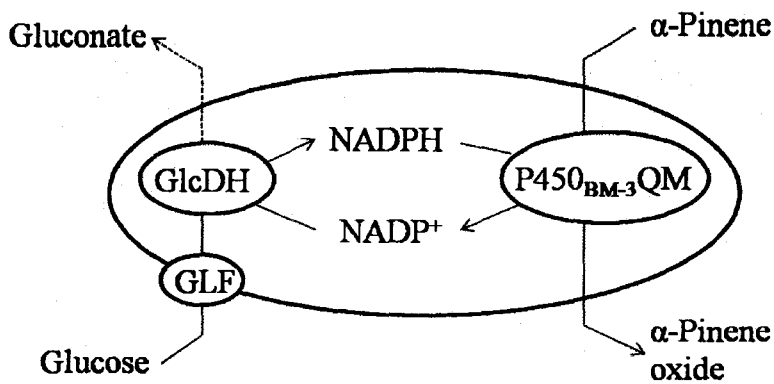


Figure 2.7: A novel whole-cell biocatalysis using genetically-engineered *E. coli* with improved co-factor regeneration driven by external glucose. The bacterial host is overexpressed with $P450_{BM-3}QM$, an enzyme that can selectively oxidise α-pinene substrate to form α-pinene oxide as the final product and requires NADPH as the co-factor. The regeneration of NADPH is driven by glucose dehydrogenase (GlcDH), which oxidises external glucose into gluconate. To maintain an effective biocatalysis system, the regular uptake of external glucose is continuously aided by facilitator-mediated diffusion of glucose via a glucose facilitator (GLF).

The major improvement brought about by this molecular approach is an increase in the level of enzyme produced by the genetically-modified organisms. Although the relationship between the rate of product formation and the level of recombinant protein (enzyme) has never been mentioned in the literatures discussed above, nevertheless, it has been undeniably proven that the percentage yield can be significantly elevated via this technique. However, the application of this technology in *S. cerevisiae* still cannot overcome the problems associated with the slow growing yeast and their disability to grow in high biomass concentration. This issue however can be addressed with the utilisation of genetically-

modified bacteria such as *E. coli* since this type of microorganism is more easily- and fastly-grown, has shorter life cycle and genome that can be easily modified.

2.2.2 Expression of yeast proteins in prokaryotic hosts

Several attempts have been carried out in order to express gene coding hydroperoxide lyse enzyme that is isolated from *Arabidopsis thaliana* (Kandzia *et al.*, 2003) and green bell pepper (Delcarte *et al.*, 2003) for the biocatalytic synthesis of green note compounds (Figure 2.6) in *E.coli.* However, both groups of researchers found that high level of expression of foreign cDNAs coding enzymes in bacteria has often resulted in inactive products found in inclusion body (Waché *et al.*, 2006). However in theory, this experiment can be extended with some further modification of their current engineering techniques so as to express a modified recombinant protein that can be produced in its active form by using the same bacterial strain as a host.

There are evidences showing that the expression of recombinant eukaryotic gene in *E. coli* host is highly achievable based on some previous experiments. For instance, (Chang *et al.*, 2001) have successfully produced the recombinant dehydrolichyl diphosphate synthase (DDPPs) from yeast in *E. coli*. The DDPP protein which acts as a biocatalyst is involved in the synthesis of dehydrolichyl diphosphate required in eukaryotic glycoprotein biosynthesis. In this system, the expression pattern of the recombinant DDPPs has been designed in such a way that they are refolded into an active enzyme from pellet. This can subsequently prevent the formation of inclusion body. This system was shown to perform approximately 20-fold higher specific activity in comparison with that reported for the native DDPPs prepared from *S. cerevisiae* (Adair and Cafmeyer, 1987).

2.2.3 An improved production of YADH enzyme through a recombinant DNA technique using *E. coli* as a host

A different strategy of enzyme production is pursued by creating a strain of *E. coli* that can produce YADH enzyme as a reductase for the biocatalytic reduction of geraniol to citronellol (Figure 2.3). This bacterial expression system serves as an advantage in terms of the level of enzyme production which is benefited by the ability of *E. coli* to grow more quickly and easily. This will ultimately alleviate

the efficiency of bioconversion of geraniol into citronellol using the engineered *E. coli* strain as the catalyst. Additionally, the use of the whole cells prominently eliminates the need for enzyme purification and co-factor regeneration (Schewe *et al.*, 2008), which finally reduce the cost of overall biotransformation (Etschmann *et al.*, 2008).

MATERIALS AND METHODS

3.1 Yeast, bacterial strains and plasmids

Yeast, bacterial strains and plasmids used in this study are listed in table 3.1.

Table 3.1: List of yeast, bacterial strain and plasmids.

Strain or plasmid	Genotype of phenotype	Source
Yeast strains:		
Saccharomyces cerevisiae	Wild type	Bioprocess Laboratory
Bacterial strains:		
Escherichia coli JM 109	endA1, recA1, gryA96, thi, hsdR17, (rk-, mk-), relA1, supE44,	Promega
Escherichia coli TOP10		Invitrogen
Plasmids:		
*p*GEM-T		Promega
*p*TrcHis		Invitrogen

3.2 General methods

3.2.1 Sterilisation

Equipments, media, and solutions were autoclaved using Sun Clave at 15 psi (121 °C) for 15 minutes.

3.2.2 Spectrophotometry

Optical density of bacterial growth was determined using spectrophotometer Ultraspec 2000 UV-Visible (Pharmacia Biotech) at 600 nm wavelength. The concentration and purity of DNA was determined at 260 nm and 280 nm.

3.2.3 Growth conditions

Yeast

Saccharomyce cerevisiae was grown in yeast media containing glucose, peptone and yeast extract at pH 7.0. Incubation was carried out in a rotary shaking incubator (B. Braun Certomat R&H) at 150 rpm and 30 °C.

Bacteria

Escherichia coli was grown in Luria bertani (LB) and SOC at 37 °C. Incubation were carried out in Incubator (Memmert) and orbital shaker (B. Braun Certomat R&H).When necessary, ampicillin was added to the media to maintain the plasmids.

3.2.4 DNA restriction and modification enzymes

All restriction and modification enzymes were purchased from Promega, New England Biolab, Fermentas, and Amersham. Utilisation of these enzymes was performed according to the suppliers' recommendations.

3.2.5 Chemicals and reagents

Utilisation and preparation of the chemicals, reagents and kits in this study were done according to the manual provided by the suppliers. Chemical and reagents used are listed in table 3.2

Table 3.2: Chemicals and reagents used in this study

Chemicals and reagents	Suppliers
General:	
Chemicals such as antibiotics, IPTG, X-gal, salts, acids etc.	Sigma Aldrich, Merck,
Microbiological:	
LB, SOC, yeast media, bacteriological agar	Sigma Aldrich, Fluka,
DNA manipulation:	
1 kb DNA ladder	Fermentas
HindIII DNA marker	Fermentas
Agarose	Promega
Oligonucleotide primer	First Base Laboratory
QIAquick gel extraction kit	Qiagen
Expression and purification of protein:	

3.3 Medium preparation

3.3.1 Yeast media

The yeast media was prepared by adding glucose (10 g), peptone (6 g) and yeast extract (3 g) into 1 L distilled water. The pH was then adjusted to 7.0 with NaOH. The medium was autoclaved at 15 psi (121 °C) for 15 minutes.

3.3.2 Luria Bertani

Luria bertani (LB) powder (16g) was dissolved with 1 L distilled water. The pH was then adjusted to 7.0 with NaOH. The medium was autoclaved at 15 psi (121 °C) for 15 minutes. Sterile

Ampicillin (100 ug/ml) was added into the media when necessary. To prepare agar media, 1.5% bacteriological agar was added to the media prior to autoclaving. The molten agar medium was cooled down to approximately 55 °C before pouring onto plates under aseptic conditions.

3.4 Genomic extraction and gene manipulation

3.4.1 Extraction and purification of genomic DNA from *Saccharomyces cerevisiae*.

The yeast genomic extraction procedure was carried out according to the Current Protocol in Molecular Biology (John Wiley and Sons, Inc). Firstly, 10 ml culture of yeast was grown overnight to stationary phase at 30 °C with agitation at 160 rpm by orbital shaker. The overnight culture was harvested by centrifugation using KUBOTA 2100 at 3 000 rpm for 5 minutes at room temperature. The supernatant was aspirated off and the cell pellet was resuspended in 0.5 ml. The resuspended cell was transferred to a microcentrifuge tube and spun 5 seconds using Eppendorf Centrifuge 5415 C. The supernatand was decanted and the cell pellet was disrupted by vortexing briefly.

Secondly, the technique to break open the cell was performed by treating the cells with 200 µl breaking buffer, 200 µl phenol, and chloroform and isomyl alcohol prior to vortexing at the highest speed for 3 minutes. Then, the sample was vortexed briefly again before the addition of 200 µL TE buffer. After 5 minutes microcentrifugation at high speed, the aqueous layer was transferred to a clean microcentrifuge tube and added with 100% ethanol. Following another microcentrifugation for 3 minutes at high temperature, the supernatant was collected and resuspended in 0.4 ml TE buffer. The suspension was added with 30 µl 1 mg/ml DNase-free RNase A and incubated at 37 °C in HOTECH water bath for 5 minutes. 10 µL of 4 M ammonium acetate and 100% ethanol were then added. After a final microcentrifugation, the dry pellet containing the desired DNA was resuspended in 100 µl TE buffer.

3.4.2 Gel electrophoresis of yeast genomic DNA

To determine the size of large chromosomal DNA, 0.1% (w/v) of agarose gel was used. Approximately 20 g of agarose powder was dissolved in 20 ml of 0.5X TBE buffer (see appendix..). The slurry was heated in a microwave until the agarose dissolved. It was then cooled down to 60 °C and poured into the mini gel cast. 3 µL of HindIII DNA marker (0.1 µg/mL) and approximately 20 ng of DNA sample were loaded into the well. The gel was run in the 0.5X TBE buffer at 75 V and 350 mA. It was run until the loading dye has migrated at the appropriate distance through the gel. The gel was then soaked in ethidium bromide (0.5 µg/ml) solution for 15 minutes before rinsing in distilled water for 10 minutes. Finally, the gel was examined by ultraviolet illumination and photographed.

3.4.3 Amplification of *adh1 gene* by Polymerase Chain Reaction (PCR)

PCR mix was prepared by adding 1 µL of 10 ng/ µL of genomic DNA (genomic DNA of *S.cerevisiae*), 4 µL of MgCl2 (25 mM), 4 µL of 10X of Taq Polymerase buffer, 1.5 µL of dNTP's mix (10 mM), 1 µL of forward (100 µM) and reverse (100 µM) primer, and 0.3 µL of Taq Polymerase (5 U/ µL). Finally, the PCR mixture volume was topped up to 20 µL with deionised distilled water. Both specific primers are listed in Table...

Table 3.3: Specific primers used in amplification of *adh1 gene*

Primer	Sequence
Forward	*Bam*HI site 5'-GGC**GGATCC**TATCCCAGAAACTCAAAAAGGTG-3'
Reverse	*Eco*RI site 5'-GGG**GAATTCC**AACAACGTATCTACCAACGA-3'

Subsequently, PCR reaction was performed using PCR machine manufactured by MJ Research Inc. The cycle and conditions for the PCR reaction are shown in table 3.4. To determine the success of the PCR reaction, PCR products obtained were electrophoresed as described in section 3.4.2. The PCR product is then purified using QIAquick™ Gel Extraction kit.

Table 3.4: PCR cycle and conditions for the amplification of *adh1 gene*

Step	Reaction	Temperature (°C)	Time	Cycle
1	Initial	95	5	1
2	Denaturation	95	1	1
3	Annealing	59	1	1
4	Extension	72	1	1
5	Go to step 2	95		30
6	Final Elongation	95	10	1
7	Cooling	10	∞	1

RESULTS

4.1 Isolation of *adh1 gene*

Isolation of *adh1 gene* was carried out by extracting genomic DNA of *S. cerevisiae* and amplification of *adh1 gene* with direct PCR technique. The genomic DNA with the concentration approximately 50 ng/uL (Plate 4.1) was successfully extracted using the conventional (phenol/choloroform) extraction method.

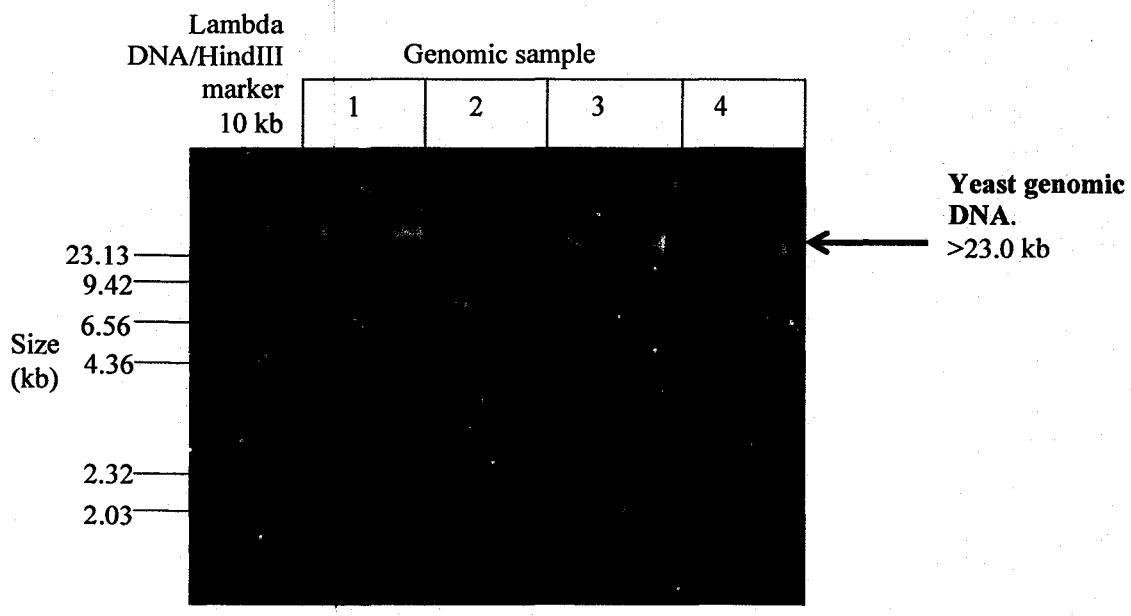


Plate 4.1: Analysis of genomic DNA extraction of *S. cerevisiae*

The expected size of *adh1 gene* is approximately 1.05 kb as shown in plate 4.2.

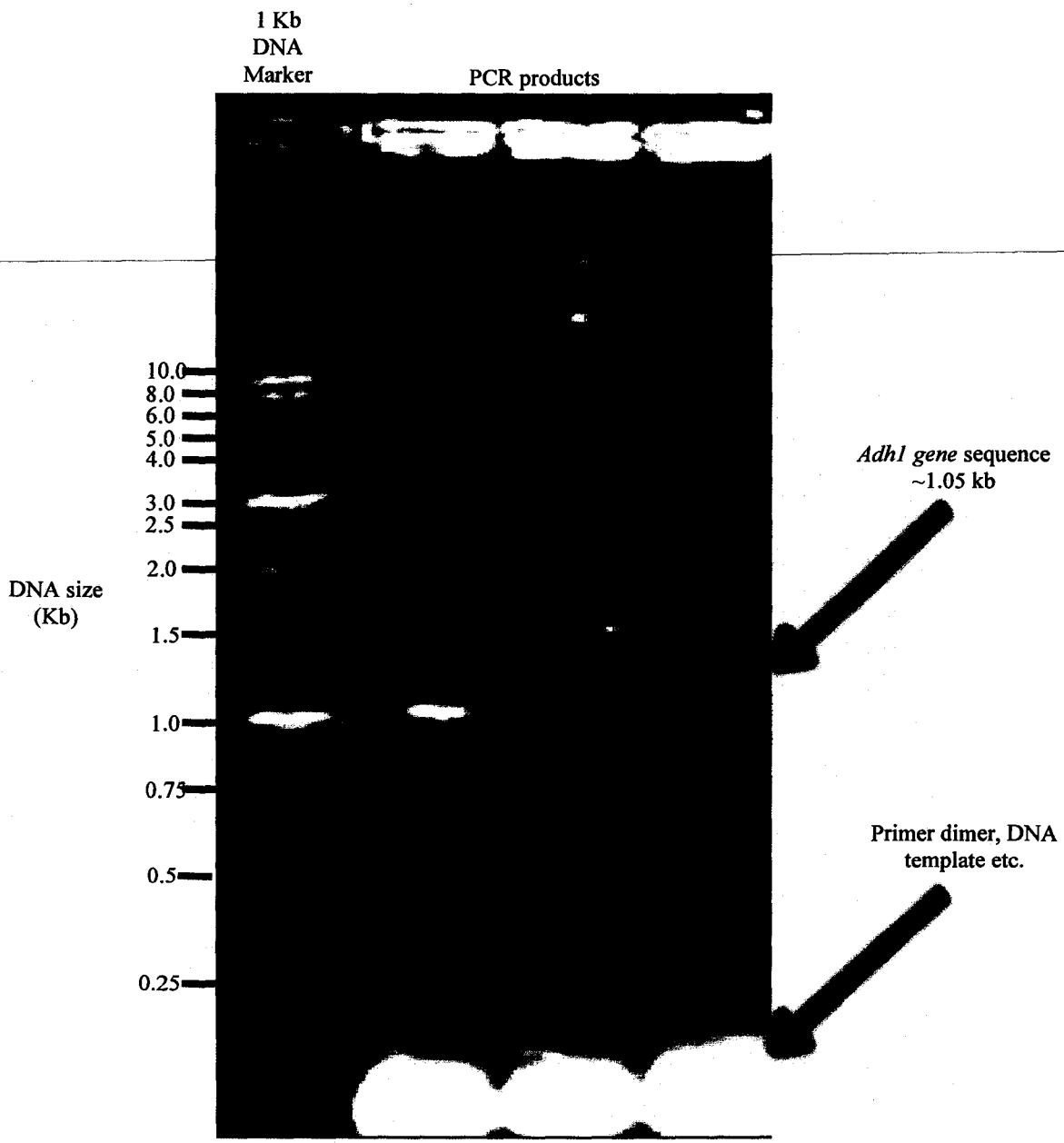


Plate 4.2: Analysis of the PCR product

The PCR product was purified using QIAquick gel extraction kit. The purified gene was then sent to First Base laboratory for sequencing analysis. The sequencing result is shown in figure 4.1.

CTNGGACTTTTCATCTTTTCGTAATTTCTGGGCAAGGTAGACAAGCCGACAACCTTGATTGGAGACTTGATCAA
ACCTCTGGCGAAGAAGTCCAAAGCTTCTCTGGTGTCAGCTCTGTTACCGACGTAAGAACCAACAATAGAGATG
GACTTGACGACTTGGTTGAAGACATCAGAACAACACTTGGCACCAGCTGGCATACCGACCAAAACGGTGGTAC
CGTTAGCTCTAACGTATCTGGTGGAAGCTTCAATAGCGGCTTCGGAAACGGAAACGTTGATGACACCGTGAGC
ACCACCGTCAGTGGCCTTTAGAACAGCACCGACAATGTCCTTTTCCTTAGTGAAGTCAATGAAGACTTCACCAC
CGATGGATCTGAATAATTCTTCCTTACCTTCACCACCGTCAATACCCAAGACTCTGTAACCCATAGCCTTGGCG
TATTGAACAGCCAAAGAACCTAGACCACCAGCAGCACCGGAGATAGCAACCCAGTGACCGGCCATCAAGTTA
GCAGACTTCAAAGCCTTGTAGACGGTGATACCAGCACACAAAACTGGGGCGACTTCAGCCAAGTCAGTACCTT
GAGGAATGTGAGCGGCTTGAACAGCGTCAGCGGTAGCGTATTCTTGGAAAGAACCGTCGTGGGTGTAACCAGA
CAAGTCAGCGTGAGGACAGTTGGATTCGTTACCCAATTCACAATATTTTTATGTCATATAATAACCTTTCAACC
ATTTGATATTATTGTATAATTTAATATTTAA

Figure 4.1: Sequencing result of the PCR product. This result was obtained from the First Base Laboratory. This sequence is 764 bp in length.

The nucleotide sequence was then analysed using the Nucleotide Blast software available on the National Centre for Biotechnology Information (NCBI) website [http://www.ncbi.nlm.nih.gov/]. The data is presented below.

```
Score = 1242 bits (672),  Expect = 0.0
Identities = 717/738 (97%), Gaps = 5/738 (0%)
Strand=Plus/Plus

Query  7       CTTTT-CATCTTTTCGT-AATTTCTGGGCAAGGTAGACAAGCCGACAACCTTGATTGGAG  64
|||||  |||||||||||  |||||||  ||||||||||||||||||||||||||||||||||||||||||
Sbjct  159590  CTTTTCCATCTTTTCGTAAATTTCT-GGCAAGGTAGACAAGCCGACAACCTTGATTGGAG  159648

Query  65      ACTTGATCAAACCTCTGGCGAAGAAGTCCAAAGCTTCTCTGGTGTCAGCTCTGTTACCGA  124
||||||  ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  159649  ACTTGACCAAACCTCTGGCGAAGAAGTCCAAAGCTTCTCTGGTGTCAGCTCTGTTACCGA  159708

Query  125     CGTAAGAACCAACAATAGAGATGGACTTGACGACTTGGTTGAAGACATCAGAACAACACT  184
||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  159709  CGTAAGAACCAACAATAGAGATGGACTTGACGACTTGGTTGAAGACATCAGAACAACACT  159768

Query  185     TGGCACCAGCTGGCATACCGACCAAAACGGTGGTACCGTTAGCTCTAACGTATCTGGTGG  244
||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||| |||
Sbjct  159769  TGGCACCAGCTGGCATACCGACCAAAACGGTGGTACCGTTAGCTCTAACGTATCTGGTAG  159828

Query  245     AAGCTTCAATAGCGGCTTCGGAAACGGAAACGTTGATGACACCGTGAGCACCACCGTCAG  304
||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  159829  AAGCTTCAATAGCGGCTTCGGAAACGGAAACGTTGATGACACCGTGAGCACCACCGTCAG  159888

Query  305     TGGCCTTTAGAACAGCACCGACAATGTCCTTTTCCTTAGTGAAGTCAATGAAGACTTCAC  364
||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  159889  TGGCCTTTAGAACAGCACCGACAATGTCCTTTTCCTTAGTGAAGTCAATGAAGACTTCAC  159948

Query  365     CACCGATGGATCTGAATAATTCTTCCTTACCTTCACCACCGTCAATACCCAAGACTCTGT  424
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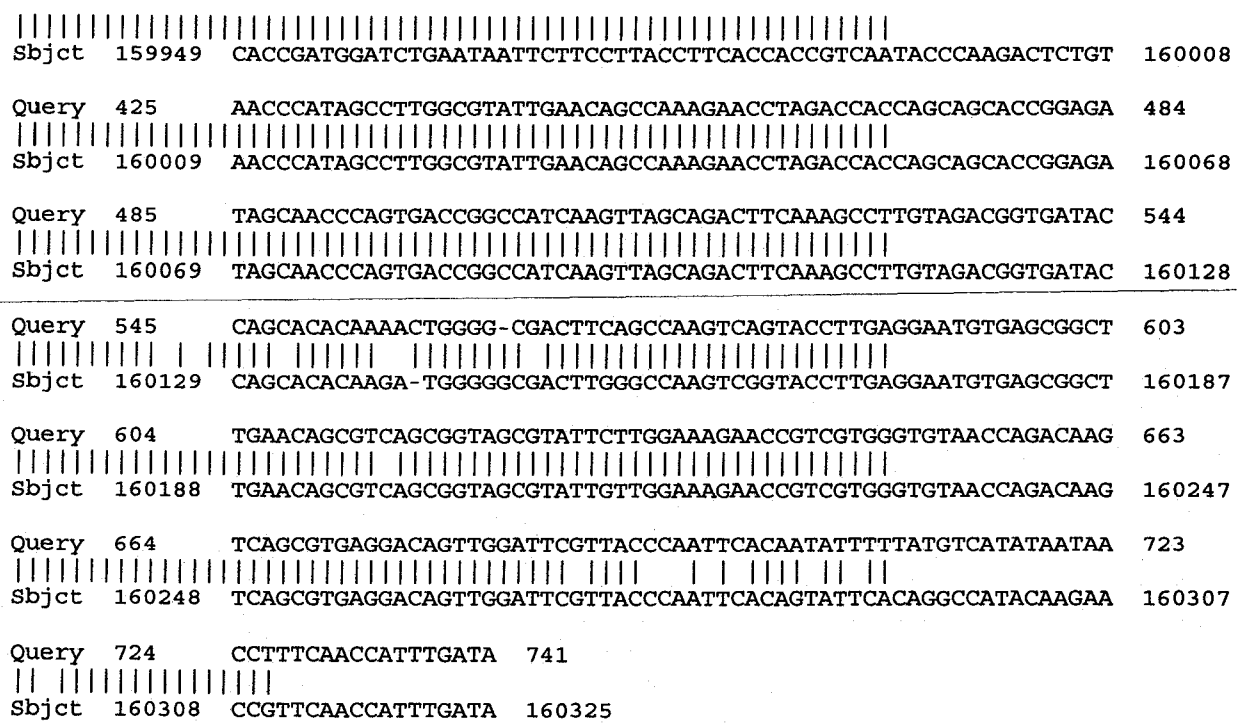

Figure 4.2: The nucleotide blast analysis of the PCR product sequencing result.

The sequence of PCR product possesses 97% similarities in identities in comparison with the Adh1p sequence of *Saccharomyces cerevisiae* chromosome XV, designated as NC_001147.5 on the NCBI and SGD (*Saccharomyces cerevisiae* Genome) databases.

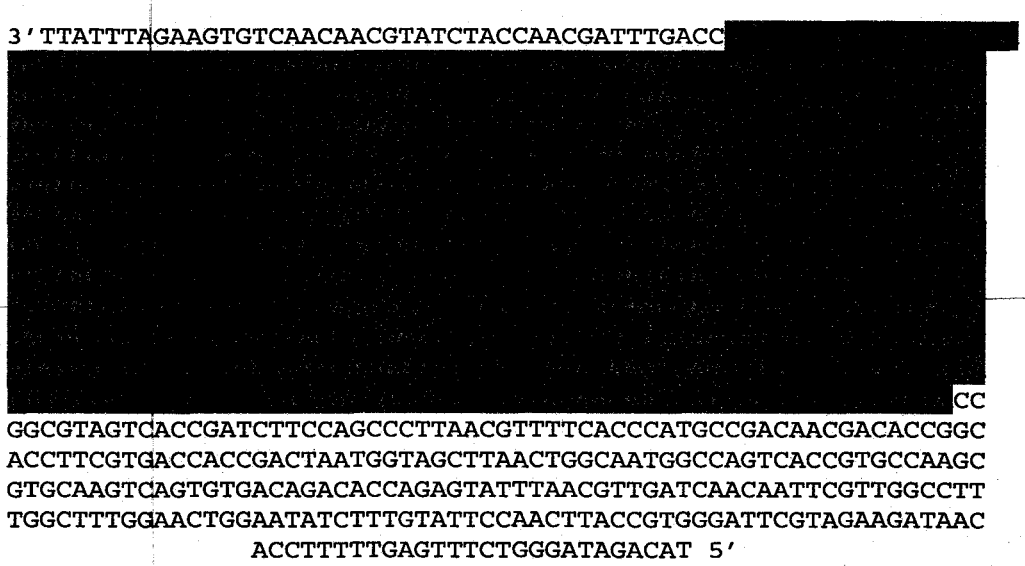

Figure 4.3: The nucleotide sequence of *adh1* gene from coordinate 159548 to 160594 on chromosome XV of the *Saccharomyces cerevisiae* genome. This was retrieved from the SGD database [http://www.yeastgenome.org/]. The yellow highlighted letters indicate the sequence of PCR product identified using the nucleotide blast shown in figure 4.1 and 4.2, from coordinate 159590 to 160325.

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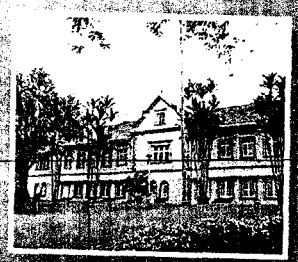

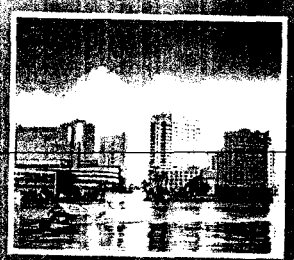

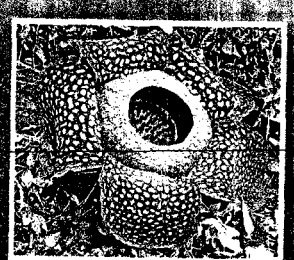

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CAPM-65

EFFECT OF EPOXIDIZED NATURAL RUBBER ON MECHANICAL PROPERTIES OF EPOXY REINFORCED KENAF FIBRE COMPOSITES

M A Abu Bakar, S Ahmad and W Kuntjoro

Kenaf fibre that is known as Hibiscus cannabinus, L. family malvaceae is an herbaceous plant that can be grown under a wide range of weather condition. The uses of kenaf fibres as reinforcement in the polymeric matrix have been widely investigated. It is known that epoxy has a disadvantage of brittleness and exhibit low toughness. In this research, epoxidized natural rubber (ENR) was introduced to the epoxy to increase the toughness of this epoxy. Kenaf fibres with five different fibre loadings of 5%, 10%, 15%, 20% and 25% by weight were used to reinforce the epoxy resins (with and without addition of epoxidized natural rubber) as the matrices. The flexural strength, flexural modulus and fracture toughness of rubber toughened epoxy reinforced kenaf fibre composites was investigated. The results showed that the addition of epoxidized natural rubber (ENR), had improved the flexural modulus, flexural strength and fracture toughness by 31%, 30%, and 28% respectively at 20% fibre loading. The fractured surfaces of these composites were investigated by using scanning electron microscopic technique (SEM) to determine the interfacial bonding between the matrix and the fibre reinforcement.

CAPM-66

CLONING OF GENE ENCODING YEAST ALCOHOL DEHYDROGENASE 1 (YADH 1) IN *Escherichia coli* TOP10 FOR FURTHER USE IN BIOCATALYSIS

Mohd Rezuan M Aspar, Rashidah Abdul Rahim, Mohamad Hekarl Uzir

Yeast producing alcohol dehydrogenase 1 (YADH 1) enzyme has been used as a biocatalyst for the synthesis of an optically active flavouring compound known as citronellol. However, the slow growth of yeast (Saccharomyces cerevisiae) has deterred the progress of biotransformation with only 1.8g/L product obtained. Therefore, the main purpose of this work is to clone the genes producing YADH1 enzyme from yeast into a faster growing organism, which in this case, Escherichia coli. Initially, the sequence of the gene encoding this protein has been identified in the S. cerevisiae Genome Databases (SGD). The so-called Yadh1 gene sequence is located from coordinate 159548 to 160594 on chromosome XV of the organism. Based on this information, two primer sequences (Forward and Reverse) were constructed. Each of these primers will bind to either end of the adh1 gene. The yadh1 gene was then amplified using Polymerase Chain Reaction (PCR) technique. The amplified Yadh 1 gene was 1050 base pair in length. The gene was successfully ligated into a cloning vector, TOPO TA plasmid. This plasmid also contains a gene which confers resistance to ampicillin. The recombinant plasmid was then inserted into Escherichia coli TOP 10 using heat shock protocol at 42°C. Finally, the cloned bacteria which possessed the recombinant TOPO TA plasmid harbouring Yadh1 gene was able to grow on Luria Bertani (LB) media supplied with antibiotic.

CAPM-67

EFFECTS OF CATALYTIC PARTICLES MASS ON FLUIDIZED BED CHEMICAL VAPOR DEPOSITION SYNTHESIS OF CARBON NANOTUBES

Firoozeh Danafar, Fakhru'l Razi Ahmadun, Dayang Radiah Awang biak, Mohd amran mohd Salleh

In fluidized bed chemical vapour deposition synthesis of carbon nanotubes (FBCVD), the initial amount of catalyst provides the operability characteristics of fluidized beds. Therefore, the challenge associated to the catalyst mass placed in the reactor is not only its effects on carbon nanotubes (CNT) formation but also on fluidization condition. Accordingly, in this study the effects of the amount of the catalytic particles were investigated. Result showed that dense bed lessen the process efficieny as well as CNTs quality.

CAPM-68

ANODISATION OF TITANIUM AND TUNGSTEN IN VISCOUS FLUORINE CONTAINED BATH FOR THE FORMATION OF NANOTUBULAR ANODIC OXIDE

Zainovia Lockman, Syahriza Ismail

Anodisation processes of titanium and tungsten foils were studied for the formation of anodic oxides with porous, nanotubular structure. Anodisation was conducted in a typical 2-electrode electrochemical bath. The bath was consisted of excess fluorine in organic viscous electrolyte as well as in aqueous bath. The formation of the nanotubular arrays was successful when anodisation of titanium was carried out at 20V for 30 min in both

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Cloning of Gene Encoding Yeast Alcohol Dehydrogenase 1 (YADH 1) in *Escherichia coli* TOP10 for Biocatalysis

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ABSTRACT

Yeast producing alcohol dehydrogenase 1 (YADH 1) enzyme has been used as a biocatalyst for the synthesis of an optically active flavouring compound known as citronellol. However, the slow growth of yeast (*Saccharomyces cerevisiae*) has deterred the progress of biotransformation. The main purpose of this work is to clone the genes producing YADH1 enzyme from yeast into a faster growing bacteria, *Escherichia coli*. Initially, the sequence of the gene encoding this protein has been identified in the *S. cerevisiae* Genome Databases (SGD). The so-called *Yadh1* gene sequence is located from coordinate 159548 to 160594 on chromosome XV of yeast. Based on this information, two primer sequences (Forward and Reverse) were constructed. Each of these primers will bind to either end of the *Yadh1* gene. The *Yadh1* gene was then amplified using Polymerase Chain Reaction (PCR) technique. The amplified *Yadh 1* gene was successfully cloned into a cloning vector, TOPO TA plasmid. This plasmid also contains a gene which confers resistance to ampicillin. This recombinant plasmid was then inserted into *Escherichia coli* TOP 10 using heat shock protocol at 42°C. Finally, the cloned bacteria containing the recombinant TOPO TA plasmid harbouring *Yadh1* gene was able to grow on Luria Bertani (LB) media supplied with antibiotic.

Keywords: Yeast alcohol dehydrogenase 1 (*Yadh 1*) gene, Polymerase Chain Reaction (PCR), cloning vector, TOPO TA plasmid

NOMENCLATURE

X-gal	bromo-chloro-indolyl-galactopyranoside
kb	Kilobases

INTRODUCTION

Specific Bioreduction of Geraniol *into* Citronellol

Bakers' yeast has been used to catalyse the stereospecific, asymmetric reduction of geraniol forming citronellol (a compound that constitutes the odour of rose). It was demonstrated that the conversion of *trans*-3,7-dimethyl-2,6-octadiene-1-ol (geraniol) into 3,7-dimethyloct-6-en-1-ol ((*R*)-(+)-citronellol). The product formed was reported to be of high optical purity, which is more than 98% enantiomeric

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excesses and is significantly higher than that available from natural pool (70- 80% e.e) (Gramatica *et al.*, 1982). Hence, this method promises a greater commercial value for the production of (*R*)-citronellol. The ability of bakers's yeast to catalyse bioreduction of geraniol forming citronellol in remarkable degree of regio-specificity and stereo-specificity lies in the ability of this organism to produce yeast alcohol dehydrogenase (YADH) enzyme (Reynolds & Holland, 1997).

Yeast Alcohol Dehydrogenase (YADH) Protein

Yeast alcohol dehydrogenase (YADH) is one of the first enzymes to be purified and isolated (Negelein *et al.*, 1937). Prior to the 20th century, only five ADHs were known, namely, ADH1, ADH2, ADH3, ADH4, and ADH5 (de Smith *et al.*, 2008). Following the completion of the sequencing of the *S. cerevisiae* genome in April 1996, additional number of ADH's or sequences related to these enzymes were revealed, and this led to the identification and characterization of ADH6 (Larroy *et al.*, 2003; Jornvall *et al.*, 1999) and ADH7 (de Smith *et al.*, 2008).

YADH1 is believed to be involved in the specific bioreduction of geraniol into citronellol due to several reasons. Firstly, it is located in the cytoplasm, the region where the transformation takes place (de Smith *et al.*, 2008). Secondly, it can catalyse a stereospecific reduction of acetaldehyde to ethanol during fermentation. Fisher *et al.* (1953) reported that YADH catalyses the transfer of hydrogen from NADH (co-factor) to acetaldehyde (substrate) forming NAD^+ and ethanol as the product. In this reaction, the enzyme is shown to be able to discriminate between the diastereotopic hydrogens attached at a C4 of the co-factor.

Furthermore, the specificity of YADH is also given by a particular arrangement of substrate and co-factor at the active site (Weinhold *et al.*, 1991). In the context of geraniol as the substrate, the active site of YADH is positioned in a way that the hydrogen atom from NADH can be transferred to the *Si* faces of the central carbon of the geraniol *(Fig. 1)*. As a result, the methyl group attached to the central carbon is protruded to the *Re* face, forming (*R*)-citronellol instead of the (*S*)-isomer.

Therefore, the purification of ADH1 from yeast is crucial to demonstrate whether this enzyme is the one involves in the reduction of geraniol into citronellol. In this experiment, the first step to obtain the isolated and purified ADH1 was carried out by identifying and isolating the gene that encodes for ADH1. The gene of yeast alcohol dehydrogenase 1 (*Yadh1*) was cloned and recombinant *Escherichia coli* was constructed. This strain can express the recombinant YADH1 protein and will be subsequently used as a possible biocatalyst that can perform the bioreduction of geraniol into (*R*)-citronellol.

MATERIALS AND METHODS

Microorganism, Plasmid and Cultivation Conditions

In this study, *Saccharomyces cerevisiae* strain used in the fermentation of geraniol was used as the genetic source. Meanwhile, *Escherichia coli* TOP10 was used as the host cell, whereas, TOPO TA 2.1 plasmid was the cloning vector. *S. cerevisiae* and *E. coli* were grown at 30°C and 37°C, respectively.

Yeast Genomic Extraction

The yeast genomic extraction procedure was carried out according to Sambrook *et al.* (2000). The overnight 10 ml yeast culture was harvested by centrifugation at 3000 rpm for 5 minutes at room temperature. The supernatant was discarded and the cell pellet was transferred to a microcentrifuge tube and spun for 5 seconds. The supernatant was decanted and the cell pellet was disrupted by a brief vortex.

The technique to break open the cell was then performed by treating the cells with 200 µl breaking buffer, 200 µl phenol, and chloroform and isoamyl alcohol, prior to vortexing at the highest speed for 3 minutes. The sample was vortexed and added with 200 µL TE buffer. After 5 minutes of centrifugation at a high speed, the aqueous layer was transferred into a microcentrifuge tube and 100% ethanol was added. This was followed by another 3 minutes of high speed centrifugation and then, the supernatant was collected and resuspended in 0.4 ml TE buffer. The suspension was added with 30 µl 1 mg/ml DNase-free RNase A and incubated at 37°C for 5 minutes. 10 µL of 4 M ammonium acetate and 100% ethanol were also added. After a final microcentrifugation, the dry pellet containing the desired DNA was resuspended in 100 µl TE buffer.

Identification and Amplification of Yadh 1 *Gene*

Saccharomyce cerevisiae Genome Databases (SGD) [http://www.yeastgenome.org/] and National Centre for Biotechnology Information (NCBI) [http:// www.ncbi.nlm. nih.gov/] were used as the sources of the genetic information of this particular organism.

Primer Construction

The forward and reverse primer was constructed (Table 1) based on the *Yadh1p* gene sequence revealed in the SGD and NCBI online databases.

TABLE 1
Specific primers used for the amplification of *Yadh1* gene

Primer	Sequence
Forward	5'-GGCGGATCCTATCCCAGAAACTCAAAAAGGTG-3'
Reverse	5'-GGGGAATTCCAACAACGTATCTACCAACGA-3'

Polymerase Chain Reaction (PCR)

PCR was performed using the cycle and conditions shown in Table 2. The time for cooling process in step 7 was set infinite because the PCR product was very unstable and must always be kept at a very low temperature. The PCR products obtained were analyzed using gel electrophoresis. The PCR product was then purified using QIAquick™ Gel Extraction kit.

TABLE 2
PCR cycle and condition for the amplification of *Yadh1* gene

Step	Reaction	Temperature (°C)	Time (min)	Cycle
1	Initial	95	5	1
2	Denaturation	95	1	1
3	Annealing	59	1	1
4	Extension	72	1	1
5	Go to step 2	95		30
6	Final Elongation	95	10	1
7	Cooling	10	∞	1

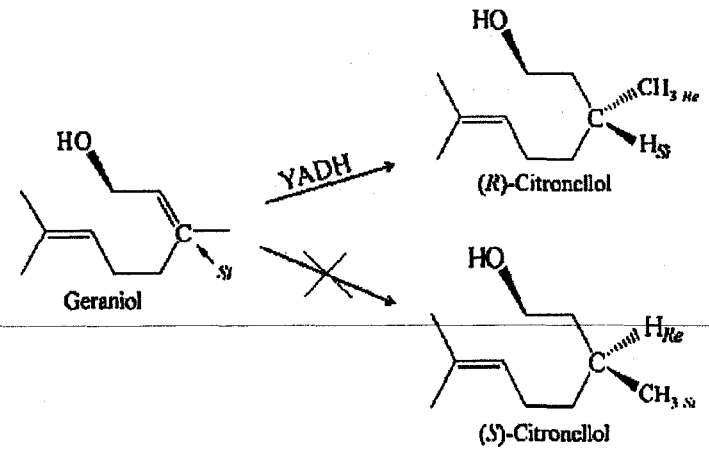

Fig. 1: Stereospecific reduction of geraniol into (R)-citronellol catalysed by yeast alcohol dehydrogenase (YADH) enzyme

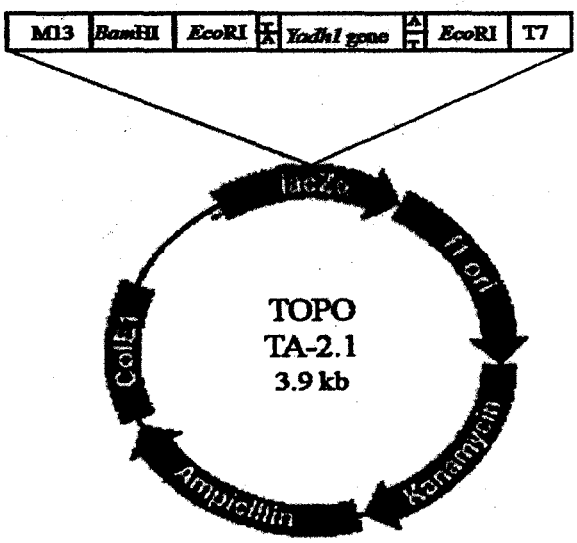

Fig. 2: Schematic representation of TOPO TA 2.1 plasmid map (Invitrogen) containing Yadh 1 *gene insert*

Gene Sequencing and Analyses

The purified PCR product (*Yadh1* gene) sample was sent to First Base Laboratories for sequencing analysis. Finally, the sequencing results obtained was analyzed using the Nucleotide Blast Software (NCBI).

Construction of Recombinant Plasmid and Cloning of Yadh 1 *Gene into* E. coli *TOP10*

The *Yadh 1* gene was ligated into TOPOTA 2.1 plasmid (*Fig. 2*). The recombinant plasmid was inserted into *E. coli* TOP10 using heat shock protocol at 42°C for two minutes as described by Sambrook *et al.* (2000). The transformed cells were grown overnight at 37°C on Luria Bertani (LB) agar media supplied with ampicilin and bromo-chloro-indolyl-galacto- pyranoside (X-gal).

Analyses of the Recombinant Plasmid carrying Yadh 1 *Gene*

The recombinant TOPO-*Yadh1* plasmid was extracted from the transformed *E. coli* TOP10 and subjected to molecular analyses, such as digestion with restriction enzyme *Eco*RI and *Bam*HI. The purified recombinant plasmid was also sequenced to confirm the presence of *Yadh1* gene insert.

RESULTS AND DISCUSSION

The isolation of *Yadh1* gene was carried out by extracting the genomic DNA of *S. cerevisiae* and amplifying it using direct PCR technique. The genomic DNA with the concentration of approximately 50 ng/uL (*Fig. 3*) was successfully extracted using the conventional (phenol/ choloroform) extraction method.

Based on the analyses of the DNA sequence using the Nucleotide Blast software, the sequence of PCR product possesses 97% similarities in identities compared to the *Adh1p* sequence of *S. cerevisiae* chromosome XV, which is designated as NC_001147.5 on the NCBI and SGD databases.

The purified *Yadh1* gene was ligated into the multiple cloning sites (MCS) of TOPO 2.1 cloning vector. Meanwhile, *E. coli* TOP10 was transformed with the TOPO plasmid. The identification and selection of successfully transformed cells were also undertaken. Only the cells containing the plasmid can grow on ampicillin containing agar as they carry the gene conferring resistance to ampicillin. In addition, this plasmid also contains *LacZ*α which codes for β-galactosidase. β-galactosidase will cleave colourless X-gal presents in the agar media to yield galactose and 5-bromo-4-chloro-3-hydroxyindole. The latter is then oxidized into 5,5'-dibromo-4,4'-dichloro-indigo, an insoluble blue product (Bassaneze *et al.*, 2008). Therefore, the bacterial colony will appear blue.

However, the recombinant plasmid that formed, following a successful ligation, has the *Yadh1* gene being inserted in the middle of *LacZ*α. As this gene was disrupted, it would no longer produce functional β-galactosidase and thus, appeared white on the LB agar, as shown in *Fig. 5*. This finding is summarized in Table 3.

TABLE 3
Expression of Recombinant *Yadh1* in *E. coli* TOP10

Strain	Growth on LB media containing ampicilin	X-gal indication
E. coli TOP10 (without TOPO TA)	No	-
E. coli TOP10 (TOPO TA plasmid)	Yes	Blue
E. coli TOP10 (TOPO TA plasmid plus *Yadh1* gene)	Yes	White

The extraction of recombinant TOPO-*Yadh1* plasmid was performed from an overnight culture of a white colony. The extracted plasmid was analysed by digestion with restriction enzyme *Eco*RI and *Bam*HI. In *Fig. 6 (a)*, two distinct bands were produced when the TOPO-*Yadh1* was digested with *Eco*RI. This enzyme cuts at three sites on the plasmid, as illustrated in *Fig. 2*. The resulting fragments are 3.9 kb (contains the plasmid vector only), 1.05 kb (consists of 1.047 kb *Yadh1* gene inserts). Meanwhile, the digestion with *Bam*HI, shown in *Fig. 6b*, produced only one linear band of 4.95 kb since there is only one *Bam*HI recognition site present in this plasmid.

In order to prove the success of the cloning of *Yadh1* gene into *E. coli* TOP10, the purified recombinant TOPO-*Yadh1* plasmid had to undergo a sequencing analysis. The sequencing results

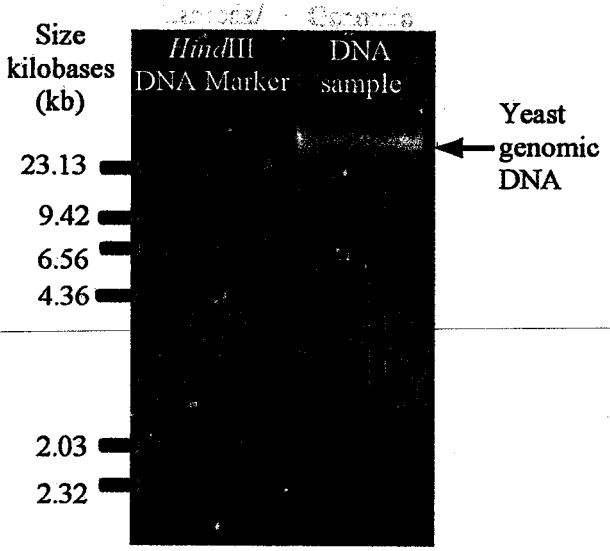

Fig. 3: A photograph of electrophoresis gel showing the analysis of genomic DNA extraction of S. cerevisiae

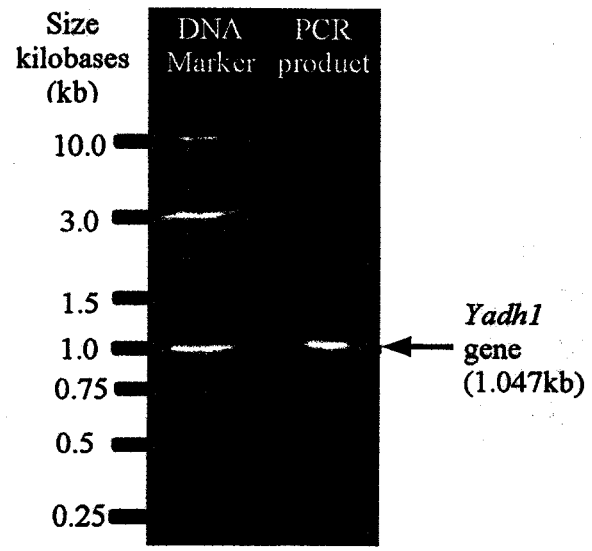

Fig. 4: A photograph of electrophoresis gel showing the analysis of the PCR product (amplified Yadh1 *gene)*

Fig. 5: Formation of blue and white E. coli *TOP10 colonies on selective LB agar media containing ampicilin*

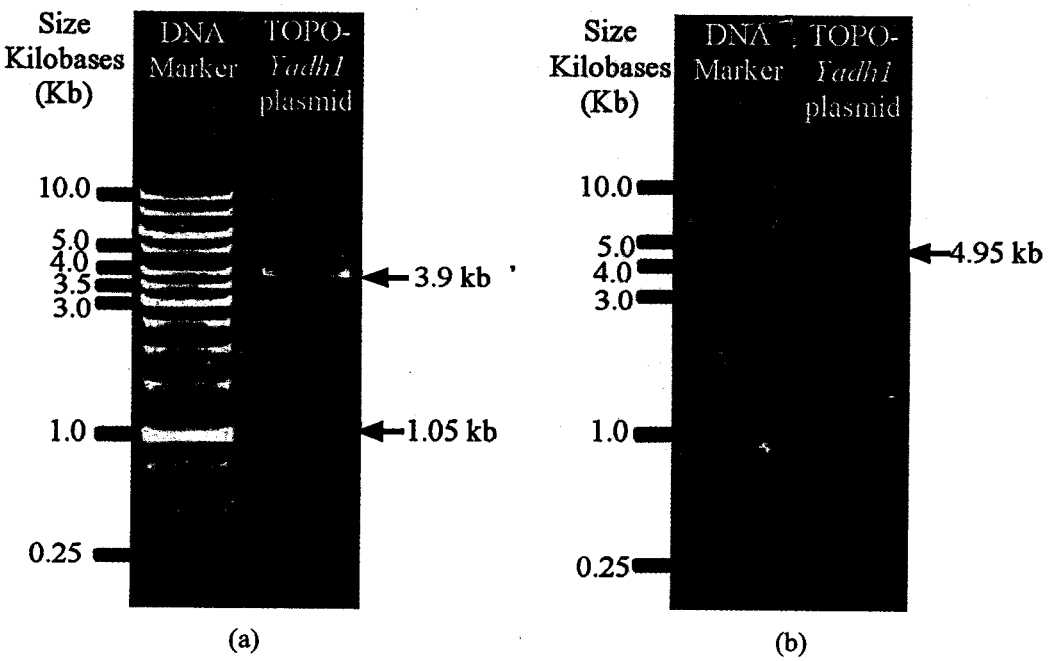

Fig. 6: Recombinant TOPO-Yadh linear fragments digested with restriction enzyme; EcoRI and (b) BamHI

were then analyzed using the Nucleotide Blast software. The sequence of the PCR product possessed 100% similarities in identities in comparison with the *Adh1p* sequence of *S. cerevisiae* chromosome XV, designated as NC_001147.5 on the NCBI and SGD databases.

CONCLUSIONS

Yadh1 gene has been successfully cloned from *S. cerevisiae* into *E. coli* TOP10. The molecular studies have shown that the recombinant TOPO-*Yadh1* gene produced by the genetically engineered *E. coli* TOP10 has significant similarities in identities in comparison with the one available in the databases. The next step is to allow the cloned *E. coli* strain to produce *Yadh1* gene for further use as a biocatalyst for highly specific bioreduction of geraniol into citronellol.

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