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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^{o}C and 37^{o}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

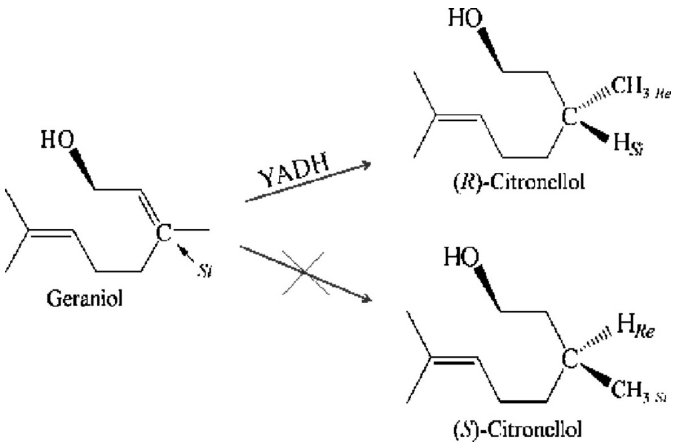

Fig. 1: Stereospecific reduction of geraniol into (R)-citronellol catalysed by yeast alcohol dehydrogenase (YADH) enzyme

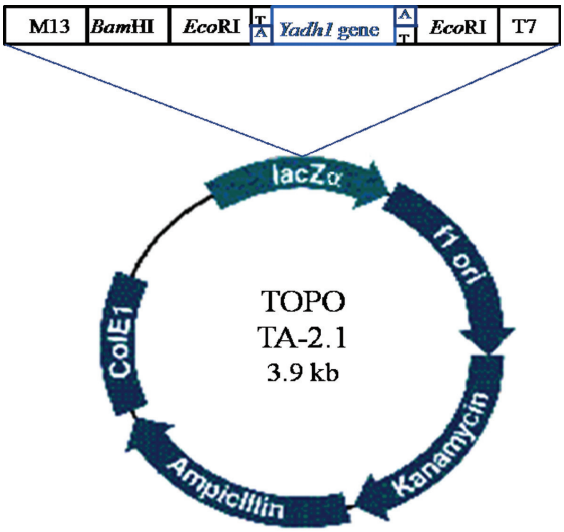

Fig. 2: Schematic representation of TOPO TA 2.1 plasmid map (Invitrogen) containing Yadh 1 *gene insert*

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

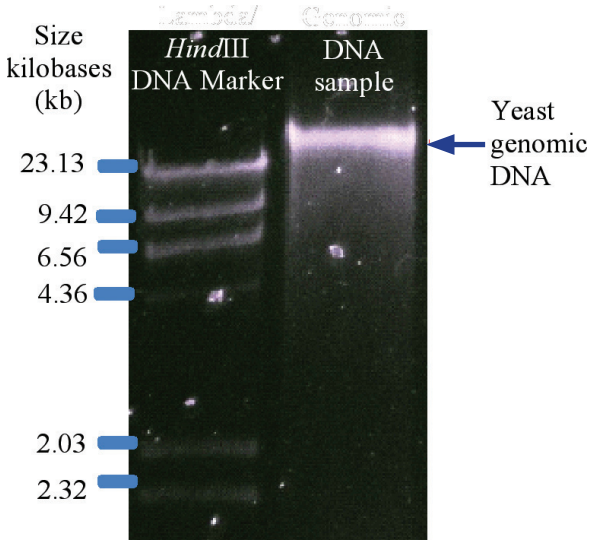

Fig. 3: A photograph of electrophoresis gel showing the analysis of genomic DNA extraction of S. cerevisiae

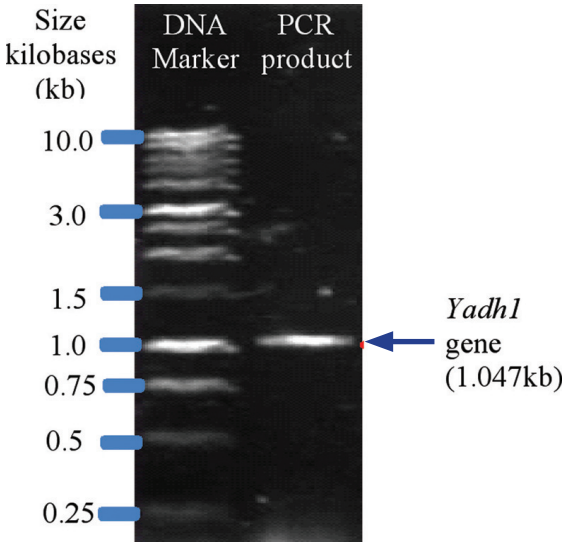

Fig. 4: A photograph of electrophoresis gel showing the analysis of the PCR product (amplified Yadh1 *gene)*

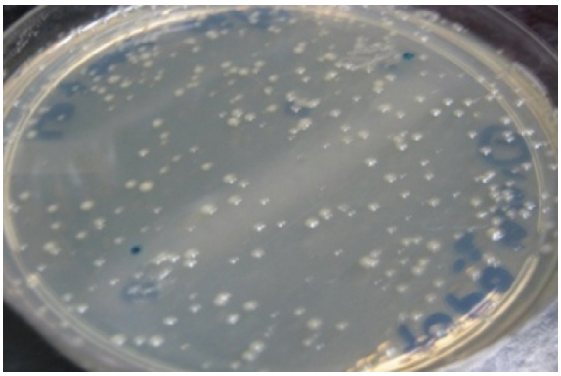

Fig. 5: Formation of blue and white E. coli *TOP10 colonies on selective LB agar media containing ampicilin*

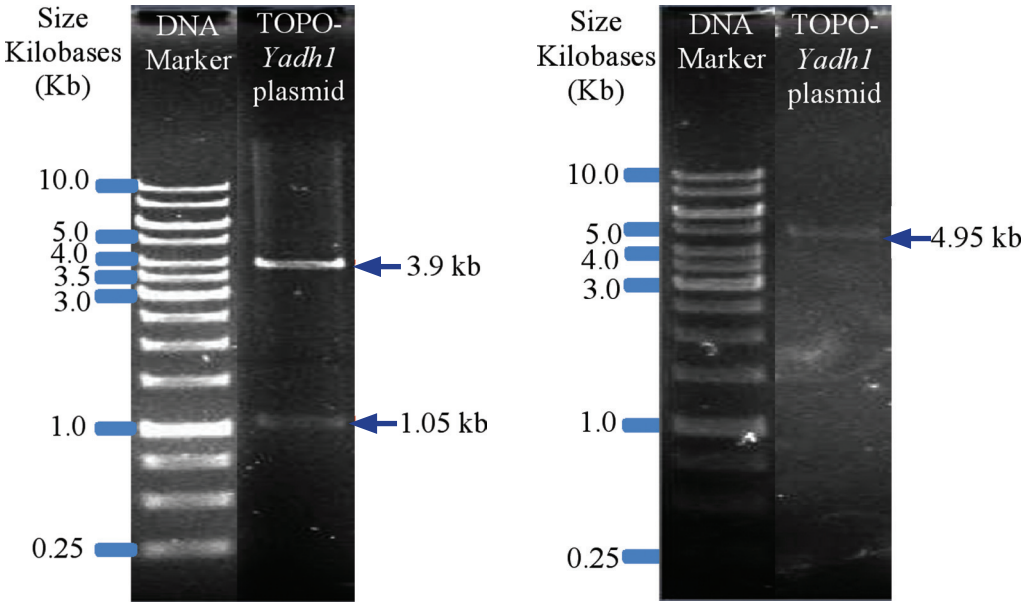

Fig. 6: Recombinant TOPO-Yadh linear fragments digested with restriction enzyme; EcoRI and (b) BamHI

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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