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BIOTRANSFORMATION OF CITRAL BY FREE AND IMMOBILIZED *Saccharomyces cerevisiae*

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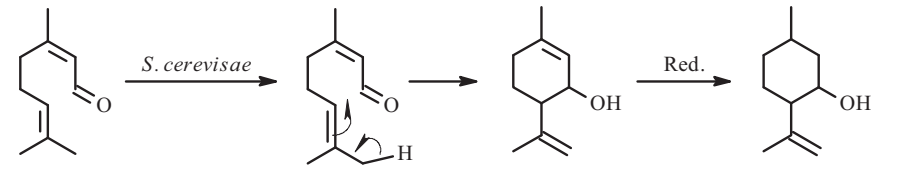
Citronellol is a fragrance ingredient used in decorative cosmetics and fine fragrances. It has many applications in flavoring, extracts, and food and drug manufacturing. Its use worldwide is greater than 1,000 metric tons per annum. Previous studies concerning the biotransformation of (L)-citronellal to (L)-citronellol using free and immobilized cells of *Rhodotorula minuta* have been reported. The objective of this research was to study the pathways involved during biotransformation of commercial citral by a free cell method (FCM) and an immobilized cell method (ICM) using *Saccharomyces cerevisiae.* Biotransformation of citral by FCM and ICM are important methods to convert cheap and plentiful organic compounds into more useful ones, and rely on the ability of plant cell cultures to specifically produce secondary metabolites.

The culture preparation was done using different microbial methods and incubation periods to obtain the maximum number of *S. cerevisiae* cells for citral biotransformation. It was found that using either method, citral was converted to citronellol in high yield. The biotransformation products were identified by their theoretical study (TS), Fourier-transform infrared spectroscopy (FT-IR), ultraviolet visible (UV) spectrometry, gas chromatography (GC), and gas chromatography/mass spectroscopy (GC-MS). Comparison of the two methods showed ICM was more effective as it produced one major product, citronellol (48.5%), while FCM produced more products, yielding citronellol at only 24.2% and 27.2% over a period of 6 and 15 days, respectively.

The literature provides several examples of biotransformations of citronellal. The biotransformation of (L)-citronellal to (L)-citronellol by free and immobilized *Hodotorula minuta* has been reported [1]. The bioconversion of geranyl and neryl acetate by *Aspergillus niger* has also has been described [2, 3]. One report showed that the main reaction of a liquefied method using *A. niger* was hydrolysis of a terpene to 8-hydroxy derivatives during ω-hydroxylation [4]. Using a sporulated surface culture method, we found that geraniol was converted to linalool and partially oxidized to citral [5]. Microbial transformation of geraniol and nerol by a sporulated surface culture method using five *A. niger* strains and three *Penicillium* strains were compared with a submerged liquid method [6]. Several groups have used different fungi to bioconvert citral [7–12]. In this article, biotransformation of the pure terpene aldehyde citral is carried out by a sporulated surface culture method, and the pathways involved in this biotransformation are investigated. Microbial transformation of citral by a sporulated surface culture method grown on 50 mL of medium in conical flasks was monitored over 3 weeks. The suspension was extracted with Et_2O three consecutive times and directly analyzed by GC and GC-MS.



Scheme 1

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TABLE 1. Possible Compounds of Bioconversion of Citral by *S. cerevisiae*

Compound	FCM, %		ICM	Compound	FCM, %		ICM
	6 days	15 days			6 days	15 days	
α-Terpineol	1.0			Geraniol		9.2	
Limonene	1.2			*p*-Mentha-1,5-dien-8-ol		4.7	
α-Pinene	1.2			4-Hydroxy-4-methyl-2-pentanone		1.0	1.0
Geraniol	8.2			1-Hydroxy-p-Mentha-3-one			1.0
Citronellol	24.2	27.2	48.5	*p*-Menth-8-en-3-ol			6.3
3,5-Dimethyl-2-cyclohexen-1-one		6.1					

The culture conditions suitable for obtaining maximum *S. cerevisiae* cell growth under shaker flask conditions were determined. The increase in cell concentrations during the growth of *S. cerevisiae* in PGE (peptone broth/glucose/yeast extract) and MYPB (malt extract/yeast extract/peptone broth) was assessed. These results are similar to those reported for *Rhodotorula minuta* [13–15]. The *S. cerevisiae* biotransformation of citral by FCM and ICM was studied under constant pH and temperature conditions. GC and GC-MS analysis were used to analyze the products. The determination of the most suitable culture age during the growth of *S. cerevisiae* in PGE for maximum product formation was done by harvesting the cells at various stages of growth and employing them for citral biotransformation. The results indicated a cell culture age of between 6 and 15 days was the most suitable for FCM and 7 days for ICM for optimum product formation. After these longer periods, the cells were at the end of the exponential growth phase and had attained maximum cell concentrations. After 6 days of citral biotransformation with *S. cerevisiae* using FCM, the main products were α-terpineol (1.0%), limonene (1.2%), α-pinene (1.2%), geraniol (8.2%), and citronellol (24.2%) (Table 1). After a total of 15 days, the biotransformation of citral yielded 3,5-dimethyl-2-cyclohexen-1-one (6.1%), geraniol (9.2%), *p*-mentha-1,5-dien-8-ol (4.7%), 4-hydroxy-4-methyl-2-pentanone (1.0%), and citronellol (27.2%) (Scheme 1). The biotransformation of citral using ICM, yielded 1-hydroxy-*p*-mentha-3-one (1.0%), citronellol (48.5%), *p*-menth-8-en-3-ol (5.3%), and 4-hydroxy-4-methyl-2-pentanone (1.0%). The optimum values of pH and temperature for biotransformation were found to be approximately pH 5.5 and 27°C for all three incubation periods.

The optimum initial medium pH and temperature values for biotransformation by FCM are similar to those found in the literature for the biotransformation of the other optical isomer, (D)-citronellal [16]. The optimum substrate concentration for obtaining maximum product concentrations was determined by adding different substrate concentrations to *S. cerevisiae* cells grown as discussed above. The study of the microbial transformation of a monoterpene by a sporulated surface culture method using *Aspergillus niger* and *Penicillium* sp. showed that biotransformation of *cis*-*p*-menthan-7-ol yielded limonene, *p*-cymene, and γ-terpinene [17]. Leuenberger reported that product yields could be effectively increased by solubilizing/emulsifying immiscible substrates [18]. However, careful selection of the nature and concentration of solvent is necessary since many miscible solvents are cytotoxic at lower concentrations [19]. The biotransformation of volatile monoterpenoids by fungal FCM and ICM was also examined. Using the FCM method, we identified 10 components on different days (6 and 15 days of biotransformation) representing 35.8% and 48.2% total yields respectively, but using ICM, four compounds were produced with a total yield of 55.8%.

From the data given in Table 1, it can be concluded that citral was primarily converted to citronellol, 3,5-dimethy-l-2-cyclohexen-1-one, and geraniol. The pathway responsible for this conversion involved the formation of a C=C bond followed by the transfer of an electron to produce a *p*-menth-8-en-3-ol compound (Scheme 1).

By comparison with the literature, bioconversion of citral and nerol by spores of *Penicillium digitatum* resulted in the production of 6-methylhept-5-en-2-one [7]. Microbial transformation of geraniol, nerol, and citral by *Aspergillus niger* resulted in linalool and α-terpineol. Bioconversion of nerol with *Penicillium chrysogenum* yielded mainly α-terpineol and some unidentified compounds. With *Penicillium rugulosum* the major bioconversion product from nerol and citral was linalool [6].

The microbial transformation of monoterpenes by a sporulated surface culture method using *Aspergillus niger* and *Penicillium* sp. produced *cis*-*p*-menthan-7-ol from *A. niger* and showed that the main products were limonene, *p*-cymene, and γ-terpinene [17]. The two main products of microbial transformation of citral are similar to those obtained by others [20]. The main bioconversion products of (–)-menthol by *Mucor ramannianus* using a sporulated surface culture method were *trans*-*p*-menthan-8-ol, *trans*-menth-2-en-1-ol, sabinane, *p*-menthane-3,8-diol, isomenthol, and 1,8-cineole [20]. The main biotransformation products obtained from menthol by surface *Penicillium sp.* were α-pinene (18.0%), *trans*-*p*-menthan-1-ol (10.6%), *p*-menth-1-ene (5.8%), sabinene (3.9%), 1,8-cineole (6.4%), and limonene (3.2%) [21]. Two recent articles suggested

that monoterpene biotransformation with *Penicillium* and *Aspergillus* resulted in oxidation and transformation of monoterpenes to a more stable product.

Experiments with FCM and ICM. *S. cerevisiae* cells were separated by centrifugation at 800 rpm at 10°C for 30 min. The cells were washed and resuspended in sterile 0.85% (w/v) saline. Equivalent cell concentrations (4.45 g/L) were immobilized by the following entrapment and adsorption methods.

Agarose Entrapment. Agarose solution prepared by dissolving agarose (15% w/v) in water at 100°C was cooled to 40°C and mixed with separated *S. cerevisiae* cells. The mixture was allowed to solidify by standing at 4°C. The hard gel was shredded in a Waring blender and nonentrapped cells removed by washing with saline [22].

Biotransformation. Biotransformation was started after adding 100 mL of medium containing 4.47 g/L myrcene; 0.1 g/L methanol was used as a solubilizing agent.

Optimum Conditions for Biotransformation. *S. cerevisiae* was added into the media, and a suitable growth phase was obtained by examining different phases of growth. An agitation speed of 150 rpm with biotransformation times of 30, 72 and 120 h was employed.

Identification of the constituents of the oil was made by comparison of their mass spectra and retention indices (RRI) with those given in the literature and with authentic samples [23, 24, 12]. The mass spectra of a mixture of geraniol, citronellol, *p*-menth-8-en-3-ol, α-pinene, and limonene had five main peaks.

Geraniol. MS *m/z*: 154 [M^+] 41 (100), 69 (75), 81 (15), 53 (10), 93 (7), 123 (7), 111 (6), 139 (5).

Citronellol. MS *m/z*: 156 [M^+] 41 (100), 69 (82), 55 (55), 82 (30), 67 (37), 81 (32), 57 (28), 95 (20).

***p*-Menth-8-en-3-ol**. MS *m/z*: 170 [M^+] 43 (100), 69 (45), 41 (45), 58 (40), 109 (30), 71 (22), 97 (20), 82 (20).

α-Pinene. MS *m/z*: 136 [M^+] 93 (100), 91 (43), 92 (39), 77 (29), 79 (24), 105 (17), 121 (12), 139 (5).

Limonene. MS *m/z*: 136 [M^+] 68 (100), 67 (86), 93 (78), 79 (43), 39 (36), 53 (34), 107 (30), 121 (30).

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