Asian Journal of Chemistry Vol. 21, No. 3 (2009), 2412-2418

Biotransformation of Camphor Using Free and Immobilized *Saccharomyces cerevisiae*

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The biocatalytic reduction of camphor was carried out using Baker's yeast (*Saccharomyces cerevisiae*) as microbial catalyst in free Baker's yeast (FBY) as well as immobilized Baker's yeast (ImBY) form in aqueous ethanolic (1:1v/v) medium. The reduction product was isolated and purified by chromatographic techniques and characterized on the basis of its spectral analysis.

Key Words: Baker's yeast, Immobilized baker's Yeast, Camphor.

INTRODUCTION

Monoterpenes are widely distributed in nature and find extensive applications in the flavour and fragrance industry depends on the absolute configuration of the compounds because enantiomers present different organoleptic properties. Their simple structures make them ideal targets for microbial biotransformation to yield several commercially important products[1].

Biotransformation allows the production of regio- and stereoselective compounds under mild conditions. These products may be labeled as natural and commands higher value in the market than it's chemically produced counterpart.

Commercially useful chemical building-blocks and pharmaceutical stereo isomers can also be produced by bioconversion of terpenes. Enzymes and extracts from bacteria, cyanobacteria, yeasts, microalgae, fungi, plants and animal cells have been used for the production and or bioconversion of terpenes. In addition, whole cell catalysis has also been used.

Reduction by *Saccharomyces cerevisiae*[2-4] have been reported for various carbonyl compounds. However, information on the biotransformation of camphor to borneol remains limited. The optimization of process parameters remains a challenging task due to several limitations posed by monoterpenes such as toxicity and volatility, by-product formation, immiscibility and low yields of the product[5]. Immobilized cells have been used for carrying out biotransformation of various substrates. They offer advantages such as easier separation and reuse of cells, maintenance of higher cell concentrations and stabilization of several cell functions[6], micro-organism entrap-

ment in a gel or a membrane or within microcapsules are applicable for laboratory as well as industrial use (urethane, cellulose, agar, alginate, *etc.*[7]. Collagen, chitosan, k-carragenan[8] and montmorillonite-K10[9], have been used as polymers porous networks for entrapment. Steroid hydroxylations by immobilized *Curvularia lunata*[10] and biotransformation of glycerol by alginate immobilized *Gluconobacter oxydans*[11] have been reported earlier. The biotransformation reactions can be carried out by using various types of immobilized cells.

Different types of immobilization are represented diagrammatically as follows:

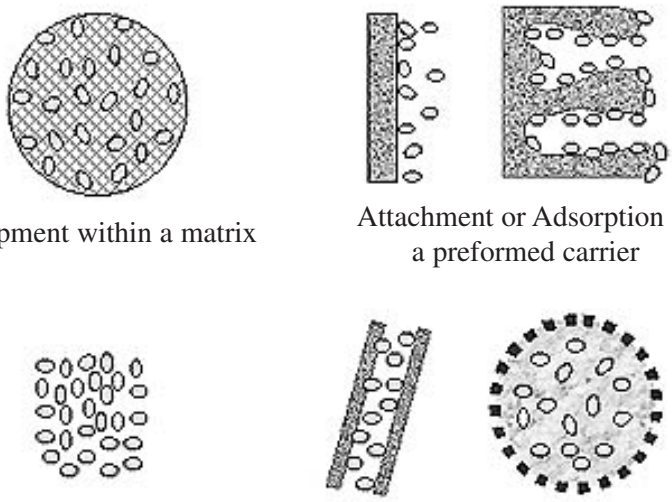

Fig. 1

Recently, it has been shown that immobilization of whole cells results in lowering of substrate toxicity in case of β-ionone biotransformation by immobilized *Aspergillus niger*[12] and ethyl 3-oxobutanoate reduction by alginate immobilized bakers yeast[13]. During *Saccharomyces cerevisiae* and *Candida utilis* mediated biotransformation of benzaldehyde to L-phenylacetylcarbinol, the toxic effects of the substrate on immobilization of the yeast cells were minimized[14].

In the present work, the optimal conditions for application of *Saccharomyces cerevisiae* in biotransformation of camphor to borneol by free and immobilized cells is reported. The reuse of immobilized cells is presented in this work. The suitable culture conditions such as pH, temperature and agitation were studied to maximize the product concentration.

EXPERIMENTAL

Acrylamide, Ammonium per sulphate, *Tris*(trihydroxy methyl amino ethane), N,N,N′, N′′-tetra methyl ethylenediamine (TEMED) were purchased from Sigma Chemicals Co., USA.), N,N′-methylene *bis*acrylamide, yeast extract, sucrose were

purchased from Hi-Media Laboratories Pvt. Ltd. Mumbai, India, Other chemicals of analytical grade were obtained from standard sources.

Reduction of camphor using free Baker's yeast: In a 1 L round-bottom flask, equipped with magnetic stirrer (2LH Remi make) 200 mL water, 10 g fresh Baker's yeast and 4 g glucose were placed and the suspension was stirred for 0.5 h. The camphor (2 mmol) was separately dissolved in to ethanol (50 mL) and ethanolic solution was poured into Baker's yeast suspension. The resulting mixture was make up with water to about 750 mL and was magnetically stirred for a suitable period. The suspension changes its colour from white to dull creamish.

After the completion of the reaction, the Baker's yeast was separated from the mixture by vaccum filtration. The filtrate was extracted with methylene chloride and methylene chloride extract was dried over sodium sulphate and was allowed to evaporate, an offwhite crystalline product was obtained. The purity of product was checked by single spot TLC developed in iodine chamber. The m.p. of the product came out to be 208 °C (reported 206-207 °C) and it was then characterized by spectral analysis.

Reduction of camphor using immobilized Baker's yeast by polyacrylamide gel: (a) The (5 % cross linked) polyacrylamide gel was prepared using following solutions: 1.0 mL of solution 'E', 0.5 mL of solution 'F', 0.5 mL of solution 'G', 2.0 mL of solution 'H'.

Where the composition of above solutions was as follows:

Solution E = 10 g acrylamide and 2.5 g N,N′-methylene *bis*acrylamide in 100 mL double distilled water.

Solution F = 5.98 g *tris*(trihydroxy methyl amino ethane), 0.46 mL TEMED and 48 mL 1 N HCl to 100 mL solution.

Solution G = 560 mg ammonium per sulphate (APS) in 100 mL DDW.

Solution H = 34.2 g sucrose in 100 mL DDW.

After preparing the above solutions they were added in the sequence

E + F + H (5 g Baker yeast) → (dearation for 0.5 h) + G

Polymerization was allowed to take place at 20 ºC for 1h and the resulting gel was cut into even pieces. The pieces were washed with saline (0.85 % w/v) to remove unbound cells[15].

Sectional representation of cross linked copolymer of acrylamide and *bis* acrylamide is depicted in the Fig. 2.

Activation of immobilized pieces: After the yeast cells undergo the immobilization, they were transferred to glass assembly having required quantity of camphor dissolved in aqueous:ethanol (50:50 v/v), that was used as the reaction media. For determination of the yeast cells immobilized cell effectivity during biotransformation in low nutrient surroundings supplements of reactant and immobilized gel pieces were added as decided.

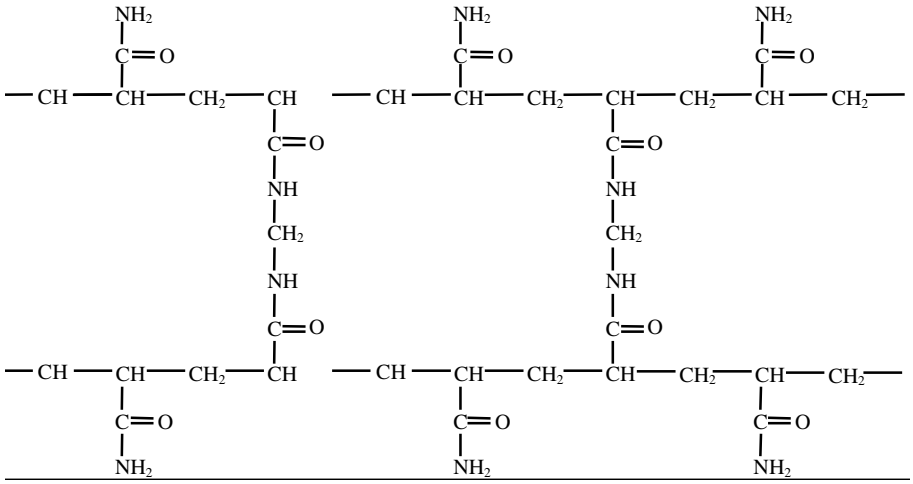

Fig. 2. Structure of gel matrix

Optimum conditions for biotransformation: The optimum values of pH and temperature were estimated in the range of pH 4-9 and by varying temperature from 20-40 ºC and it was found that at pH 7 and 27 ºC temperature the reduction process was faster with high yield. The time required for maximum product formation was determined by analyzing the product after every 0.5 h interval. An agitation speed of 150 rpm and biotransformation time of 48 h was employed. Optimum solvent/emulsifier type and concentration to be employed was standardized by addition of these to baker yeast cells.

Extraction and analysis: On completion, the contents of the flasks were extracted twice with petroleum ether, de-moisturized by addition of 1 g L^{-1} sodium sulphate. The products were further identified by IR, NMR, mass analysis.

RESULTS AND DISCUSSION

The biotransformation of camphor to borneol by free cells of *Saccharomyces cerevisiae* was studied under different pH and temperature conditions. IR analysis revealed the presence of -OH bond. Biotransformation at pH values in the range of pH 4-9 showed a gradual increase in the product upto pH 7. No significant change in the product concentrations were seen at pH's above 7 at substrate (camphor) concentration of 2 mM. No change in the pH value was seen during the course of biotransformation at the end of 24 h. Studies on the effect of temperature showed that the product concentration increased from 20 to 27 ºC and then decreased. Time period of 40-48 h was found to be suitable for optimum product formation. On addition of the substrate, the concentrations of the product obtained were 40 % in 2 h, 55 % in 6 h, 70 % in 24 h and 80 % in 48 h.

Re-use of immobilized cell: Immobilized cell reuse in a batch wise manner has been done earlier *e.g.* steroid biotransformations using polyacrylamide immobilized

Clostridium paraputrificum cells where a gradual decrease in activity was seen on reuse of immobilized cells in spite of subjecting them to intermittent growth medium[16]. Immobilized *Actinoplanes teichomyceticus* could be reused 5 times in a batch wise manner (30 d) during biotransformation of lipoglycopeptide antibiotic A40926[17]. During present investigations on biotransformation of camphor, it was observed that immobilized baker yeast cells could be re-employed twice after the first use. Gel pieces were activated in nutrient rich media (sucrose solution) at pH 7 and 27 ºC for 12 h, before the first use. The product concentrations during the first and second reuse were 65 and 50 %.

Immobilization enhances the operational stability of free Baker's yeast and isolation of the products becomes easier. In addition, reuse of the catalyst is often possible under these conditions the product formation rates are usually high[5], not only because of the inhibitory influences but also high cell population. It is also permits easy continuous operation since immobilized cells can be easily removed from the reaction medium and can be repeatedly reused although with decreasing activity of the immobilized cells.

The actual reducing agent in present system is nicotinamide adenine dinucleotide hydride (NADH). NADH donates $H^{\bullet}$ (hydride ion) to aldehydes and ketones (and thereby reduces them). The electron lone pair on a nitrogen atom of NADH pushes out H- which adds to a carbonyl group in another molecule to cause a reduction. The amount of NADH in the yeast cell is limited to a quite low level. In order to allow the reduction continuously, it is therefore necessary to active another biological pathway to reduce (nicotinamide adenine dinucleotide ion) NAD^+ in to NADH. Yeast contains some saccharides in the cell, which reduce NAD^+ to NADH *via* pentose-phosphate pathway. The addition of glucose to the reaction mixture ensure simultaneous feeding of the yeast cells which ultimately results in enhanced concentration of NADH, which is regenerated from NAD^+ *via* pentose phosphate pathway.

The spectroscopic results of product summarized in following Table-1, which suggest that the product is alcohol.

TABLE-1

Substrate name	Reaction time (h)	FBY yield (%)	ImBY yield (%)	IR data (cm^{-1})	NMR data (δ-value)	Mass data	Confirmed product
Camphor	48	78	83	2950 3350 1475 1060	3.87(s) 3.58 (s) 4.08 (d) 3.72 (s)	97, 67, 81, 55, 110, 121, 136	Borneol

As compared with classical methods which generally involve use of either corrosive reagent or yield product which are burden to the ecosystem. The use of Baker's yeast offer alternative to carry out reduction a quite simple installation, at room temperature with an easy work-up of products and good yields which is essentially green.

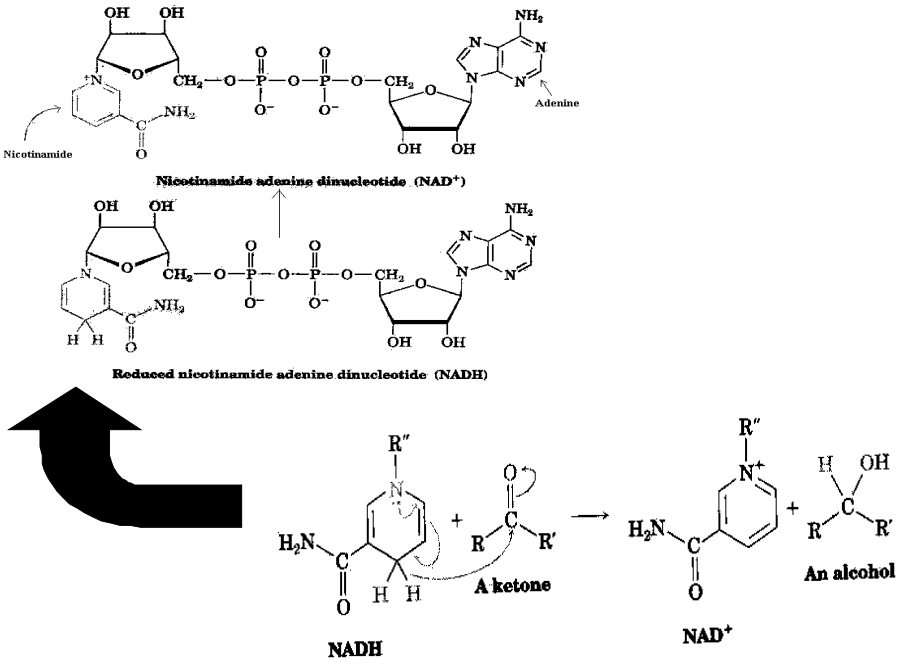

Fig. 3. Depicting biological pathway for reduction of camphor by NADH

ACKNOWLEDGEMENTS

The authors are grateful to Head, Department of Chemistry to provide necessary facilities for carrying out this investigation. Thanks are also due to CSIR, New Delhi, for providing fellowships to Vijay Chhimpa and Manu Gupta.

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(*Received*: 3 July 2008; *Accepted*: 8 December 2008) AJC-7072