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RESEARCH ARTICLE

Biotransformation of (L)-citronellal to (L)-citronellol by free and immobilized *Rhodotorula minuta*

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This paper reports biotransformation of (L)-citronellal to (L)-citronellol using free and immobilized cells of *Rhodotorula minuta*. The culture preparation variables such as pH, temperature and incubation period for obtaining maximum cell growth of *R. minuta* were optimized. The optimized culture conditions for free and immobilized cells of *R. minuta*. have been compared for (L)-citronellal biotransformation. The various factors such as the optimum substrate concentration and the time of substrate addition at varying cell concentrations during the growth of yeast culture were also studied. Highest (L)-citronellol concentration of 3.5 gl^{-1} was obtained with free cell catalyzed biotransformation at pH 5.5, 27ºC and 150 rpm after 8 hrs using initial (L)-citronellal concentration of 4.47 gl^{-1}. Alginate immobilized *R. minuta* cells could optimally biotransform similar substrate concentration to 3.3 gl^{-1} (L)-citronellol at pH 6, 27ºC and 150 rpm after 8 hrs. Immobilized cells could be reused twice after the first run and the product concentrations of 2.63 gl^{-1} and 1.52 gl^{-1} were obtained during the first and the second reuse.

Monoterpenes are widely distributed in nature and find extensive applications in the flavor and fragrance industry. Their simple structures make them ideal targets for microbial biotransformations to yield several commercially important products (Werf et al. 1997). Citronellal (3,7-Dimethyl-6-octanal), a monoterpene which occurs in the L or D form, bears distinct odor characteristics and also occurs as a constituent of essential oils in *Eucalyptus citriodora* (Betts, 2000). Citronellal can be further hydrogenated to produce citronellol (3,7-Dimethyl-6-octanol), which is a commercially important product due to the peculiar rose -like odor characteristics of the product (Guenther, 1950).The chemical synthesis of citronellol has been reported via hydrogenation of citronellal using polymer-stabilized noble metal colloids (Weiyong et al. 2000). It would be interesting to generate (L)-citronellol via bioconversion from (L)-citronellal as (L)-citronellol generated via biotransformation is labeled as a 'natural' product and commands higher value in the market than it's chemically produced counterpart. The biotransformation of other optical isomer *i.e.* (D)-citronellal to (D)-citronellol using *Pseudomonas aeruginosa* (Joglekar and Dhavalikar, 1969) and *Saccharomyces cerevisiae* (Ward and Young, 1991; Chatterjee et al. 1999) have been reported. A patented report using *Candida reukaufii* mediated (L)-citronellal biotransformation to (L)-citronellol with 80% optical purity is also available (Takasago, 1974). However, information on the biotransformation of (L)-citronellal to (L)-citronellol 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 (Krasnobajew, 1984). Immobilized viable 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 (Freeman and Lilly, 1998). Steroid hydroxylations by immobilized *Curvularia lunata* (Sonomoto et al. 1983) and biotransformation of glycerol by alginate immobilized *Gluconobacter oxydans* (Adlercreutz et al. 1985) have been reported earlier. Recently, it has been shown that immobilization of whole cells has resulted in lowering of substrate toxicity in case of β-ionone biotransformation by immobilized *Aspergillus niger* (Larroche et al. 1995) and ethyl 3-oxobutanoate reduction by alginate immobilized bakers yeast (Kanda et al. 1998). During *Saccharomyces cerevisiae* and *Candida*

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utilis mediated biotransformation of benzaldehyde to L-phenylacetylcarbinol, the toxic effects of the substrate on immobilization of the yeast cells were minimized (Oliver et al. 1999). In the present work, the optimal conditions for *R. minuta* growth and its application in biotransformation of (L)-citronellal to (L)-citronellol by free and immobilized cells is reported. The reuse of immobilized cells is presented in this work. The biotransformation reaction using *R. minuta* cells is schematically presented in Figure 1. The suitable culture conditions such as pH, temperature and agitation were studied to maximize the product concentration. Immobilization was examined as a means to offset substrate toxicity.

MATERIALS AND METHODS

Malt extract, peptone, yeast extract, agarose and sodium alginate were purchased from Hi-Media Laboratories Pvt. Ltd. Mumbai, India. Gluteraldehyde, Polyethyleneimine, Acrylamide, Bis and TEMED were purchased from Sigma Chemicals Co., USA. The substrate and the product, (L)-citronellal and (L)-citronellol were purchased from Sigma Chemicals Co., USA. Other chemicals of analytical grade were obtained from standard sources. 0.1 M Acetate buffer (pH 5.5) [40.85 g sodium acetate + 462.5 µl HCl (1N) + 1200 ml distilled water] was prepared.

Microorganism and cultural conditions

Strain of *Rhodotorula minuta* (NCIM 3359) was obtained from National Collection of Industrial Microorganisms, NCL, India and maintained on potato/dextrose/agar slants (pH 5.5) at 4ºC. For cultivation in liquid media, growth from slants was inoculated in 250 ml sterile PDB in 500 ml Erlenmeyer flasks and incubated at 27ºC, pH 5.5, 150 rpm for 44 hrs.

Growth

The best media amongst PDB, YEPD, MEB (malt extract broth), MDP (malt extract/dextrose/peptone broth) and GYPB (glucose/yeast extract/peptone broth) for growth was selected on the basis of dry cell weights calculated as below. The media compositions were as given in the literature (Atlas, 1997). *R. minuta* cell suspension was prepared by suspending growth from slants into 0.85% (w/v) sterile saline. The cell concentration of saline suspension was adjusted to optical density 0.6 at 550 nm. 250 ml media (pH 5.5) in 500 ml Erlenmeyer flasks was inoculated with *R. minuta* suspension (8% v/v) and the flasks were incubated at 27ºC and 150 rpm.

Cell growth in all the media was determined by estimating the cell dry weight after every 2 hrs interval. This was done by separation of cells from the broth by centrifugation at 10ºC, 800 rpm for 30 min.

The cells were added to pre-weighed aluminium foil and dried at 100ºC for 24 hrs. The difference in the initial and the final weights gave the dry weights. On obtaining the dry weight values, the growth curves [Dry wt. (gl^{-1}) vs. Time (hrs)] were plotted. The optimum pH for growth was determined by growing *R. minuta* culture at 27ºC in PDB at different pH values (4 - 9). Suitable temperature for growth was determined by growing *R. minuta* cells at pH 5.5 and different temperatures (20ºC – 30ºC). All the growth determination experiments were performed in triplicates.

Immobilization of *R. minuta* cells

R. minuta cells were separated by centrifugation at 800 rpm at 10ºC for 30 min. The cells were washed and re-suspended in sterile 0.85% (w/v) saline. Equivalent cell concentrations (13.1 gl^{-1}) were immobilized by the following entrapment and adsorption methods.

Agarose entrapment. Agarose solution prepared by dissolving agarose (6% w/v) in water at 100ºC was cooled at 40ºC and mixed with separated *R. minuta* cells. The mixture was allowed to solidify by standing at 4ºC. The hard gel was shredded in a Warring blender and non-entrapped cells removed by washing with saline (D'Souza and Nadkarni, 1980).

Hen egg white (HEW) entrapment. HEW (5 ml) was mixed with yeast cells and treated with gluteraldehyde to a final concentration of 2%. The mixture was stirred and allowed to stand for 2.5 hrs at 25ºC. The hard gel was passed through a syringe and broken into shreds. The shreds were washed to remove unbound cells (D'Souza et al. 1985).

Polyethyleneimine (PEI) treated Glasswool adsorption. Glasswool was immersed overnight in chromic acid, washed with tap water and sterile distilled water. Cleaned glasswool was dried at 100ºC for 2 hrs. Dried glasswool was immersed in 0.2% (v/v) PEI solution prepared in distilled water. Separated yeast cells were added to PEI solution containing glasswool and allowed to stand for 3 hrs under mild agitation. PEI treated glasswool was washed with gentle stream of saline to remove unbound cells (D'Souza et al. 1986).

Acrylamide entrapment. Yeast cells were suspended in 0.85% (w/v) saline and 1 g acrylamide monomer, 30 mg Bis, 0.2 ml, 6% (w/v) ammonium persulphate solution and 3.5 µl TEMED was added to the mixture in a total volume of 5 ml. Polymerization was allowed to take place at 20ºC for 1h and the resulting gel was shredded through a Waring blender. The shreds were washed with saline (0.85% w/v) to remove unbound cells (D'Souza and Nadkarni, 1980).

Alginate immobilization. Yeast cells were mixed homogeneously with 2.5% (w/v) sodium alginate solution. The mixture was passed through a syringe into cold CaCl2 (2% w/v) solution and left to harden for 1 hr. The alginate beads of diameter 2-3 mm were washed with 0.85% (w/v) saline and used when required. (Rotmann and Rehm, 1990).

Activation of immobilized beads

After the yeast cells undergo the immobilization treatments, they were incubated in nutrient rich medium (PDB) at 27ºC, 100 rpm for 12 hrs. This was done with a view to stabilize and promote cell growth within/over the immobilized matrix.

On completion of cell incubation, the immobilized cells were washed twice with 0.85% (w/v) saline and transferred to yeast extract/peptone/dextrose broth (YEPD), that was used as the reaction media. For determination of the immobilized cell effectivity during (L)-citronellal biotransformation in low nutrient surroundings, the constituents of PDB and YEPD were reduced by half and used as the reaction media. These were termed as half strength media.

Biotransformation

In principle, the methodology described by Leuenberger, 1984 for biotransformations was employed. Either the free cells after 44 hrs of growth in PDB or the immobilized cells after activation (see Results and Discussion) in PDB were added to 100 ml YEPD containing 4.47 gl^{-1} (L)-citronellal. 0.1 gl^{-1} methanol was used as a solubilizing agent. 13.1 gl^{-1} was the constant cell concentration used for carrying out free and immobilized cell biotransformations.

Optimum conditions for biotransformation. 500 ml Erlenmeyer flasks were used for obtaining *R. minuta* growth in 250 ml media for studying optimum conditions necessary for biotransformation. The determination of suitable growth phase in PDB during which cells could be harvested and employed for biotransformation of 4.47 gl^{-1} (L)-citronellal with 0.1 gl^{-1} methanol was determined. This was done by harvesting cells at different phases of growth. The optimum values of pH and temperature were estimated in the range of pH 4 – 9 and 20ºC-30ºC. The time required for maximum product formation was determined by analysing the product after every 30 min interval till 12 hrs. An agitation speed of 150 rpm and biotransformation time of 8 hrs was employed. Optimum solvent/emulsifier type and concentration to be employed was standardized by addition of these to *R. minuta* cells (44 hrs) cultivated in 250 ml PDB present in 500 ml Erlenmeyer flasks. Solvents/emulsifiers such as methanol, octane, DMSO and isopropanol were each used in concentrations of 0.1 gl^{-1}, 0.2 gl^{-1}, 0.3 gl^{-1}, 0.4 gl^{-1} and 0.5 gl^{-1} and the flasks were incubated for 8 hrs at 27ºC, 150 rpm. The flasks were later harvested and the cell counts were determined microscopically on a Neubaur chamber at yeast culture dilution of 10^{-3}.

Extraction and analysis

On completion, the contents of the flasks were extracted twice with petroleum ether (40ºC – 60ºC), de-moisturized by addition of 1 gl^{-1} sodium sulphate and distilled on water bath at 60ºC. As per the method described by Speelmans et al. 1998, a method with some modifications was standardized for analysis of the product on a gas chromatograph. The products were further identified by GCMS analysis. The composition in relative percentage was computed by the normalization method from the GC peak areas and percentage conversion was used as the performance criterion. Mean of three replicates is reported.

GC and GCMS analysis. Gas Chromatography (GC) analysis was carried out on Varian 3800 Gas Chromatograph with FID, a data handling system and a vaporizing injector port along with Varian chromatographic star workstation software. Injector port was equipped with a HP-1 column cross-linked with methyl silicon (25 m x 0.33 mm i.d.; 1.05 µm film thickness). Injector No 1079 was used. Port temperature was kept at 250ºC. The GC oven temperature was programmed as follows: initial temperature 60ºC (1.0 min) with rise at the rate of 5ºC/min to 250ºC. The carrier gas used was H_2 at the rate of 1.5 ml/min at constant volume. The samples were injected using split sampling method, ratio 1:50. The composition in relative percentage was computed by the normalization method from the GC peak areas. Mean of three replicates was taken.

For the GC-MS analysis conditions were, unit: Varian Star 3400-CX series, equipped with a DB-5 fused silica column (30m x 0.25mm i.d.; film thickness 0.25 µm Supelco) interfaced with a Saturn-3 Ion Trap Detector (ITD). Oven temperature: 70ºCfor 3 min with rise at the rate of 3ºC/min up to 250ºC kept isothermal for 2 min. The transfer line temperature was 280ºC; detector temperature 300ºC.Helium was used as the carrier gas and was adjusted to linear velocity 30 cm/s, split ratio 1:20; ionisation scan range 40-450 *m/z*. The identification of compounds was performed by comparison with their retention indices relative to C_4-C_{16} *n*-alkanes and mass spectra with the corresponding data of components of reference oils.

RESULTS AND DISCUSSION

Growth conditions

The suitable culture conditions necessary for obtaining maximum *R. minuta* cell growth at shake flask conditions were determined. The increase in cell concentrations during growth of *R. minuta* cells in PDB, YEPD, MEB, GYPB and MDP was assessed and plotted as growth curves (Figure 2). *R. minuta* cells attained highest concentrations of 13.1 gl^{-1} (PDB), 12.5 gl^{-1} (YEPD), 12 gl^{-1} (MEB), 9.1 gl^{-1}(GYPB) and 9.9 gl^{-1}(MDP) in different medium compositions. The *R. minuta* cell growth rate values calculated in the exponential phases during culture in different media were 0.086 h^{-1} (PDB), 0.033 h^{-1} (YEPD), 0.053 h^{-1} (MEB), 0.041 h^{-1} (GYPB) and 0.076 h^{-1} (MDP). PDB was found to be the suitable media composition for *R. minuta* growth where maximum cell concentrations of 13.1 gl^{-1} and optimum growth rate of 0.086 h^{-1} was obtained. Determination of suitable pH values and temperatures for *R. minuta* cell growth was done by culturing cells in the range of pH 4-9

and 20ºC – 30ºC respectively. Higher cell concentrations of 13 gl^{-1} were obtained at pH 5.5. The cell concentrations gradually decreased at lower or higher pH values. Studies on different cultivation temperatures showed that the cell growth increased gradually from 20ºC till 27ºC, which was found to be most conducive for growth where optimum cell concentration of 13.2 gl^{-1} was obtained. Cells cultured at 30ºC showed lowered optimum cell concentrations (12 gl^{-1}). These results are similar with those reported for *R. minuta* (Lund, 1958; Morris, 1958; Clinton, 1968).

Culture conditions for biotransformation with free cells

The biotransformation of (L)-citronellal to (L)-citronellol by free cells of *R. minuta* was studied under different pH and temperature conditions. GC analysis revealed the presence of the substrate, (L)-citronellal and a single major product, (L)-citronellol along with a minor product hydroxyl-citronellol [2-4% (w/v)]. Biotransformation at pH values in the range of pH 4-9 showed a gradual increase in the product (L-citronellol) concentrations from 2.3 ± 0.1 gl^{-1} at pH 4 to 3.4 ± 0.1 gl^{-1} at pH 5.5. No significant change in the product concentrations were seen at pH's above 6 at substrate (L-citronellal) concentration of 4.47 gl^{-1} (Figure 3).No change in the pH value was seen during the course of biotransformation at the end of 8 hrs. Studies on the effect of temperature showed that the product concentration increased from 2.4 ± 0.14 gl^{-1} at 20ºC to a maximum of 3.2 ± 0.2 gl^{-1} at 27ºC and then decreased (Figure 4).

For ascertaining the suitability of PDB as growth and reaction media, (L)-citronellal biotransformation was carried out in different media combinations by *R. minuta* cells previously grown in PDB. Biotransformation in other media resulted in decreased product formation. The product formation did not improve even when these media were used as both, the growth and reaction media. PDB was found to be the suitable media composition for obtaining maximum (L)-citronellol concentration. The determination of suitable culture age during growth of *R. minuta* in PDB for maximum product formation was done by harvesting cells in various stages of growth and employing them for (L)-citronellal biotransformation. The results indicated the cell culture age of 40 hrs – 44 hrs to be suitable for optimum product formation. Here, the cells were at the end of the exponential phase and had attained maximum cell concentration.

The optimum initial medium pH and temperature values for biotransformation by free cells are similar with those found in the literature for the biotransformation of the other optical isomer, (D)-citronellal (Chatterjee et al. 1999). The optimum substrate concentration to be employed for obtaining maximum product concentrations was done by adding different substrate concentrations to *R. minuta* cell growth and the results are presented (Figure 5). The maximum substrate concentration of 4.47 gl^{-1} (L)-citronellal could be optimally biotransformed to 3.5 gl^{-1} ± 0.1(L)-citronellol. The product formation decreased on further increase in substrate concentrations. Recent studies indicate (L)-citronellal to be toxic against some types of fungi at concentrations of 10 – 20 ppm (Ramezani et al. 2002). The log*P* values for (L)-citronellal and (L)-citronellol have been estimated to be 3.53 and 3.56 respectively (Syracuse Research Corporation, SRC, Environmental Research Website).

(L)-citronellol formation with respect to time was studied. On addition of the substrate, the concentrations of the product obtained were 0.9 gl^{-1} in 2 hrs, 2.01 gl^{-1} in 4 hrs, 2.45 gl^{-1} in 6 hrs and 3.4 gl^{-1} in 8 hrs. Marginal decrease in product concentration to 3.2 gl^{-1} was observed on further incubation to 12 hrs.

Leuenberger, 1984 reported that product yields could be effectively increased by solubilizing/emulsifying immiscible substrates. However, careful selection of the nature and concentration of solvent is necessary since many miscible solvents are cytotoxic at lower concentrations (Salter and Kell, 1995). The whole cell stability in presence of an organic solvent mainly depends on its log*P* value and is affected in presence of solvents having log*P* values up to 2-4, beyond which the solvent toxicity decreases (Laane et al. 1985; Mozhaev et al. 1989). Schneider, 1991 has further shown that enzymatic activity in both miscible and immiscible aqueous/non-aqueous systems was much better correlated with the important solvent solubility parameters namely dispersive, polar and hydrogen-bonding interactions than with log*P* alone. During our studies, solvents/emulsifiers having log*P* values and aqueous solubilities (aq. sol.) distributed over a wide range (Osborne et al. 1990) were selected to determine their suitability. Dimethyl-sulfoxide (log*P*: –1.35, aq. sol.: 3,311 mM), methanol (log*P*: -0.79, aq. sol.: 24,540 mM), isopropanol (log*P*: 0.15, aq. sol.: 13000 mM), octane (log*P*: 4.58, aq. sol.: 57.78 x 10^{-6} mM were used for increasing (L)-citronellol formation. 71% (w/w) conversion was obtained in the control flasks where no solvent/emulsifier was added. Suitable solvent/emulsifier concentration to be added along with the substrate was estimated by determining yeast cell viability at different solvent/emulsifier concentrations without addition of the substrate. The optimum concentration of solvent/emulsifier to be employed along with the substrate at non-toxic levels to 44 hrs phase yeast cell culture in PDB was 0.1 gl^{-1}. The yeast cell count at this stage was 10^8 cells/ml. At increased concentrations of solvents/emulsifiers the cell counts decreased to 10^4 – 10^6 cells/ml. Amongst the different solvents/emulsifiers used, the addition of octane did not increase the product yields, whereas DMSO and isopropanol lowered (L)-citronellol formation. The addition of 0.1 gl^{-1} methanol was found to be suitable for increasing the product yields to 3.5 gl^{-1}± 0.1(Figure 6).

Choice of immobilization method

Amongst the different matrices used for *R. minuta*

immobilization, alginate entrapped cells could effectively biotransform 4.47 gl^{-1} (L)-citronellal to 3.2 gl^{-1} ± 0.3 (72% w/w) (L)-citronellol. Other methods used for immobilization of *R. minuta* included entrapment in hen egg white andagarose, gluteraldehyde aided adsorption to cotton cloth and adsorption to polyethyleneimine treated glasswool.Although, adsorption offered the advantage of operational simplicity, the product concentrations on employment of *R. minuta* cells adsorbed to cotton cloth and glasswool were1.4 gl^{-1} ± 0.3 (32% w/v) and 1.06 gl^{-1} ± 0.3 (24% w/v) respectively. This was due to low retention of the cells on the immobilization support. Slight variations in culture conditions such as pH, ionic strength and temperature cause disorption of the cells from the immobilization matrix (D'Souza, 1989). (L)-citronellol concentrations of 0.98 gl^{-1} ±0.5 (23% w/v)and 0.6 gl^{-1} ± 0.2 (14% w/v) were obtained on use of hen egg white and agarose as cell entrapment matrices. Diffusional barriers set up by the gel against the transport of substrates and products (D'Souza, 1989) may be responsible for lowered product formation.

The use of half strength medium as reaction media and a nutrient rich complete medium for cell regeneration have been used for improving product concentrations during steroid biotransformations (Freeman and Lilly, 1998). In our studies, nutrient rich media (YEPD) was used for regeneration of immobilized cells before the first run and subsequently before each reuse. This was intended to prolong cell activity during biotransformation. However, it was found that intermediate activation decreased the product concentration to 2.2 gl^{-1} ± 0.2 and 0.9 gl^{-1} ± 0.3 on the first and the second reuse respectively. Single regeneration treatment in nutrient rich medium (YEPD) before the first run proved useful and the product concentrations of 3.3 gl^{-1} ± 0.3, 2.6 gl^{-1} ± 0.2, and 1.6 gl^{-1} ± 0.1 were obtained in the first, second and the third run respectively.

Culture conditions for biotransformation with immobilized cells

The biotransformation of (L)-citronellal to (L)-citronellol was studied under different pH and temperature conditions employing alginate immobilized *R. minuta* cells. Studies on biotransformation with immobilized cells at different pH's showed that the optimum pH value was in the range of pH 5.5 - 6 where a maximum product formation of 3.1 gl^{-1} ± 0.3 was obtained (Figure 3). 27ºC was found to be suitable for obtaining 3.3 gl^{-1} ± 0.3 product concentration (Figure 5). Similar to free cells, the optimum substrate concentration that could be maximally biotransformed to the product (3.2 gl^{-1} ± 0.1) was found to be 4.47 gl^{-1} (0.5%). Further increase in substrate concentrations decreased the product formation (Figure 5).

Comparing the data of product concentrations obtained at increased substrate concentrations during free and immobilized cell catalysed biotransformation, the product formation decreased sharply at increased substrate concentrations during use of immobilized cells. Increased (L)-citronellal concentrations during employment of free cells resulted in a gradual decrease in product concentrations (Figure 5). The reason for this could be attributed to substrate diffusional barrier posed by the immobilization matrix.

The time required for optimum product formation for immobilized cells did not vary and was 8 hrs. Amongst the different nutrient media used for conducting immobilized cell biotransformation, YEPD was found to be suitable for obtaining product concentrations up to 3.2 gl^{-1} ± 0.2 in the first run. On use of half strength PDB and YEPD as the reaction media, the product concentrations decreased to 2.5 gl^{-1} ± 0.3 and 2.6 gl^{-1} ± 0.2 in the first run. On use of 0.1 M acetate buffer (pH 5.5) + 2% (w/v) glucose as the reaction media 3.1 gl^{-1} ± 0.2 (L)-citronellol was obtained in the first run (Table 1).

The addition of solvents/emulsifiers for increasing (L)-citronellol formation was more effective for alginate-immobilized cells as compared to that for free cell catalysed (L)-citronellal biotransformation.

The product concentrations were higher during the use of all solvents/emulsifiers as compared to control flasks where no solvent/emulsifiers was used. The highest product concentration of 3.3 gl^{-1} ± 0.1 was obtained in case of 0.1 gl^{-1} methanol, followed by 0.1 gl^{-1} octane and 0.1 gl^{-1} DMSO where a product concentration of 3.2 gl^{-1} ± 0.2 was obtained (Figure 6). Although, octane and DMSO were equally effective as methanol for increasing product concentrations, their use imparted a typical undesired odour to the product. The overall effectivity of solvents/emulsifiers for immobilized cell catalysed (L)-citronellal biotransformation could be attributed to increased availability of the substrate through the immobilization matrix.

Re-use of immobilized cell beads

Immobilized cell reuse in a batch wise manner has been done earlier for steroid biotransformations using polyacrylamide-hydrazide 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 (Abramov et al. 1990). Immobilized *Actinoplanes teichomyceticus* could be reused 5 times in a batch wise manner (30 days) during biotransformation of lipoglycopeptide antibiotic A40926 (Jovetic et al. 1998). During our investigations on (L)-citronellal biotransformation, immobilized cells could be re-employed twice after the first use. Beads were

activated in nutrient rich media (PDB) at pH 5.5 and 27ºC for 12 hrs, before the first use. The product concentrations during the first and second reuse were 2.63 gl^{-1} ± 0.2 and 1.56 gl^{-1} ± 0.1 respectively. Product formation was studied on use of different media compositions such as half strength media and buffer as the reaction media during reuse of immobilized *R. minuta* cells for (L)-citronellal biotransformation. The (L)-citronellol concentrations obtained during the first and second reuse were 1.1 gl^{-1} ± 0.3and 0.7 gl^{-1} ± 0.2 gl^{-1} ± 0.1 on use of half strength PDB and 1.3 gl^{-1} ± 0.2 and 0.8 gl^{-1} ± 0.2 during use of half strength YEPD respectively.

When 0.1 M acetate buffer (pH 5.5) + 2% (w/v) glucose was used as the reaction media, the product concentrations during the first and second reuse were 1.6 gl^{-1} ± 0.3 and 0.5 gl^{-1} ± 0.3 respectively. The decrease in yeast biotransformation activity during successive runs was studied by determining the cell counts of the alginate bead and the surrounding medium. The cell counts gradually decreased from 10^6 cells/bead in the first run to 10^4 cells/bead in the second run and the third run. The cell count of the surrounding medium ranged between $10^2 – 10^3$ in all the runs. Thus the gradual decrease in biotransformation activity could be attributed partly to cellular inhibition due to the substrate and the product as well as due to the cell leakage through the immobilization matrix.

We report the culture conditions of 27ºC, pH 5.5, 150 rpm and incubation period as 44 hrs to be optimum for *R. minuta* growth in PDB. The suitable conditions for (L)-citronellal biotransformation are the same as standardized for growth and the time taken for reaction completion is 8 hrs. Amongst the solvents used, use of methanol proved beneficial for improving product concentrations. For immobilized cell systems, alginate proved to be the best matrix for cell immobilization and the optimum culture conditions of 27ºC, pH 6 and 150 rpm and 8 hrs were determined for maximum product formation. Although use of methanol, octane and DMSO improved product concentrations, the application of methanol was found to be useful in retaining the characteristic rose-like odour of (L)-citronellol. The important advantage of immobilized cell reuse for two times after the first run with gradual decrease in biotransformation activity has been studied. The data is important considering the scale-up potential of (L)-citronellal biotransformation.

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APPENDIX

Tables

Table 1. Effect of different media combinations during biotransformation of (L) –citronellal by alginate immobilized *R. minuta.*

Type of media	Product conentration (gl^{-1})
PDB	3.1 ± 0.2
YEPD	3.2 ± 0.2
½ PDB	2.5 ± 0.3
½ YEPD	2.6 ± 0.2
0.1 M acetate buffer + 2% glucose	3.1 ± 0.2

Figures

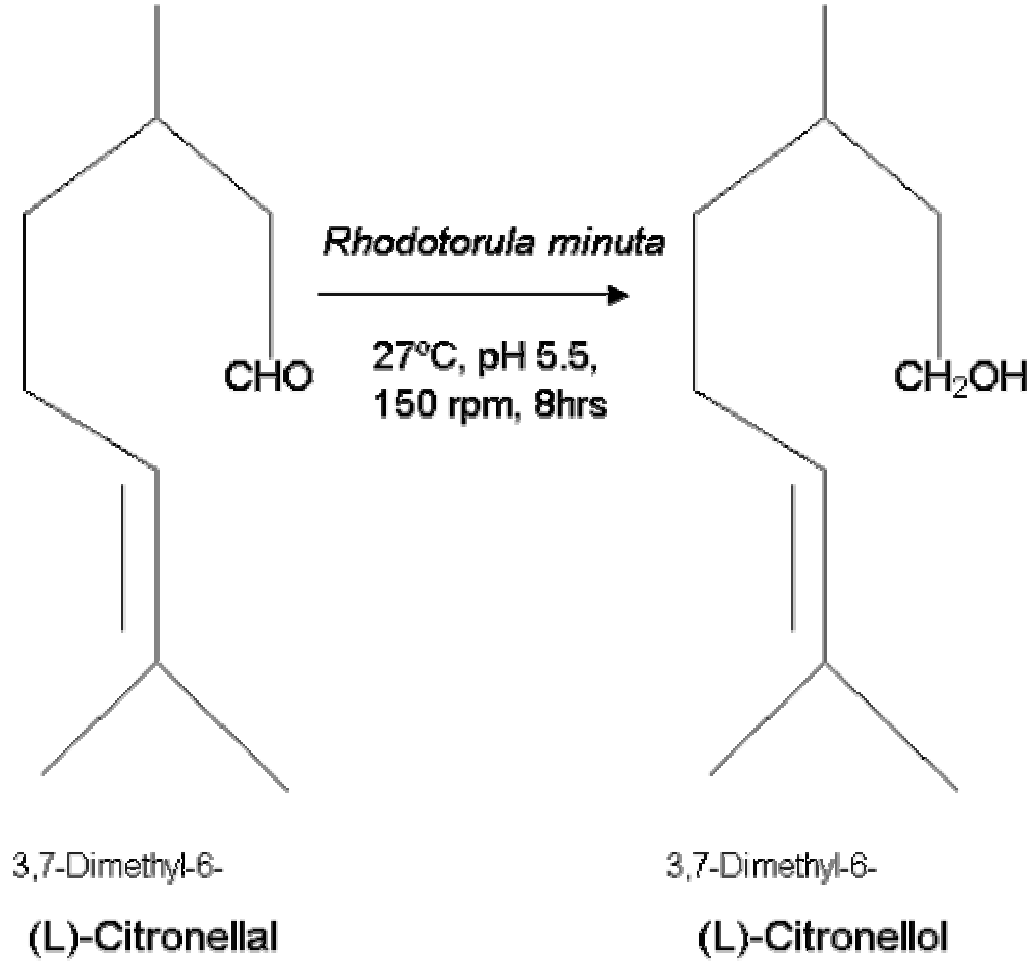

Figure 1. Schematic representation of (L)-citronellal biotransformation to (L)-citronellol mediated by *R. minuta*.

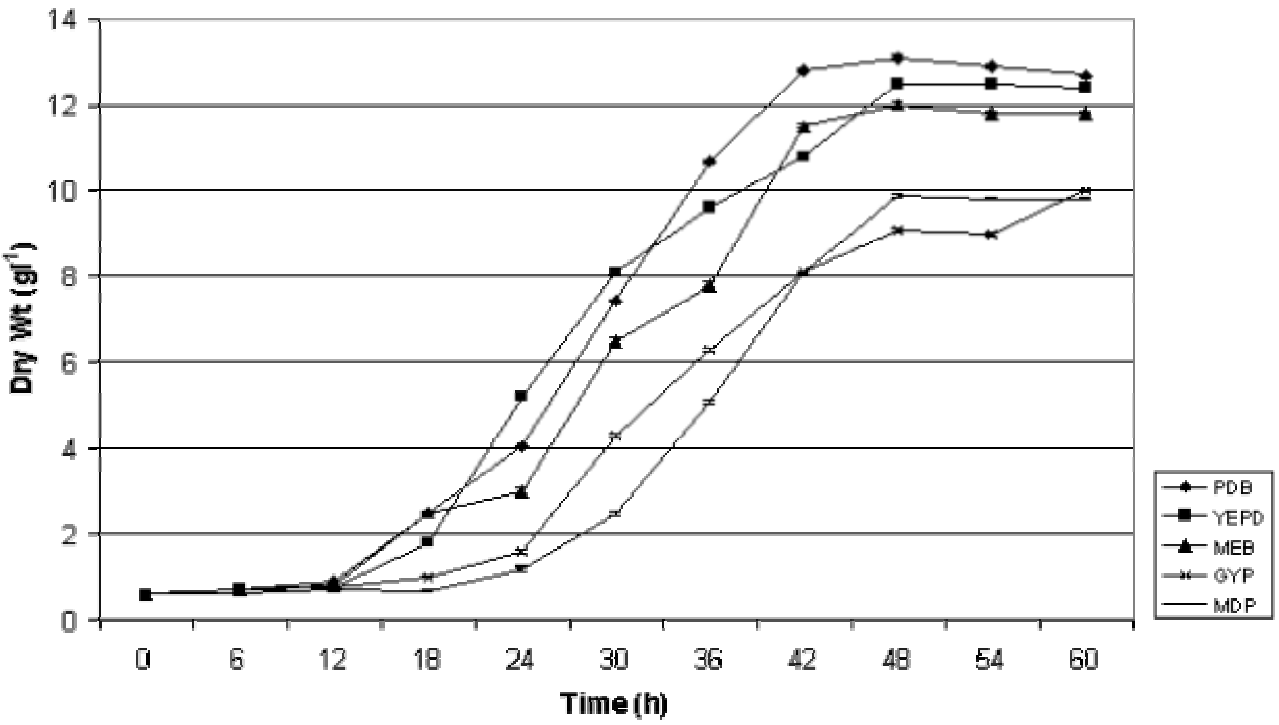

Figure 2. Growth of *Rhodotorula minuta* in different media composition at pH 5.5, 27ºC, 150 rpm for 44 hrs.

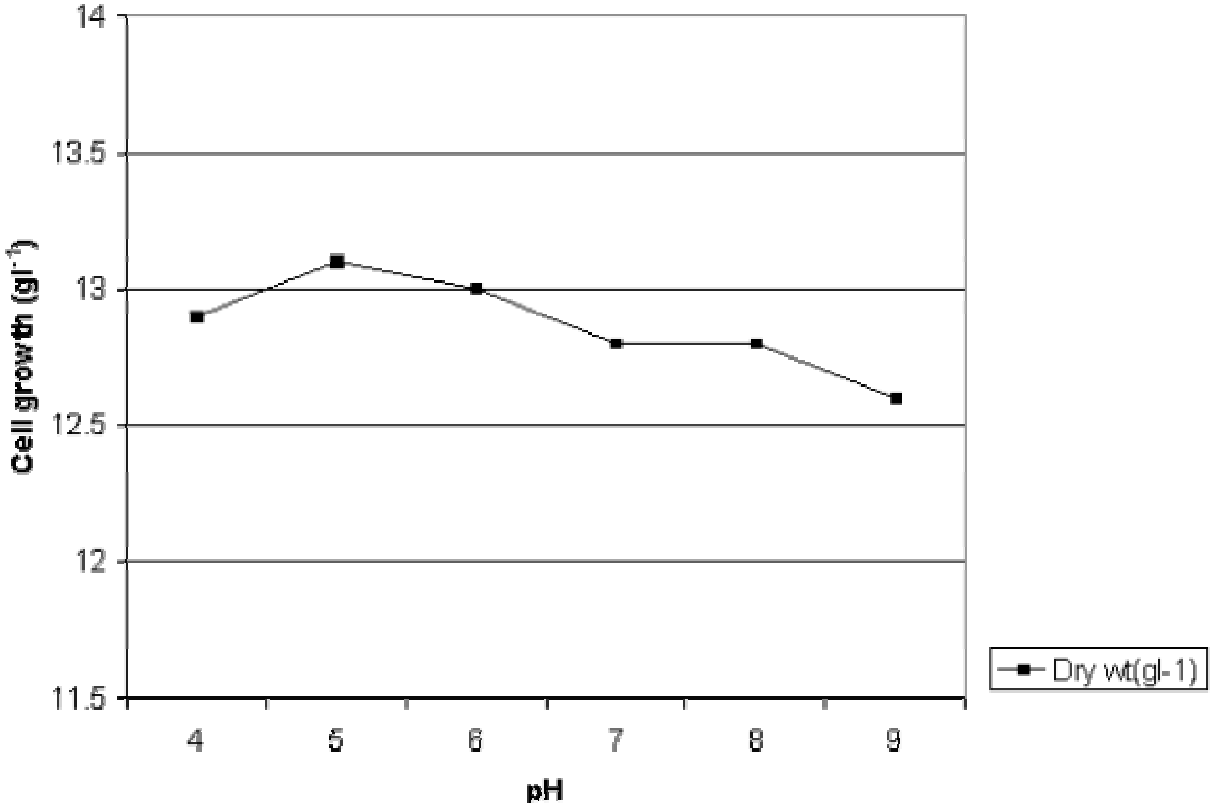

Figure 3. Determination of *R. minuta* cell growth (gl^{-1}) at different initial pH values.

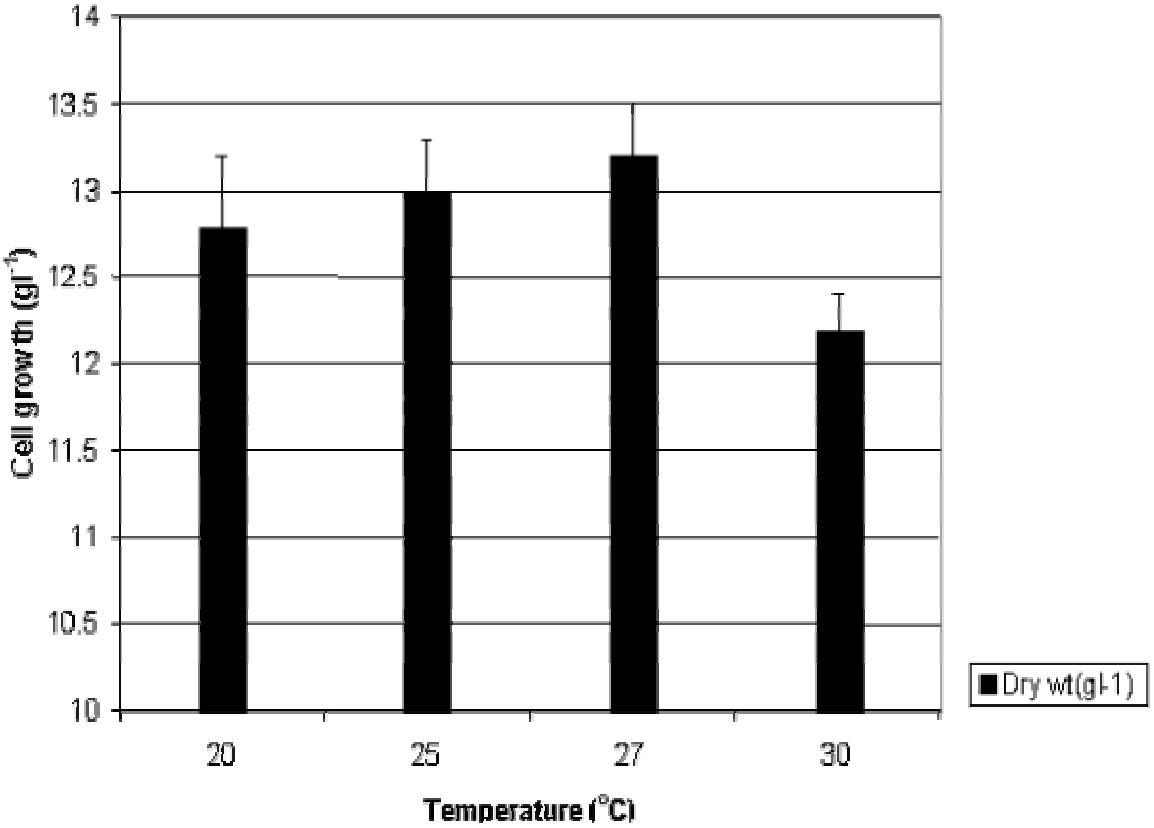

Figure 4. Determination of *R. minuta* cell growth (gl^{-1}) at different temperatures.

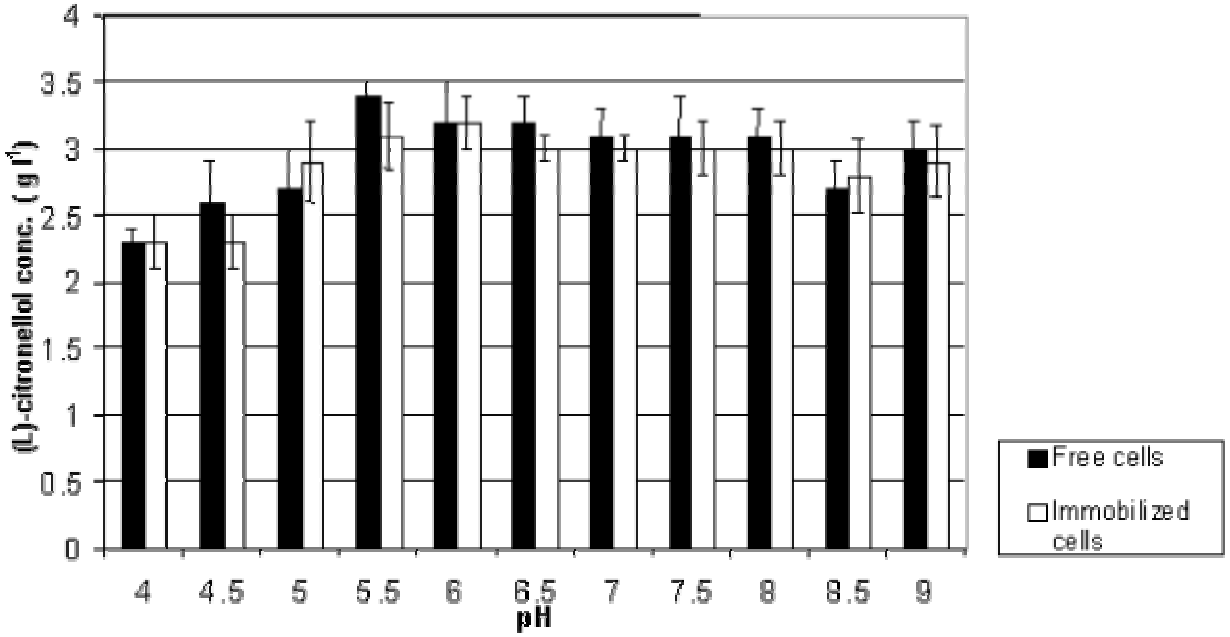

Figure 5. (L)-citronellol concentrations at different pH values during biotransformation of (L)-citronellal biotransformation by free and immobilized *R. minuta* at 27ºC, 150 rpm, and 8 hrs.

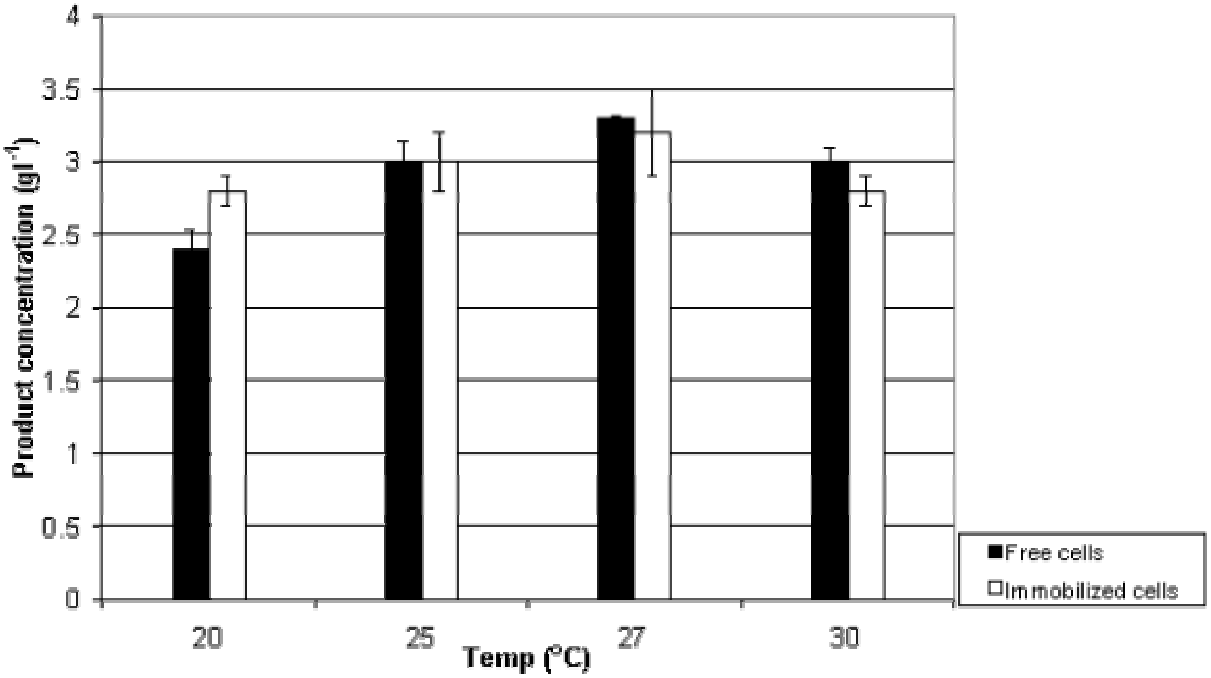

Figure 6. (L)-citronellol concentrations at different temperatures during free and immobilized *R. minuta* mediated (L)-citronellal biotransformation at pH 5.5, 150 rpm and 8 hrs.