Microbial conversion of isoeugenol to vanillin by *Rhodococcus rhodochrous*

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Optimum conditions for this biotransformation such as temperature, pH, substrate concentration and time course are also established. The strain *Rhodococcus rhodochrous* MTCC 289 is shake-cultured for 3 days in a medium containing beef extract, yeast extract, peptone and NaCl at 30°C. The bioconversion is carried out with single substrate addition, so that the substrate concentration in the medium do not exceed 1.5% (w/v). The structure of the biotransformation product is established by analytical methods, such as, FTIR, 1H NMR, 13C NMR and GC-MS spectrometry. Under optimal conditions the yield of vanillin is found to be 58% (mol), demonstrating the efficiency of *Rhodococcus rhodochrous* for this biotransformation.

The role of aromatic aldehydes as flavor base compound in food industries is well-established. These aldehydes impart fruity smell in foods and beverages\(^1\). Vanillin (4-hydroxy-3-methoxybenzaldehyde) is one such commercially extremely important aromatic aldehyde, which is responsible for a vanilla-type flavor in many food products\(^2\).

Presently global demand for vanillin is met by synthetic chemical method from eugenol, guaiacol or lignin\(^3\). However, in recent years, there is a trend for growing demand for bioflavors because sole microbial biotechnological route can produce "natural" flavor\(^4\). Due to these inherent benefits of the bioprocess and some inevitable demerits of the chemical synthetic pathway, like stereoechemically impure product formation, poor organoleptic property etc. the microbial transformation process for the synthesis of vanillin has been explored during the past few years\(^2\). Several investigators have examined the microbial production of vanillin from ferulic acid using different types of microorganisms\(^5,6\). Plant cell culture can also produce vanillin but there are some technical processing problems and the yield is also low\(^7\). The literature reveals that the genus *Rhodococcus* are able to degrade short and long chain hydrocarbons and numerous aromatic compounds. Many authors also report that biotransformations catalyzed by *Rhodococci* include enantioselective synthesis which make them excellent candidates for biomediation treatments\(^8\).

The present study has therefore aimed at the synthesis of vanillin starting from an easily available compound isoeugenol (2-methoxy-4-propenylphenol) by means of microbial oxidation catalyzed by *Rhodococcus rhodochrous* MTCC 289 (Scheme 1). Our purpose was also to determine the best experimental conditions to achieve the desired bioconversion.

**Materials and Methods**

*Microorganism, maintenance and growth*

The strain *Rhodococcus rhodochrous* MTCC 289 was purchased from the Institute of Microbial Technology (Chandigarh, India). Stock cultures of *Rhodococcus rhodochrous* MTCC 289 were grown at 30°C on agar plates for 2 to 3 days and were stored at 5°C. For growth in liquid medium, the stock culture was used to inoculate twenty 250 mL of Erlenmeyer flasks, each containing 100 mL of sterile growth medium. The liquid medium contained 1.0 g of beef extract, 0.7 g of yeast extract, 1.7 g of peptone and 1.7 g of NaCl per litre. The pH of the medium was maintained at 5.8 for storage and maintenance, and...
5.0 to 6.0 for treatments. The flasks containing the inoculated broth were incubated at 30°C with shaking for 72 hr.

Fermentation procedure

The resulting culture broths (contained in twenty 250 mL Erlenmeyer flasks) were divided into five sets of four flasks. To each set 0.1, 0.2, 0.5, 1.0 and 1.5% (w/v) of isoeugenol I was added to find out the optimum concentration of isoeugenol, and was incubated for five days under the same condition as mentioned above. Samples from the treated cultures were collected from each set after 2, 3, 4 and 5 days respectively, and analyzed by thin layer chromatography and FTIR spectra. Simultaneously, a control experiment was carried out without microorganism by adding substrate directly into the sterile broth.

Extraction and purification of products

After cultivation, the cells were removed by centrifugation (6000 rpm, 10 min) and the supernatant was extracted thrice with ethyl acetate (30 mL each time). The combined extract was washed with distilled water (3×100 mL) and the organic phase was dried over anhyd. MgSO₄. The solvent was then removed under reduced pressure (30 mm Hg at 35°C) to obtain crude reaction products. The crude reaction products were subjected to column chromatography using glass column (20 mm) on silica gel (60-120 mesh). Column was packed with a slurry of silica acid (10 g) in n-hexane and eluted with 120 mL of n-hexane-diethyl ether (80:20 v/v) for isolation of bioconversion product. Rechromatography on fresh silica acid column yielded pure reaction product 2.

Analytical methods

The reaction product was analyzed by TLC on glass plates (20×20 cm), using a 0.2 mm layer of silica gel G. The plate was developed in 100 mL of n-hexane-diethyl ether (40:60 v/v) and the spot was identified by iodine absorption (Rf 0.42). No conversion of isoeugenol was observed in control sets as evidenced from TLC (Rf 0.76).

Chemical structure of the biocconversion product was identified by FTIR spectroscopy, NMR and GC-MS. FTIR spectra was obtained by a Perkin-Elmer 1600 Fourier transform spectrometer on KBr disk for solid bioconversion product and NaCl plate for isoeugenol. Proton and 13C-NMR spectra in CDCl₃ were obtained by the use of a Bruker AM-300L spectrometer operating at the frequency of 300 MHz. TMS was used as reference compound. GC-MS spectra of the compound was obtained from a Hewlett Packard Model HP 5890 series II GC-MSD apparatus, equipped with flame ionization detector and a HP-1 capillary column. The carrier gas used was helium with a flow rate of 30 mL/min.

Results and Discussion

Identification of isoeugenol bioconversion product

The bioconversion product of isoeugenol, which was obtained as off-white crystals after crystallization in chloroform (0.54 g, 58%, m.p. 79-80°C at 76 cm of Hg), was characterised by FTIR, 1H-NMR, 13C-NMR and GC-MS spectroscopy (Table I). FTIR spectrum indicated the presence of a hydroxy (OH, 3320 cm⁻¹) and aldehyde carbonyl (CH=O, 1675 cm⁻¹) groups. The 1H-NMR spectrum suggested the presence of aldehyde functional group (CH=O, δ 9.80 ppm, s), aromatic hydroxy (OH, δ 6.41 ppm, s), methoxy (OCH₃, δ 3.94 ppm, s) groups. The aromatic protons, ortho to the aldehyde functional group, were found to resonate at δ 7.40 ppm (s, 1H) and 7.41 ppm (d, 1H) whereas the meta proton resonated at δ 7.02 ppm (d, 1H, J=8.0 Hz). The 13C-NMR spectrum also showed the formation of aldehyde functional group (δ 190.93 ppm). All the spectral data were identical with 4-hydroxy-3-methoxybenzaldehyde (vanillin), compared with authentic sample to confirm the identity.

Growth of Rhodococcus rhodochrous on isoeugenol

The growth medium inoculated with R. rhodochrous MTCC 289 resulted in a visible increase in biomass with time, and a growth curve was constructed by measuring the dry weight of the biomass formed at intervals (Figure 1). Sharp decline in the amount of isoeugenol from the medium in the first three days with the growth of microorganism, as measured by complete extraction of the substrate-product mixture followed by column chromatographic

Table I—Spectral data of isoeugenol bioconversion product by the strain Rhodococcus rhodochrous MTCC 289

<table>
<thead>
<tr>
<th>Compound</th>
<th>FTIR v_max (cm⁻¹)</th>
<th>1H NMR δ (ppm)</th>
<th>13C NMR δ (ppm)</th>
<th>GC-MS m/z</th>
</tr>
</thead>
<tbody>
<tr>
<td>MTCC 289</td>
<td>3320, 2926, 1675</td>
<td>7.41, 9.80, 6.41</td>
<td>147.11, 151.68, 1</td>
<td>190.93 (CH=O)</td>
</tr>
<tr>
<td></td>
<td>1260,1030,702</td>
<td>129.73, 108.72</td>
<td>114.35, 56.03</td>
<td>81(6), 110(20)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>6.41 (br.s, 1H)</td>
<td>127.50 (CH=O)</td>
<td>51(18)</td>
</tr>
</tbody>
</table>
isolation of the substrate to determine its consumption, was indicative of the ability of *R. rhodochrous* MTCC 289 to metabolize isoeugenol. After three days, although there was an increase in the quantity of biomass with time (measured up to five days), the bioconversion activity of the microorganism falls as measured by decline in its consumption, indicating the maximum activity of the microorganism after three days of inoculation.

**Influence of substrate concentration**

The growth of *R. rhodochrous* MTCC 289 in the medium was studied with varying concentration of isoeugenol between 0.1 to 1.5 g/L in the medium (Figure 2). It was found that growth of the microorganism increased steadily with the increasing concentration of isoeugenol, and reached maximum at the concentration of 1.0 g/L. However, still higher isoeugenol concentration suppressed the growth of this microorganism and use of 1.5% (w/v) of growth substrate lead to poor growth of *R. rhodochrous*, which may be due to toxicity of isoeugenol on the growth of the said biomass.

**Effect of pH on bioconversion**

It is important to know the optimal pH for the activity of a particular microorganism for a specific bioconversion, since different microorganisms have different pH optima. The effect of pH on the molar percentage conversion of isoeugenol by the strain *R. rhodochrous* MTCC 289 is shown in Figure 3. The optimum pH was found to be 6.0, at which the molar percentage yield of vanillin was maximum. Reactions carried out at pH higher than 6.0, decreased the yield as evident from Figure 3.

**Time course of biotransformation of isoeugenol**

To optimize the yield of production of vanillin the time course of biotransformation of isoeugenol was studied. The results obtained is shown in Figure 4. The molar percentage conversion of isoeugenol to vanillin was found to maximize (58%) in three days of incubation (after the addition of the substrate). After three days the molar percentage yield of vanillin was found to decline, indicating no further conversion of isoeugenol by the strain *R. rhodochrous* to vanillin.

**Optimum temperature for biotransformation**

The effect of temperature for biotransformation of isoeugenol is shown in Figure 5. It is evident from the figure that the optimum reaction temperature is 30°C. Increase of incubation temperature to 35 or 40°C found to reduce the yield of the biotransformation product.

The strain *Rhodococcus rhodochrous* MTCC 289 is therefore found to be capable of metabolizing isoeugenol and thereby producing vanillin (4-hydroxy-3-methoxybenzaldehyde) in acceptably good yield. The product is valuable in creating food flavours and hence may be potentially important from the viewpoint of commercial production.
Figure 3—Effect of pH on the bioconversion of isoeugenol by Rhodococcus rhodochrous MTCC 289.

Figure 4—Time dependence of the biotransformation of isoeugenol to vanillin by Rhodococcus rhodochrous MTCC 289.

Figure 5—Temperature dependence of the biotransformation of isoeugenol to vanillin by Rhodococcus rhodochrous MTCC 289.

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References