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Eugenol Biotransformation by *Pseudomonas fluorescens* NCIM 2100

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Abstract—*The demand for natural food additives has resulted in a number of processes for producing natural vanillin. Although there are chemical procedures and plant sources for vanillin production, microbial bioconversions are being sought as a suitable 'natural' alternative. The exploitation of microbial biosynthetic pathways is both feasible and cost effective in the production of natural compounds. The present paper describes the biotransformation of eugenol as sole source of carbon, into vanillin and coniferyl aldehyde, by Psuedomonas fluorescens NCIM 2100. Optimization of process parameters for enhanced product formation was also performed. Products formed were analyzed by high pressure liquid chromatography. Maximum vanillin production observed was 7.10 mg/L, whereas coniferyl aldehyde production was found to be 2.04 mg/L.*

1. INTRODUCTION

Eugenol is an economic "natural" substrate that can be isolated from the essential oil of the clove tree, *Syzygium aromaticum* on an industrial scale. Eugenol plays a prominent role in dental and oral hygiene preparations. It is used as flavour compound and sensitizer [1]. Vanillin (4-hydroxy-3-methoxybenzaldehyde) is the characteristic aroma component of the vanilla pod and is used in a broad range of flavours for foods, confectionery, and beverages; as a fragrance ingredient in perfumes and cosmetics; and for pharmaceuticals. The main production of vanillin is done via chemical synthesis from guaiacol and lignin [2]. The price of the chemically synthesized vanillin is very low (about US $12/kg) as compared to the price of natural vanillin (between US $1200 to 4000/kg) [3]. Recently the increasing customer-led demand for natural flavours has stimulated the exploration of biotechnological routes for the production of natural vanillin. Due to the demand for natural products, various food and pharmaceutical industries are searching for novel metabolites obtained from microbial biotransformation. The exploitation of microbial biosynthetic pathways is both feasible and cost effective in the production of natural compounds [4]. Among different methoxyphenol compounds, the production of vanillin via biotransformation has been the most extensively reported [5]. Eugenol is a cost effective substrate for the production of methoxyphenol compounds [6]. A method for biotransformation of eugenol to vanillin was developed by Rabenhorst and Hopp (1991, patent application number EP0405197), from a new *Pseudomonas* sp. strain, HR199, which degrades eugenol via coniferyl alcohol (4-hydroxy-3-methoxycinnamyl alcohol), coniferyl aldehyde(4-hydroxy-3methoxycinnamylaldehyde), ferulic acid, vanillin, vanillic acid (4-hydroxy-3-methoxybenzoate), and protocatechuic acid (3,4- dihydroxybenzoate) [7]. The initial step of eugenol degradation was the double bond-transferring hydroxylation catalyzed by eugenol dehydrogenase enzyme [8, 9]. The genes encoding proteins of the oxidative eugenol catabolism pathway have been investigated in detail in different *Pseudomonas* strains [10, 11, 12, 13, 14, 15, 16, 17, 18].

2. MATERIALS AND METHODS

2.1 Chemicals and reagents

Eugenol (99%), coniferyl Aldehyde (98%), ferulic acid (99%), vanillin (99%) and vanillic acid (99%) were obtained from Sigma Aldrich. HPLC grade methanol was used for high pressure liquid chromatography (HPLC) analysis and was purchased from Merck.

2.2 Microbial strain maintenance and seed culture preparation

Pseudomonas fluorescens NCIM 2100 strain was maintained on nutrient agar slants by subculturing it, after a regular interval of one month and stored at 4 °C for future use. The seed culture was prepared by transferring the loop full of microbe from the slant into the 25 mL sterile nutrient broth, followed by incubation in aerobic condition at 37 °C for 24 h. Minimal media was used, having the following composition (g/L), K_2HPO_4 (4.0), NaH_2PO_4 (1.0), NaCl (0.2), $MgSO_4.7H_2O$ (0.2), $CaCl_2$ (0.05), yeast extract (0.5), pH 7.0-7.25.

2.3 Analytical procedures

Culture supernatant was acidified (pH, 1–2) and extracted with an equal volume of ethyl acetate. The aqueous phase was discarded and organic phase was evaporated by rotary vacuum

evaporation under reduced pressure. The extract was resuspended in 1 mL of methanol (50% v/v). Quantification of different samples was examined by using HPLC (Waters, USA). The HPLC conditions were set as follows: column symmetry ®C_{18} (4.68×150mm, 5µm, waters); mobile phase used for eluting the biotransformed products comprised of aqueous trifluoroacetic acid (solvent A) and methanol (solvent B). The analysis of the sample was performed using the following gradient method: 0-11 min; 65% of solvent A and 35% of solvent B. From 11 to 21 min, the mobile phase composition was constant that is, 30% of solvent A and 70% of solvent B. From 21 min to 25 min, the mobile phase composition was reequilibrated to 65% of solvent A and 35% solvent B. Each HPLC run was of 25 min, analyzed at wavelengths of 254 nm (eugenol, vanillic acid) and 310 nm (coniferyl aldehyde, ferulic acid and vanillin). Products were quantified by comparison with standard compounds.

2.4 Optimization of cultural parameters for maximum vanillin production

Optimization of different parameters was conducted for the maximum vanillin production by *Pseudomonas fluorescens* NCIM 2100 [19]. Different concentrations of eugenol (0.01, 0.03, 0.05, 0.07 and 0.09%) were checked. Optimization of additional carbon source was performed with glucose, sucrose, lactose and glycerol. Optimization of nitrogen source was carried out by replacing yeast extract in the minimal medium with beef extract, peptone, ammonium nitrate and sodium nitrate. Different pH (5, 6, 7, 8 and 9) and temperature (5, 25, 37 and 45 °C) was checked for maximum production. Inoculum size (0.5, 0.75, 1, 1.25 and 1.5 mL) was also optimized. The medium with inoculated culture was incubated at aerobic condition of 37 °C or at mentioned temperature, for different incubation period of 2, 4 and 6 days.

3. RESULTS AND DISCUSSION

Optimization was performed by *Pseudomonas fluorescens* NCIM 2100 for various parameters. Carbon and nitrogen sources are essential constituents of minimal medium as they are required for the proper cellular growth and metabolism. Different concentrations of eugenol were checked in the range of 0.01 to 0.09%, for the production of value-added compounds. 0.07% eugenol, when used as the sole carbon source, produced maximum amount of vanillin (7.1 mg/L) on 2nd day of incubation (Fig. 1). However, when concentration of eugenol was increased from 0.07%, vanillin production decreased. Coniferyl aldehyde showed maximum production at a concentration of 0.09% (Fig. 2), after 2 days of incubation. Giedraityte and Kaldiene observed that the bacterium was able to biotransform eugenol into value-added compounds at the eugenol concentration of 0.01% (v/v).

The use of additional carbon, nitrogen sources, pH, temperature variations and inoculums size did not show a significant increase in the product formation.

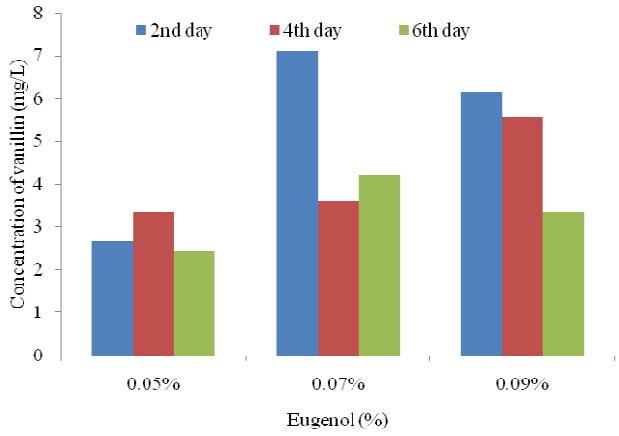

Fig. 1: Effect of various concentration of eugenol on vanillin production by *Pseudomonas fluorescens* NCIM 2100

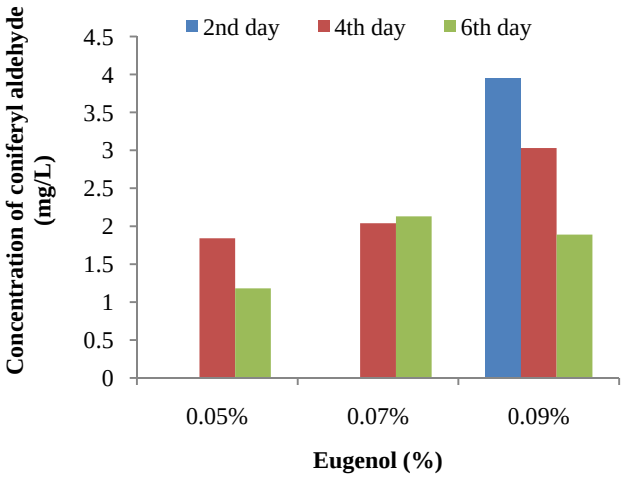

Fig. 2: Effect of various concentration of eugenol on coniferyl aldehyde production by *Pseudomonas fluorescens* NCIM 2100

4. CONCLUSION

Biotransformation ability of *Pseudomonas fluorescens* NCIM 2100 was investigated in the present study. It has the potential to convert eugenol into vanillin and coniferyl aldehyde. Aroma compounds produced may find possible applications in food, beverage, pharmaceutical, perfume and medicinal industries.

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