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Research Article

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Biotransformation of isoeugenol to vanillin by beneficial bacteria isolated from the soil of aromatic plants

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ABSTRACT

There is a greater demand for natural additives when compared with artificial additives. One of the important flavouring agents is vanillin, which is expensive and the demand is high for natural vanillin. Microbial bioconversion, a suitable alternative, was explored in the present work to produce vanillin from isoeugenol using various bacterial strains from aromatic plant roots. Among the eighteen strains, nine isolates showed maximum tolerance to isoeugenol and vanillin analysed by minimum inhibitory concentration and thin layer chromatography. The strains Bacillus spp. C1 and Pseudomonas spp. OSC1 were found to produce maximum yield of vanillin 1.52g/l and 2.43g/l respectively, and resulting in molar yield of 15.2% and 24.3%.Gram negative rod strain, OSC1 showed capability of producing high amount of vanillin in presence of isoeugenol. The strain OSC1 was optimized and was able to produce higher amount of vanillin; 2g/l of vanillin was produced when the bacterial strain OSC1 was subjected to 10g/l of isoeugenol at 96 hours by bioconversion. Vanillin formation was analysed by spectrophotometer and evaluated accurately by gas chromatography. On the basis of 16S rRNA sequence, the strain OSC1 was identified as Pseudomonas spp.

Key words: Bioconversion, Isoeugenol, Vanillin, OSC1 strain -*Pseudomonas* spp.

INTRODUCTION

Vanillin is an aromatic aldehyde $C_8H_8O_8$ and is one of the most widely used flavouring compound in the food sectors, chemical and pharma companies[1]. The production of vanillin from the pod of *Vanilla planifolia* is a laborious, slow process and is expensive [2]. The alternative process is chemical synthesis from lignin hydrocarbon, synthetically produced vanillin. However, greater preference was given for the consumption of natural vanillin production when compared with synthetic process due to the presence of racemic mixture. The worldwide demand of flavours and fragrances was estimated to be US$ 16 billion in the year 2013 [3]. The worldwide demand exceeds 15,000 tons of which approximately only 2,000 tons is met from vanilla beans and rest is produced through chemical process [4].Concurrently, there is an increase in demand of natural flavour products for industries by the FDA and European legislation[5–7]. There is need of biotechnological methods for the production of vanillin by biotransformation process [8,9].

Synthesis of vanillin by microbial conversion of various natural products have been reported in literature. Isoeugenol, ferulic acid and eugenol show a higher yield than other precursors [7]. The prerequisite for selection of precursor is its economical and eco-friendly nature. Isoeugenol is an ideal precursor as it is environmentally friendly and its intermediate in the degradation of vanillin has already been investigated[10–13]. The enzymatic conversion of isoeugenol to vanillin was also reported by enzyme lipoxygenase and a crude enzyme from soybean[14]. The other alternative method for vanillin production is the biotransformation of caffeic acid and veratraldehyde. Caffeic acid is produced from the roots of *Capsicum frutescens*[15]. In our present study, the bacterial strains capable of converting isoeugenol to vanillin were characterized and optimized.

EXPERIMENTAL SECTION

Strain Isolation and Cultivation

Soil samples were collected from the roots of *Ocimum sanctum, Syzygium aromaticum*, *Cinnamon basil* and *Eucalyptus globulus* from Sirumalai and Kodaikanal, Tamil Nadu. 1gram of each soil sample was suspended in 100ml of sterile saline water. 25 ml of nutrient broth supplemented with 0.1% isoeugenol (Sigma Aldrich,India) was inoculated with 5ml of soil suspension & incubated in a shaker at 30°C for 24hours. From this 1ml was transferred to 25ml of fresh media with 0.1% of isoeugenol and incubated at 30°C, 150rpm for 24hours. The process was repeated and 10 µl of the culture was plated on nutrient agar media by spread plate method. Individual colonies were isolated by streak plate method [16].

Identification of strains capable of high tolerance to isoeugenol and vanillin (MIC)

The Minimum inhibitory concentration (MIC) for isoeugenol and vanillin of the strain were determined based on a microdilution method in 96 multi-well microtiter plate, using resazurin dye as indicator. Briefly, bacterial strains were cultured overnight at 30 °C on nutrient broth and adjusted to a final inoculum of 1.5×108 CFU/ml. Isoeugenol and vanillin (Sigma Aldrich, India) were dissolved in N,N-Dimethylformamide (DMF) and then in nutrient broth. Serial dilutions were made in a concentration ranging from 50 to 300 mM for isoeugenol and 10 to 200 mM for vanillin. 10 µl of each 1.5×108 CFU/ml bacterial suspension was added to wells. Finally, 10 µl of resazurin (0.2%) solution was added [16].Resazurin is an oxidation-reduction indicator used for the evaluation of microbial growth. It is a blue non-fluorescent dye that becomes pink and fluorescent when reduced to resorufin by oxidoreductases within viable cells. The inoculated plates were incubated at 30 °C after 3 days. All experiments were done in triplicates.

Bioconversion Using BT Media

The culture was inoculated in the bioconversion media (Glucose 5g/l, Yeast Extract 0.5 g/l, $(NH)_2SO4$ 2.0 g/l, $CaCl_2.6H_2O$ 0.2 g/l, $MgSO_4.7H_2O$ 0.5 g/l, $FeSO_4.7H_2O$ 0.025 g/l, KH_2PO_4 0.3 g/l, $Na_2.HPO_4.12H_2O$ 1.5 g/l) and kept in shaker and incubated at 150 rpm, 30°C for 24hours after which 0.02% of isoeugenol was added and vanillin yield was calculated by thiobarbituric acid assay [17].

Thin Layer Chromatography

Equal volume of chloroform was added to 500 µl of bioconversion bacterial culture (pH adjusted to 2.0- 3.0with 5N HCl) and centrifuged at 3000rpm for 1minute to separate the organic layer (the lower layer). Standard controls were vanillin and isoeugenol. TLC was performed on 0.23mm Silica gel GF254 per coated plates using solvent proportion Hexane: Ethyl acetate (3:4). Plates were visualized with UV and by charring after spraying reagent the mixture of 250ml glacial acetic acid, 0.5ml Conc. H_2SO_4 and 250µl Anisaldehyde[16].

Thiobarbituric assay

Five ml of 24% HCl solution, 2 ml of 1% thiobarbituric acid solution and 0.5ml vanillin solution were added to distilled water to make 10 ml in a 10 ml colorimetric tube. It was heated in a 55 °C water bath for 10 minutes and subsequently stored at room temperature for 20 minutes. The absorbance was then determined with a blank solution as reference in a SPECORD S10 spectrophotometer at 434 nm[17].

Gas Chromatography

The vanillin was evaluated accurately on a flame-ionization gas chromatograph (GCFID) GC equipped with a flame ionization detector (FID),capillary column (30m x0.32mm x 0.25 µm film thickness).The oven was programmed with an initial temperature 60°C/3 minutes, followed by an increase of 20°C for 1minute to a final temperature 260°C/10minutes. The injector temperature was held constant at 250°C and detector 280°C. Helium was used as a carrier gas [17]. (Asthagiri herbal foundation, Chennai).

DNA Isolation

Bacterial culture was grown overnight in LB broth and then pelleted. It was washed with 1ml TE and then resuspended in 100 µl of the same. 50 µl of 10% SDS was added and the solution was incubated for 30 minutes at 65ºC. The lysed cell was centrifuged and the supernatant was removed. The tubes were placed in the heating block for 2 to 3 minutes. The pellet was dissolved in 200µl of TE and shaken with an equal volume of chloroform: isoamylalcohol: phenol (24:1:25) for 15 minutes. The aqueous phase was recovered by centrifugation for 20 minutes and precipitated in ethanol. The DNA was resuspended in 100– 200 µl TE buffer and stored at -80°C [18].

Optimization Process

Bacterial strains were cultured in 125ml Erlenmeyer flasks containing 25ml of BT medium supplemented with isoeugenol concentrations ranging from 1-20g/l (1, 5, 10, 15,20g/l). The basal medium was inoculated with 5% of

1.5x108 CFU/ml of the bacterial suspensions and incubated aerobically at 30°C on a rotary shaker incubator (150rpm) for 120 hours. The cells were centrifuged at 14,000rpm for 10 minutes and the supernatant was used for quantitative determination of the formed vanillin in the reaction mixture and the optical densities were measured by spectrophotometry at 434 nm (Shimadzu UV -Spectrophotometer). With thiobarbituric standard graph, vanillin was plotted and results were analysed [17].

RESULTS AND DISCUSSION

Screening of tolerant strains

Vanillin is a well-known flavouring agent and as with most food additives, the demand for the naturally produced product is high. The objective of our study was to identify microbes that could convert isoeugenol to vanillin and to develop an economically feasible process as an alternative to the conversion of ferulic acid to vanillin[19,20]. The native tolerance of strains to isoeugenol and vanillin was measured by microdilution assay. Nine out of the eighteen strains tested, which showed maximum tolerance, were shortlisted as shown in Table 1. Morphological and biochemical characterization was performed to identify the strains (data not shown) and then were named as follows: *Syzygium aromaticum* - C1, C2, C3, C4,*Oscium sanctum* - OSC1, OSC2, *Cinnamon basil*- MOC1, MOC2 and *Eucalyptus globulus* – E1. From our analysis, two strains C1 and OSC1 showed maximum tolerance to isoeugenol and vanillin. It was found to be 300mM (49.26g/l) and 125mM (19g/l) for isoeugenol and vanillin respectively.

Table 1. Screening the bacterial strains for the maximum tolerance to isoeugenol and vanillin by Minimum inhibitory concentration

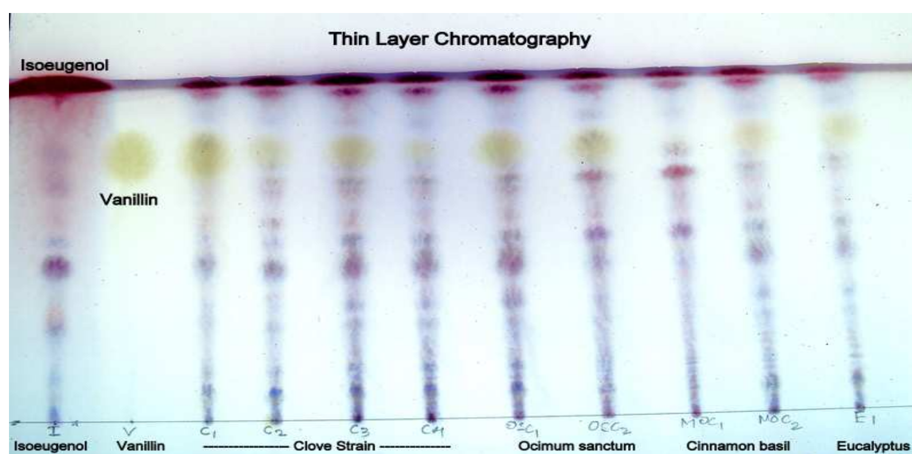
Strains	Isoeugenol MIC (mM)	Vanillin MIC (mM)
C1 (Bacillus spp.)	300	125
C2 (Bacillus spp.)	150	31.25
C3 (Bacillus spp.)	150	62.5
C4 (Bacillus spp.)	125	31.25
OSC1 (Pseudomonas spp.)	300	125
OSC2 (Bacillus spp.)	150	62.5
MOC1 (Bacillus spp.)	125	62.5
MOC2 (Bacillus spp.)	125	62.5
E1 (Bacillus spp.)	125	31.25

Conversion of isoeugenol into vanillin

The nine strains were grown in bioconversion medium supplemented with isoeugenol. The transformed products were extracted with chloroform and vanillin production was seen by TLC as shown in Figure1.

Figure 1.Vanillin production of bacteria by TLC

I –isoeugenol V-Vanillin, bacterial strains -C1, C2, C3, C4, OSC1, OSC2, MOC1, MOC2 and E1.

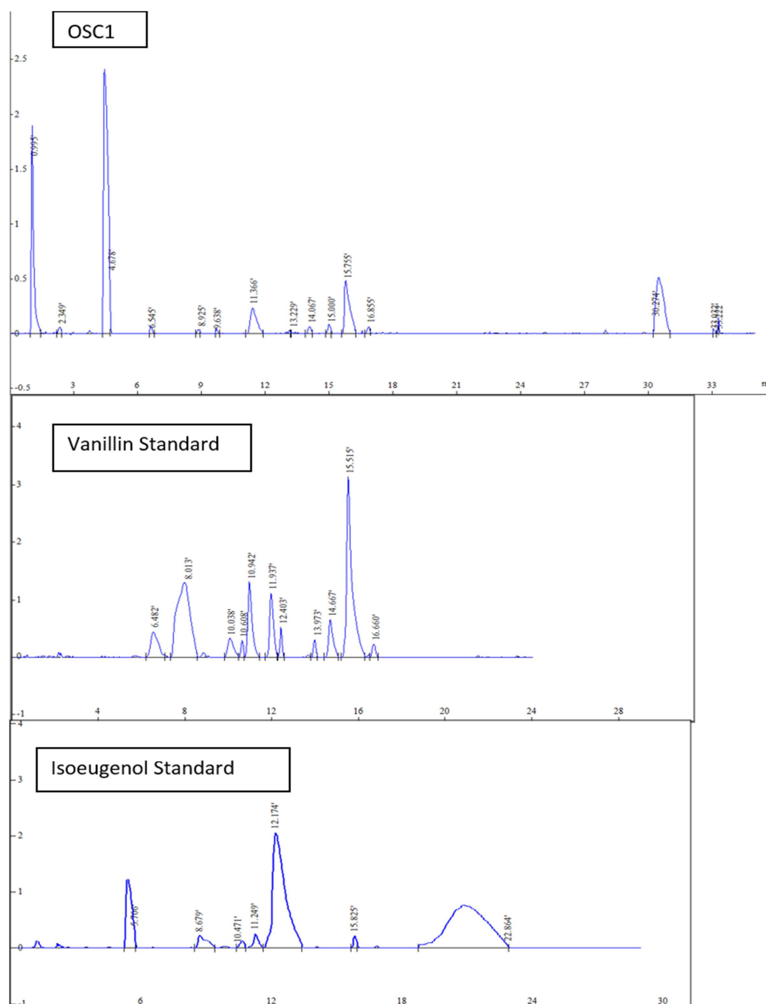


The strains OSC1 and C1 which showed the maximum tolerance to isoeugenol and vanillin were taken for further studies and the yield was estimated by thiobarbituric acid assay. C1 and OSC1 were found to produce maximum yield of vanillin 1.52g/l and 2.43g/l respectively and resulting in molar yield of 15.2% and 24.3%. The yield that we obtained was much higher when compared to previously reported data- 1.15g/l of vanillin from isoeugenol after 96 hours [17] and 0.0003g/l vanillin produced by *Bacillus* spp. from 0.0128mM eugenol with a molar yield of 15.6% after 48 hours with 0.2% of DMSO [21]. The microbial bioconversion of isoeugenol has already reported previously with *Pseudomonas spp*. and *Bacillus spp.* It was reported that the bioconversion from ferulic acid and isoeugenol

produced 1.04mg/ml and 0.64g/l vanillin respectively [22]. *Pseudomonasputida*IE27 was found to produce 16.1 g/l of vanillin (molar yield 71%) after 24 hours of incubation in the presence of 10% DMSO [23]. *Pseudomonas chlororaphis* also has shown the ability to convert isoeugenol to vanillin[24]. In accordance to these reports, the strain we identified by genotypic and phenotypic analysis was also found to be *Pseudomonas* spp.

Confirmation of vanillin production by GC-MS
The extract from the organic fraction of BT culture media was analysed by gas chromatography for strain OSC1 as it had a better vanillin yield. The metabolite retention time of control for vanillin and isoeugenol were identified as 15.51 and 12.17 minutes. Simultaneously OSC1 showed a retention peak at 15.7 minutes coinciding with vanillin control (Figure 2).

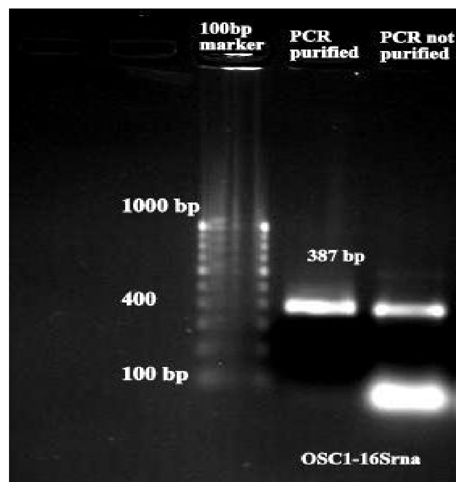
Figure 2. GCMS analysis of OSC1, Vanillin Standard and Isoeugenol Standard



Peaks for vanillin and isoeugenol were at 15.51 and 12.17. OSC1 showed a retention peak at 15.7 minutes coinciding with vanillin control

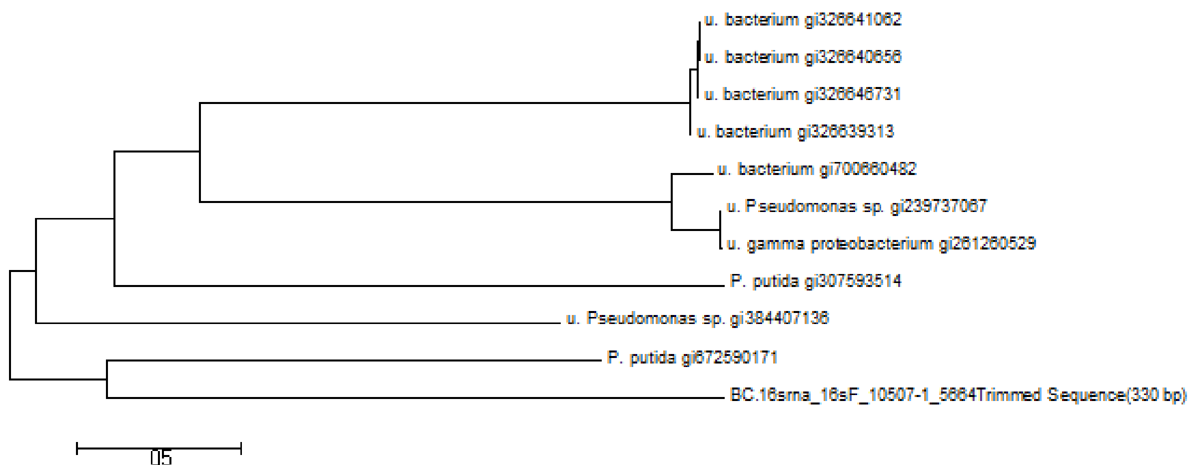
Strain identification
The strain OSC1 was subjected to morphological and biochemical studies, it was found to be gram-negative, motile and non-sporulating. It produced positive results for catalase and oxidase test and was tentatively found to be *Pseudomonasspp*. (data not shown) In order to confirm the phylogenetic relationship with the phenotypic and biochemically identified genus *Pseudomonas*, genomic DNA was isolated and PCR was performed for 16SrRNA as shown in Figure 3. The amplified product (387bp) of 16SrRNA sequenced by Scigenome, India was submitted to the GenBank with the accession number **KM975355**.The BLAST program was performed (http://www.ncbi.nlm.nih.gov/BLAST) to find the homology searches and the partial 16SrRNA showed 99% identity with *Pseudomonas* spp. Phylogenetic analysis was carried out using MEGA version 6 in order to find out the evolutionary analyses, as shown in Figure4.

Figure3. PCR amplified product of 16SrRNA of OSC1 strain



Lane 1: 100bp Marker, Lane 2 &3: Amplified purified and non-purified products of 16SrRNA of OSC1 strain(Agarose gel 1.5%).

Figure4. Phylogenetic tree of partial sequence of 16SrRNA of OSC1 strain



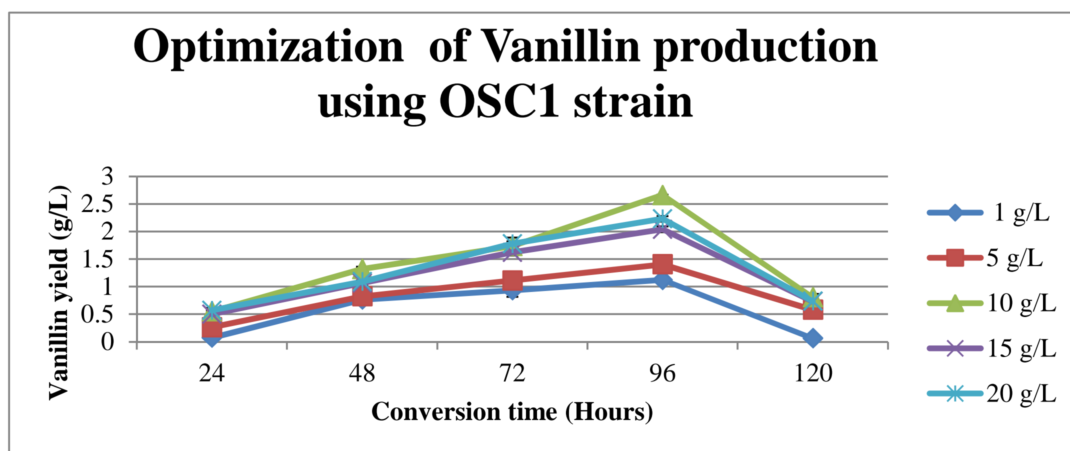
(BC-Bacterial culture OSC1 16Sr RNA). Phylogenetic tree using the neighbour-joining method shows OSC1 strain (BC.16SrRNA) close to *Pseudomonas* spp., scale bar measured as 05 along with GenBank accession numbers.

Optimization of Vanillin production using OSC1 strain

Optimization of the process to produce maximum yield was then carried out. As OSC1 which as identified as *Pseudomonas* spp. produced higher amount of vanillin it was used to optimize the process. This was done by varying the initial concentration of isoeugenol (1, 5,10,15,20g/l). It was found that 10g/l of isoeugenol gave the maximum yield of vanillin – 2.66g/l after 96 hours. The yield of vanillin was comparatively less for the remaining concentrations.

Reports have shown that the first biotransformation of isoeugenol to vanillin from *Aspergillus niger* ATCC 9142 had only 10% efficiency [10]. *Bacillus fusiformis* was found to yield vanillin concentration of 8.1g/l from 50g/lisoeugenol and product inhibition was avoided with by addition of HD-8 resin [25]. Our study showed that the strain OSC1 gave a yield of 2.6 g/l after 96 hours on addition of 10g/l of isoeugenol(Figure 5).

Figure 5. Effect of substrate concentration and conversion time of isoeugenol into vanillin by biotransformation using OSC1 strain *Pseudomonas spp*



CONCLUSION

Vanillin which is used for various foods flavouring agent, also possesses antimicrobial and antioxidant properties. In our present study, we investigated microbial production of vanillin by using isoeugenol as a precursor. As a preliminary step, native tolerance of bacterial strain to isoeugenol and vanillin was tested. From the screening process, an isolate OSC1 from soil sample (*Ocimum sanctum*) converted isoeugenol to vanillin. From the biochemical and partial 16SrRNA sequencing, the strain OSC1 was identified as *Pseudomonas* spp. When the media was optimized by the addition of 10g/lisoeugenol as a substrate, 2.6g/l of vanillin was produced in the presence of *Pseudomonas* spp. Therefore, one of the known substitutes for natural feedstock of vanillin production could be isoeugenol. Further studies in the production of vanillin could direct towards higher yield and cloning the gene response for the vanillin production.

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