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Isolation and Identification of a Novel Strain of *Pseudomonas chlororaphis* Capable of Transforming Isoeugenol to Vanillin†

Ramesh C. Kasana,[1] Upendra K. Sharma,[2] Nandini Sharma,[2] Arun K. Sinha[2]

[1]Hill Area Tea Science Division, Institute of Himalayan Bioresource Technology, Post Box No. 6, Palampur-176061, Himachal Pradesh, India
[2]Natural Plant Products Division, Institute of Himalayan Bioresource Technology, Post Box No. 6, Palampur-176061, Himachal Pradesh, India

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Abstract. Vanillin is undoubtedly one of the most popular and widely used flavoring agents in the world. Taking into consideration the worldwide demand for natural vanillin and its limited supply, alternative routes for its production including biotransformation are being constantly explored. In this regard, a novel soil bacterium capable of converting isoeugenol to vanillin was isolated by conventional enrichment process from soils of *Ocimum* field. On the basis of morphological and physiochemical characteristics and 16S rRNA gene sequence analysis, the isolate was identified as *Pseudomonas chlororaphis* CDAE5 (EMBL # AM158279). Vanillin formation was analyzed by gas chromatography (GC), and its structure was confirmed by GC-mass spectrometry and nuclear magnetic resonance. After 24-h reaction, the vanillin concentration reached 1.2 g L^{-1} from 10 g L^{-1} isoeugenol in 20-mL reaction solution at 25°C and 180 rpm. The strain showed potential to be a good candidate for biotechnological production of vanillin from isoeugenol. Further studies for standardization and optimization for higher yield of vanillin production needs to be investigated.

Vanillin (4-hydroxy-3-methoxybenzaldehyde) is one of the most widely used flavor compounds in food, beverages, pharmaceuticals, perfumes, and medicinal industries [16]. More than 12,000 tons of vanillin is produced each year, but only 1% originates from its natural source, i.e., *Vanilla planifolia*. The main portion is produced by chemical synthesis from guaiacol or lignin [24]. 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 and 4000/kg) [10]. The difference between the prices combined with the increasing customer-led demand for natural flavors has stimulated the exploration of biotechnological routes for the production of natural vanillin. One such route is microbial or enzymatic transformation of natural precursors such as ferulic acid [2, 6, 12, 14, 15, 18], vanillic acid [22], eugenol [17, 25], or isoeugenol [8, 9, 11, 21, 26, 27]. With respect to isoeugenol, there have been few studies on its degradation pathway and efficient bioconversion system. Although *Bacillus* species capable of transforming isoeugenol to vanillin have been reported earlier [21, 26, 27], little has been investigated about other microorganisms [4, 16]. With the aim of exploring the microbial wealth of the Western Himalayan region for biotransformation processes, we herein report the isolation and identification of novel strain of *Pseudomonas chlororaphis*, labeled as CDAE5, capable of transforming isoeugenol to vanillin (Fig. 1).

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Correspondence to: Arun K. Sinha; *email:* aksinha08@rediffmail. com

Materials and Methods

Materials and Microorganisms. Standard isoeugenol (98%, *cis-trans* mixture) and vanillin (99%) for the biotransformation studies were obtained from Merck (Germany). Other chemicals were of analytical grade. *P. chlororaphis* CDAE5 and other strains were isolated from soils of the Western Himalayan region.

Enrichment Culture. Soil samples collected from *Ocimum* (*Ocimum basilicum*, *O. sanctum, O. clocimum)* fields at Chandpur farm (Institute of Himalayan Bioresource Technology, Palampur, Himachal Pradesh, India) were processed for isolation of bacteria by enrichment

technique. Ten grams of each sample was suspended in 90 mL of sterile distilled water, and these suspensions were used as an inoculum for enrichment cultures. For enrichment procedures, nutrient broth (NB) medium (0.3% beef extract, 0.5% peptone, 0.5% NaCl, pH 7.0) supplemented with 0.1% isoeugenol was used. Cultures in 100-mL flask containing 20 mL of medium were inoculated with 3 mL of soil suspension and incubated shaking at 150 rpm and 28°C. Following four transfers (30 μL into 20 mL of fresh medium) after every 24 h, cultures were diluted (1 mL culture in 9 mL of distilled water) and plated on nutrient agar plates. After incubation for 24–48 h at 28°C, morphologically different colonies appearing on the plates were isolated and subjected to further purification by streaking on same medium.

Screening of Strains Transforming Isoeugenol to Vanillin. Isolates were grown in a 100-mL flask containing 20 mL of biotransformation (BT) medium (1.0% glycerol, 0.31% Na_2HPO_4, 0.25% KH_2PO_4, 0.25% $(NH_4)_2SO_4$, 0.0025% $FeSO_4.7H_2O$, 0.2% 1M $MgSO_4$, 0.3% 0.1 M $CaCl_2$) [17] at 25°C and 180 rpm for 24 h and isoeugenol was added up to a concentration of 1.5%. After incubation for 72 h, the potential biotransformation products were extracted with dichloromethane and analyzed by thin layer chromatography using hexane:ethyl acetate (80:20) as solvent system. The products were detected by developing the plates in iodine vapors [26].

Biotransformation of Isoeugenol to Vanillin by a Growing Culture. Isolates, which were found positive for biotransformation of isoeugenol to vanillin in initial screening, were grown on BT medium at 25°C and 180 rpm for 24 h, and isoeugenol was added up to 2.0% concentration. The whole culture was extracted three times with dichloromethane. Organic fractions were collected, filtered, dried over anhydrous sodium sulphate, and concentrated at 40°C to remove solvent under vacuum on a rotary vacuum evaporator (Buchi, Switzerland).

Vanillin and Isoeugenol Analysis. The above obtained extract was then dissolved in analytical grade chloroform (Merck) and analyzed by FID gas chromatograph (Shimadzu 2010) equipped with BP-1 capillary column (30m × 0.25 mm id, 0.25 μm), carrier gas N_2 at 0.5 mL/min, split ratio 40:1, injection temperature 250°C, detection temperature 280°C. The oven temperature was set at 220°C. The system was linked to a computerized integrator. Under these conditions, retention times recorded for vanillin and isoeugenol were 11.7 min and 13.6 min, respectively (Fig. 2). Gas chromatography (GC)-mass spectrometry (Shimadzu QP-2010) and nuclear magnetic resonance (Bruker Avance-300) were also used for confirmation of vanillin.

Morphological, Physiological, and Biochemical Characterization of Transforming Strains. Based on thin layer chromatography (TLC) and GC analysis, the strain giving highest vanillin yield was selected and characterized phenotypically and biochemically using standard techniques (Gram staining, motility, colony shape, size, and color on nutrient agar plate, growth on MacConkey agar, catalase, oxidase, O/F test, lipid and starch hydrolysis, etc.), according to the diagnostic table of Cowan and Steel (1974) [3] and Bergey's Manual of Determinative Bacteriology (1994) [5].

Phylogenetic Analysis. Bacterial DNA was isolated from pure culture and polymerase chain reaction (PCR) amplification of almost the entire length 16S rRNA fragment was carried out [20]. The sequence of primers used for amplification were 5′-AGAGTTTGATCATGGCT CAGA-3′ and 5′-GTTACCTTGTTACGACTT-3′ corresponding to 8 to 28 and 1493 to 1510, respectively, which are parts of 16S rRNA gene of *Escherichia coli* and are useful for amplifying 16S rRNA gene from various kinds of bacteria. The amplified 16S rRNA gene was purified from agarose gel using Qiaquick Gel Extraction Kit (Qiagen, Germany), ligated into the cloning vector (pGEM®-T easy vector) with the cloning kit (Promega, USA) and used for biotransformation of *E. coli* DH5α [20].

The nucleotide sequencing of the gene was done by using Big Dye^R Terminator Cycle Sequencing Kit (Applied Biosystems) and 3130xl Genetic Analyzer (Applied Biosystems). The BLASTN program (http://www.ncbi.nlm.nih.gov/BLAST/, NCBI, Bethesda, MD) was used for homology searches with the standard program default. Multiple alignments of the sequence were performed and a neighbor-joining phylogenetic tree [7, 19] was constructed using CLUSTAL W program [23]. The nucleotide sequence of the 16S rRNA gene of isolated bacterial strain is under EMBL Nucleotide Sequence Database Accession Number AM158279.

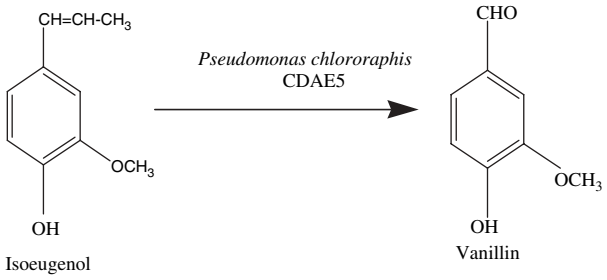

Fig. 1. Vanillin production from isoeugenol by *Pseudomonas chlororaphis* CDAE5.

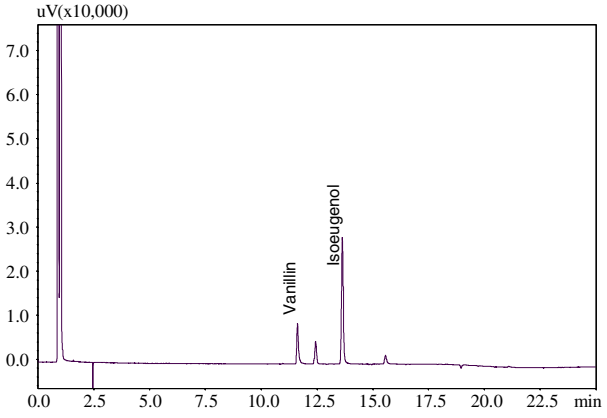

Fig. 2. Gas chromatography profile of vanillin biotransformation.

Results and Discussion

Isolation of Strains Transforming Isoeugenol to Vanillin. The present work was focused to isolate bacterial strains capable of transforming isoeugenol to vanillin. In earlier reported works, toxicity of the substrate and product to microorganisms and potential degradation of vanillin to vanillyl alcohol and vanillic acid has resulted in low yields of vanillin from isoeugenol [1, 15]. This strengthens the notion that

Table 1. Microbial characteristics of strain CDAE5

Tests	Results	Tests	Results
Colony morphology		Biochemical tests	
Configuration	Round	Growth on MacConkey agar	+
Margin	Entire	Indole test	−
Elevation	Convex	Methyl red test	−
Surface	Smooth	Voges proskauer test	−
Density	Translucent	Citrate utilization	+
Pigments	Greenish	Starch hydrolysis	−
Gram reaction	−ve	Casein hydrolysis	−
Shape	Rods	Urea hydrolysis	−
Size	Moderate	Lipid hydrolysis	+
Arrangement	Single	Tween 20 hydrolysis	+
Motility	+	Tween 80 hydrolysis	+
Fluorescence	+	Oxidase	+
Growth at temperature (°C)		Catalase	+
5	+	Oxidation/fermentation	O
10	+	Gelatin liquefaction	−
20	+	Acid production from	
30	+	Arabinose	−
37	+	Cellobiose	−
Growth at pH		Dextrose	+
4.0	−	Fructose	+
5.0	+	Galactose	+
6.0	+	Inositol	+
7.0	+	Lactose	−
8.5	+	Maltose	−
9.5	+	Mannitol	+
10.0	+	Mannose	+
11.0	−	Raffinose	−
Growth on NaCl (%)		Rhamnose	−
2.5	+	Sucrose	−
5.0	+	Trehalose	+
7.5	−	Xylose	+

strains having high tolerance to isoeugenol and lower vanillin degrading ability should be isolated [27]. Keeping this in view, microbial strains having high tolerance to isoeugenol were isolated by enrichment process from the soil samples taken from an *Ocimum* field, because *Ocimum* is a rich source of phenylpropanoids (about 75%), including isoeugenol. Based on their morphology, 21 different bacterial strains were selected and tested for biotransformation of isoeugenol into vanillin. Products were analyzed through TLC and GC (Fig. 2). Under the conditions (as mentioned under Materials and Methods), strain CDAE5 produced the highest amount of vanillin. Based on these results, strain CDAE5 was selected for further studies.

Identification and Phylogenetic Analysis of Strain CDAE5. Morphological, physiological, and biochemical characters and 16S rRNA gene sequencing was used to identify strain CDAE5. Microbial characteristics of strain CDAE5 as studied are listed in Table 1. Cells are Gram-negative, motile rods. Growth occurred at temperature of 5°C to 37°C, pH of 5.0 to 10.0, and in a medium supplemented with 2.5-5% NaCl. These characteristics indicated that the strain belongs to the genus *Pseudomonas*. To confirm its phylogenetic relationship with *Pseudomonas*, genomic DNA was isolated from the bacterium and gene coding for 16S rRNA was amplified by polymerase chain reaction. The sequencing of almost full-length 16S rDNA showed that it was closely related to genus *Pseudomonas* showing 99.2% homology to *P. chlororaphis* (Fig. 3). In accordance with these data, the isolate was included in the genus *Pseudomonas* and named as *Pseudomonas chlororaphis* CDAE5. Strain CDAE5 is kept at Microbial Type Culture Collection and Gene Bank, Chandigarh, India as *Pseudomonas chlororaphis* CDAE5.

Biotransformation of Isoeugenol to Vanillin by a Growing Culture. Bacterial culture was grown for 24 h in 20 mL of BT medium. Different concentrations of isoeugenol ranging from 0.5% to 2.0% were then added to culture, and 1.0% isoeugenol was found to give the better yield (Fig. 4). This may be because of the toxicity of substrate to microorganisms at its higher concentrations, as reported earlier [15]. The maximum vanillin yield of 1.2 gL^{-1} from 10 gL^{-1} was achieved after 24 h in 20-mL reaction mixture, resulting in a molar efficiency of 12.64%. The first BT of isoeugenol to vanillin was achieved with *Aspergillus niger* having only 10% efficiency [1]. Other bacterial strains reported include those of genera *Klebsiella*, *Enterobacter*, *Bacillus*, and *Serratia*, with *Serratia marcescens* showing maximum conversion efficiency of 20.5% [17]. Recently, a strain of *Bacillus fusiformis* has been reported for conversion of isoeugenol to vanillin, whereby product inhibition was avoided with the addition of HD-8 resin, yielding a vanillin concentration of 8.10 gL^{-1} from 50 gL^{-1} isoeugenol [27]. Further studies for improving the yield of vanillin using *Pseudomonas chlororaphis* CDAE5 by addition of resins and process optimization are under progress.

Conclusion

A novel strain, *P. chlororaphis* CDAE5, was isolated from soils of *Ocimum* field, which was found to transform isoeugenol to vanillin. In earlier studies, *Pseudomonas fluorescens* was been reported to degrade ferulic acid to vanillin [13], and *Pseudomonas putida* converted isoeugenol to vanillic acid [4]. To the best of our

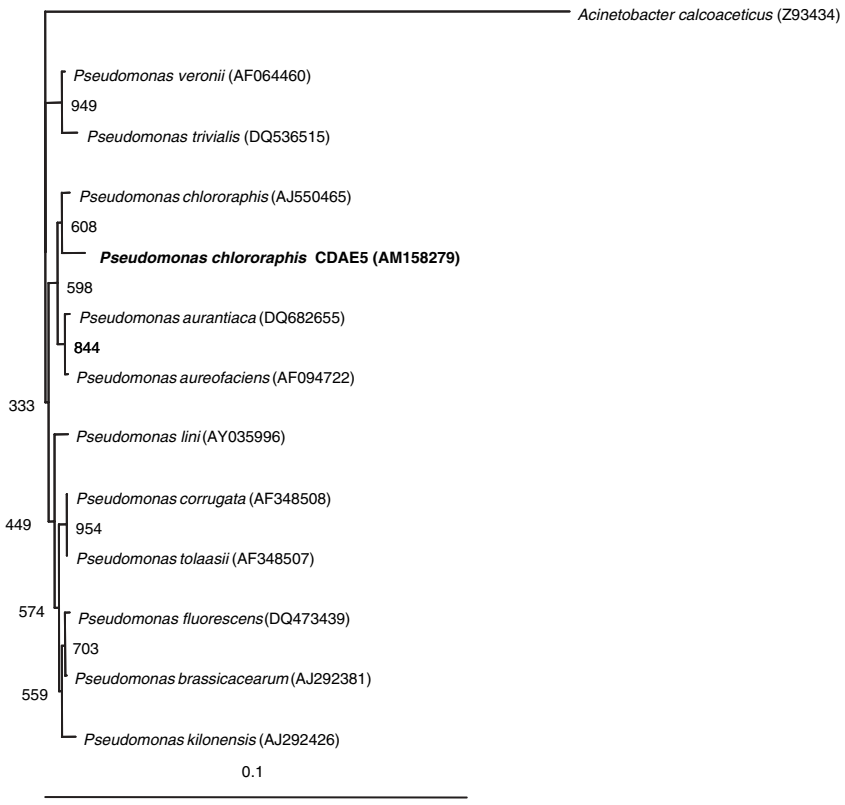

Fig. 3. Phylogenetic tree indicating the estimated relationship between strain CDAE5 (Accession no. AM158279) and species of the genus *Pseudomonas* that shared highest 16S rRNA gene sequence similarities. *Acinetobacter calcoaceticus* (Z93434) was selected as the outgroup. Numbers indicate bootstrap values. Bar, 0.1 substitutions per site.

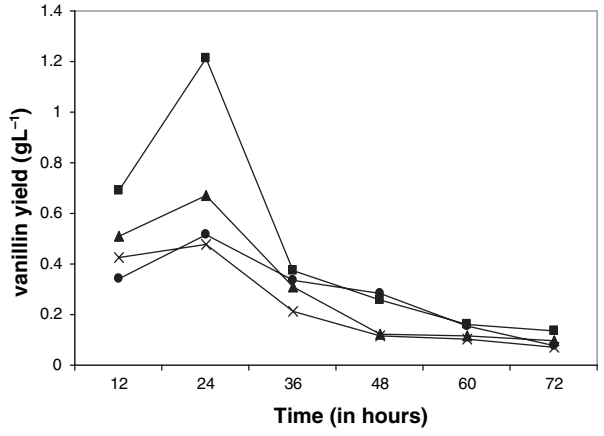

Fig. 4. Effect of substrate concentration and incubation time on biotransformation of isoeugenol to vanillin by *Pseudomonas chlororaphis* CDAE5. Isoeugenol concentrations (%): 0.5 (●), 1.0 (■), 1.5 (▲), and 2.0 (×)

knowledge, the present study gives the first evidence for conversion of isoeugenol to vanillin by *P. chlororaphis* CDAE5. Using 10 gL^{-1} isoeugenol as substrate, 1.2 gL^{-1} vanillin was produced in 20-mL reaction solution after 24 h, at 25°C and 180 rpm. Further studies in this direction for obtaining higher yields of vanillin are in progress.

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