

## A bioconversion process using a novel isolated strain of *Pseudomonas* sp. ISPC2 to produce natural vanillin from isoeugenol

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### Abstract

Vanillin is undoubtedly one of the most popular and widely used flavoring agents in the world. Natural vanillin is extracted from vanilla beans and is relatively expensive. Moreover, the consumer demand for natural vanillin highly exceeds the amount of vanillin extracted by plant sources. This had led to the investigation of alternative routes for its production such as microbial bioconversion. In this study, a novel strain bacterium capable of converting isoeugenol to vanillin was isolated by conventional enrichment process from soils of spicy plants farms. On the basis of morphological and physiochemical characteristics, the isolate was identified as *Pseudomonas* sp. strain ISPC2. Vanillin formation was analyzed by spectrophotometry with thiobarbituric acid reagent and evaluated accurately by gas chromatography. Using 10 g/l of isoeugenol as substrate in 25 ml reaction solution at 30 °C and 150 rpm, vanillin reached a maximum concentration of 1.15 g/l after 96 h reaction, corresponding to a molar yield of 12.4%. This 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.

**Keywords:** Bioconversion; Isoeugenol; *Pseudomonas* sp. strain ISPC2; Vanillin

### INTRODUCTION

Vanillin (4-hydroxy-3-methoxy benzaldehyde), one of the most important components of natural flavors, is widely used in foods, beverages, perfumes, pharmaceuticals and medical industries (1). It is currently mainly produced from petrochemicals and from lignin (2). Owing to the increasing demand for healthy and natural food, there is a growing interest in producing vanillin from natural raw materials by bioconversion (3), which can then be regarded as a natural aroma chemical (4). Production of vanillin by microbial or enzymatic conversion of natural precursors such as ferulic acid (5-7), vanillic acid (8), eugenol (9,) or isoeugenol (10-15) has been investigated. Most of the bioconversion processes studied so far resulted in low product concentrations below 1 g/l. This could be attributed to the high reactivity of vanillin that forces the applied microorganism to “detoxify” this compound by either oxidation or reduction. However, recently two actinomycetes, *Amycolatopsis*

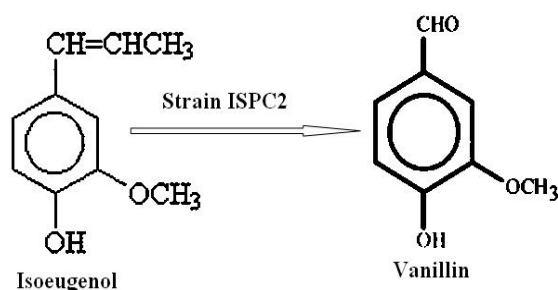
sp. HR167 and *Streptomyces setonii* ATCC 39116 were identified, which exhibited a very high tolerance towards vanillin (16-19). Based on these biocatalysts, efficient processes with yields of more than 10 g/l vanillin were established, using natural ferulic acid as the feedstock. These processes, however, suffer from the high price of natural ferulic acid, which is due to its limited accessibility from lignin by biological means. One cheap alternative feedstock for biotechnological production of natural vanillin-type aromatic compound is the phenylpropanoid isoeugenol, which is the main component of the essential oil of the clove tree *Syzygium aromaticum*. Isoeugenol (1-hydroxy-2-methoxy-4-propenylbenzene) is usually obtained from eugenol under strong basic condition or isomerization and it can also be extracted from plant directly. Isoeugenol can serve as a potential substrate for the production of valuable aromatic compounds (20,21). Bioconversion of isoeugenol has always been a hot topic because it is a natural renewable resource and the

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conversion processes are environmentally friendly.

Some microorganisms including *Aspergillus niger*, *Bacillus subtilis*, *Bacillus pumilus*, *Rhodococcus rhodochrous*, *Serratia marcescens* and *Pseudomonas chlororaphis* can degrade isoeugenol (9,11,15,22-24).

We are currently investigating a process for vanillin production, based on the bioconversion of isoeugenol, since isoeugenol is a cheap "natural" substrate that can be isolated from the essential oil *Syzygium aromaticum*. In the present study, which is done for the first time in Iran, we describe the isolation and initial characterization of a novel strain of *Pseudomonas* sp., labeled as ISPC2, capable of transforming isoeugenol to vanillin (Fig. 1).



**Fig. 1.** Representation of the bioconversion of isoeugenol in to vanillin catalyzed by strain ISPC2.

## MATERIALS AND METHODS

### Chemicals

Vanillin (99%), isoeugenol (98%, cis-trans mixture) and 2-thiobarbituric acid were purchased from Sigma-Aldrich (St. Louis, Missouri, USA). Analytical methanol for gas chromatography (GC) analysis was purchased from Merck (Merck, Germany) and was of GC grade. All other materials were of high purity commercially available.

### Isolation and Cultivation

In total, 20 bacterial strains, including strain ISPC2, were isolated from soil samples in Isfahan, Iran. Soil samples were collected from farms and greenhouses of *Ocimum*, *Dianthus* and Spicy plants, and processed for isolation of bacteria by enrichment technique. Ten grams of each sample was suspended in 90 ml of sterile physiological serum and these

suspensions were used as an inoculum for enrichment cultures. Nutrient broth (NB) medium (0.3% beef extract, 0.5% peptone, 0.5% NaCl, pH 7) supplemented with 0.1% (v/v) isoeugenol was used for enrichment procedures. Cultures in 125 ml flask containing 25 ml of medium were inoculated with 5 ml of soil suspension, incubated and shook at 150 rpm and 30 °C. Following three transfer (50  $\mu$ l into 25 ml of fresh medium) every 24 h, cultures were diluted and plated on nutrient agar plates. After incubation for 24-36 h at 30 °C, morphologically different colonies appearing on the plates were isolated and subjected to further purification by streaking on the same medium.

### Screening of strains capable of high tolerance to isoeugenol and vanillin

The tolerance patterns for isoeugenol and vanillin were determined based on a micro-dilution method in 96 multi-well microtitre plate. Briefly, bacterial strains were cultured overnight at 30 °C on nutrient broth and adjusted to a final inoculum of  $1.5 \times 10^8$  CFU/ml. Isoeugenol and vanillin were dissolved in N,N-Dimethylformamide (DMF) and then in nutrient broth to reach a final concentration of 50 mM for vanillin and 100 mM for isoeugenol as stock solutions. Serial dilutions were made in a concentration range from 5 to 80 mM for isoeugenol and 1 to 40 mM for vanillin. Amount of tolerance for isoeugenol and vanillin was determined at 30 °C after 3 days. All experiments were done in triplicates.

For primary screening of transforming strains, cells were grown (25 ml medium in 125 ml flask at 30 °C, 150 rpm) for 24 h on designed bioconversion medium (BT), containing (in g/l): glucose 5, yeast extract 0.5,  $(\text{NH}_4)_2\text{SO}_4$  2,  $\text{CaCl}_2 \cdot 6\text{H}_2\text{O}$  0.2,  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$  0.5,  $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$  0.025,  $\text{KH}_2\text{PO}_4$  0.3,  $\text{Na}_2\text{HPO}_4 \cdot 12\text{H}_2\text{O}$  1.5, and isoeugenol was added directly to a final concentration of 10 g/l. After additional 24 h of incubation, potential bioconversion products were separated by acidifying (to pH 2-3 with 5 N HCl) and extracted with equal volumes of chloroform ( $\text{CHCl}_3$ ). The organic layer was separated by centrifugation (3,000 rpm for 1

min) and used for TLC analysis in which 10  $\mu$ l samples were spotted on plates.

### Analytical methods

#### Thin layer chromatography (TLC)

TLC was performed on 0.25 mm silica gel GF254 precoated plates (20  $\times$  20 cm, Merck). The mobile phase was hexane:ethyl acetate (3:4). Plates were dried at room temperature and visualized at 254 nm.

#### Bioconversion of isoeugenol to vanillin by a growing culture

Cells were cultured in 125 ml Erlenmeyer flasks containing 25 ml of BT medium supplemented with different isoeugenol concentration (1-20 g/l). The basal medium was inoculated with 5% of  $1.5 \times 10^8$  CFU/ml of the bacterial suspensions and incubated aerobically at 30  $^{\circ}$ C on a rotary shaker (150 rpm) for 120 h. The cells were centrifuged at 14,000 rpm for 10 min and the supernatants were used for quantitative determination of the formed vanillin in the reaction mixture.

#### Vanillin analysis by spectrophotometry with thiobarbituric acid

Five ml of 24% HCl solution, 2 ml of 1% thiobarbituric acid solution and some

vanillin solution were added to distilled water to make 10 ml in a 10 ml colorimetric tube. It was heated in a 55  $^{\circ}$ C water bath for 10 min and subsequently stored at room temperature for 20 min. The absorbance was then determined with a blank solution as reference in a SPECORD S10 spectrophotometer at 434 nm (25).

#### Vanillin analysis by GC

The vanillin was evaluated accurately on a flame-ionization gas chromatograph (GC-FID).

The Agilent 19091J-413 GC equipped with a flame ionization detector and HP-5 capillary column (30 m  $\times$  0.32 mm  $\times$  0.25  $\mu$ m film thickness). The oven temperature was programmed with an initial temperature of 60  $^{\circ}$ C held for 3 min, followed by an increase of 20  $^{\circ}$ C/min to a final temperature of 250  $^{\circ}$ C. The injector temperature was held constant at 250  $^{\circ}$ C. Helium was used as a carrier gas at a constant column flow rate of 1.7 ml/min. Under these conditions, retention times recorded for isoeugenol and vanillin were 9.7 min and 10.1 min, respectively (Fig. 2).

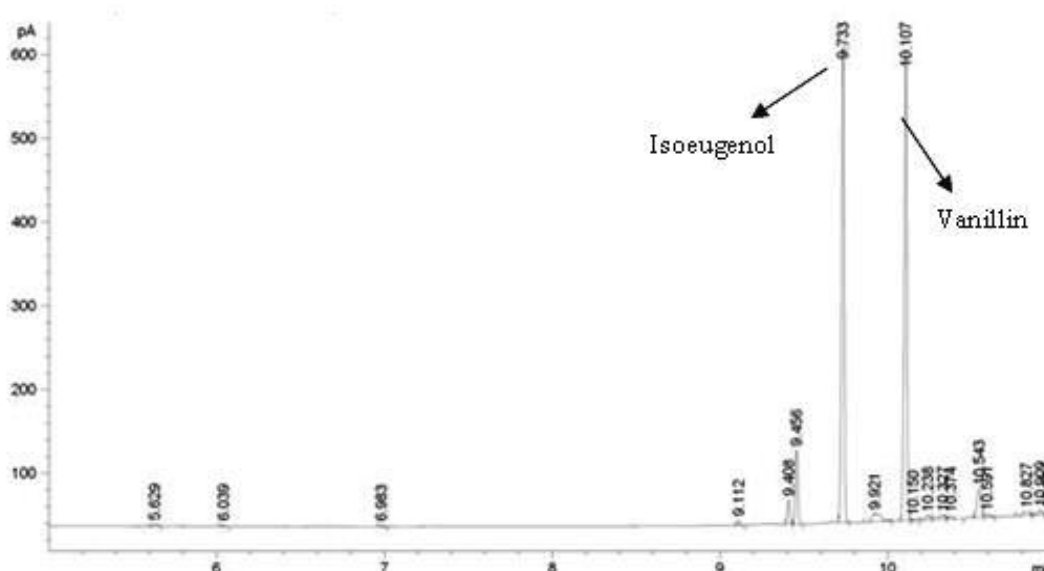


Fig. 2. Gas chromatography profile of isoeugenol and vanillin.

### Identification of the isolate

Based on TLC and GC analysis, the strain giving highest vanillin yield was selected and characterized on the basis of morphological, physiological and biochemical characteristics using standard techniques (Gram staining, motility, colony shape, color on nutrient agar, tween 20 hydrolysis, citrate utilization, MR/VP test, Indole test, acid production from sugars, etc.), according to Bergey's Manual of Determinative Bacteriology.

### Statistical methods

Data from each experiment was statistically analyzed using SPSS 11.5th software. For analyzing data, t-test and analysis of variance (ANOVA) methods were applied. t-test indicates differences between the samples in each treatment while, ANOVA analysis shows, variations between several treatments in each test.

## RESULTS

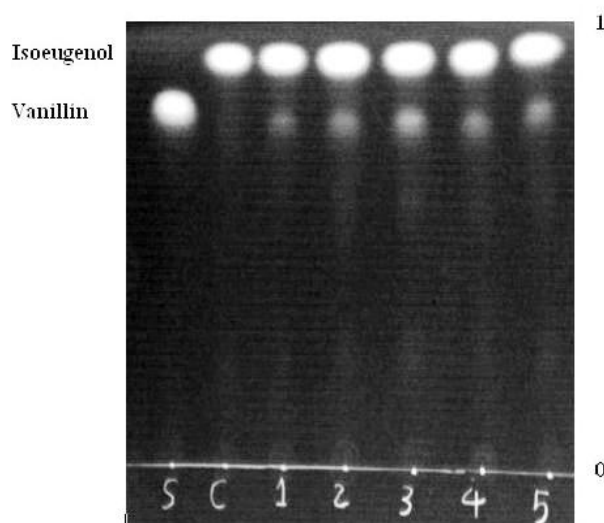
### Isolation of high-tolerance strains capable of transforming isoeugenol to vanillin

Due to the toxicity of the substrate and product to microorganisms, and the potential oxidation of vanillin to vanillic acid, the productivity from isoeugenol to vanillin was relatively low (21). This suggested that as a first step, strains having a high tolerance to isoeugenol and vanillin should be sought. Keeping in view, the isolation of the high-resistant bacterial strains to isoeugenol and vanillin performed using an enrichment culture. In total, 20 strains from different soil samples in Iran, were isolated on NB medium supplemented with isoeugenol by enrichment process. The intrinsic tolerance of strains to isoeugenol and vanillin was measured by microdilution assay (as mentioned under Materials and Methods) (Table 1).

Among the 20 strains of bacteria, 5 strains (ISPC2, IMPC12, ISPC1, IOC9, KDC18), which showed the maximum tolerance to isoeugenol and vanillin (more than 10 and 3 g/l for isoeugenol and vanillin, respectively) were selected and tested for bioconversion of isoeugenol into vanillin. Products were analyzed through TLC. As shown in Fig. 3, all

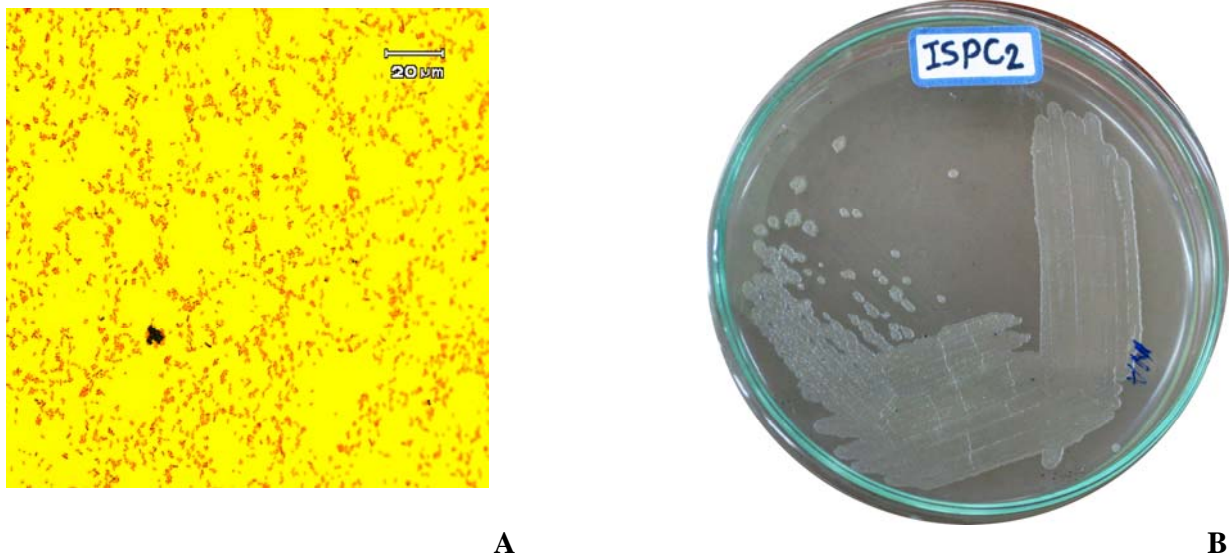
**Table 1.** Screening of bacterial strains having high-level tolerance to isoeugenol and vanillin.

Strain	Tolerance (mM)	
	Isoeugenol	Vanillin
ISPC2	>80	>40
IMPC12	75	35
ISPC1	70	35
IOC9	65	30
KDC18	65	25
Other 15 strains	<50	<20

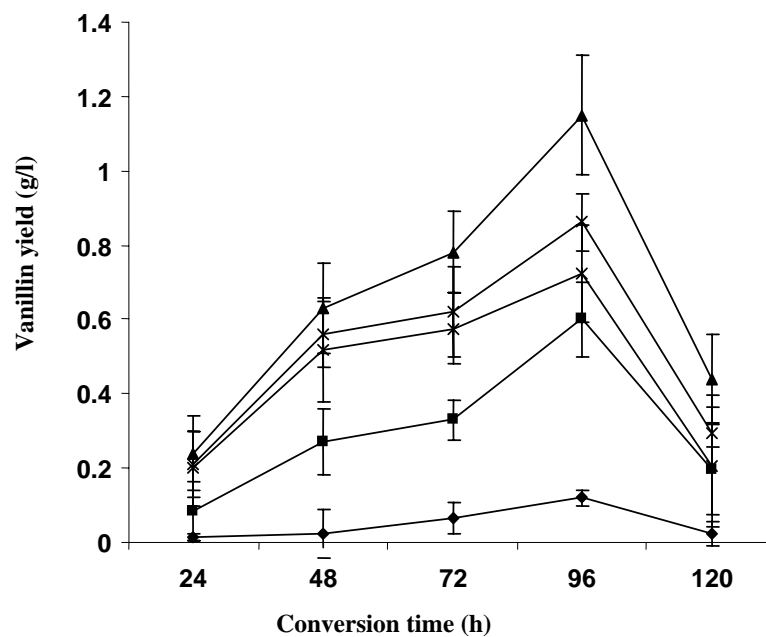


**Fig. 3.** Bioconversion of isoeugenol to vanillin. Cultures were grown in BT medium plus 0.5% glucose and 10 g/l isoeugenol at 150 rpm and 30 °C for 48 h. The transforming products were extracted with chloroform (CHCl<sub>3</sub>) and analyzed by TLC [hexane: ethyl acetate (3:4)]. (S: standard (vanillin); C: control (no-bacteria); 1: IMPC12; 2: ISPC1; 3: ISPC2; 4: IOC9; 5: KDC18).

of the tested strains produced vanillin when grown in the presence of isoeugenol (lanes 1-5). Under the conditions (as mentioned under Materials and Methods), strain ISPC2 (lane 3) produced the highest amount of vanillin. Strain ISPC2 was shown to be a gram-negative, non-sporulating, motile, aerobic rod, and produced catalase and oxidase. On the basis of cultural and morphological characteristics (Fig.4) and biochemical characterization (data not shown), the isolate was tentatively placed in the genus of the *Pseudomonas*.



**Fig. 4.** Morphological and cultural characteristics of strain ISPC2. A: Light microscopic B: growth on nutrient agar plate.



**Fig. 5.** Effect of substrate concentration and conversion time on bioconversion of isoeugenol to vanillin by strain ISPC2. Isoeugenol concentration (g/l): 1 (◆), 5 (■), 10 (▲), 15 (×), 20 (✱). Results represent the means of three separate experiments, and deviation bars indicated.

#### ***Bioconversion of isoeugenol to vanillin by strain ISPC2***

The formation of vanillin by strain ISPC2 was assessed by addition of different concentrations (1-20 g/l) of isoeugenol to a ISPC2 culture previously grown for 24 h on BT medium plus glucose. Potential products were separated by acidifying (to pH 2-3 with

H<sub>2</sub>SO<sub>4</sub>), and extracting the whole culture five times with chloroform. The organic fractions were collected, dried over anhydrous sodium sulfate, filtered, and dried by rotary evaporator. The extract was then dissolved in methanol and analyzed by a FID gas chromatograph (as mentioned under Materials and Methods).

**Table 2.** Vanillin production from isoeugenol by different microorganisms.

Microorganism	Yield (mg/l)	Molar yield (%)	Refs
strain ISPC2	1150	12.4	
<i>Aspergillus niger</i>	80	10	(22)
<i>Bacillus subtilis</i> B2	610	12.4	(11)
<i>Rhodococcus rodochrous</i>	1000	58	(23)
<i>Bacillus subtilis</i> HS8	1360	14.7	(26)
<i>Bacillus pumilus</i> S1	3750	40.5	(24)
<i>Serratia</i> sp.	3800	20.5	(9)
<i>Pseudomonas cholovoraphis</i>	1200	12.9	(15)

As shown in Fig. 5, 10 g/l isoeugenol was found to give the better yield. The maximum vanillin yield of 1150 mg/l from 10 g/l isoeugenol was achieved after 96 h in 25 ml reaction mixture, resulting in a molar yield of 12.4%.

## DISCUSSION

Vanillin (C<sub>8</sub>H<sub>8</sub>O<sub>3</sub>) is one of the principle components responsible for the characteristic aroma and flavor of vanilla extract. Synthetic vanillin, most often produced by the treatment of sulfite waste liquors from paper mills, is typically used as a low cost substitute for vanilla and indeed may even be presented as an adulterant in vanilla extract. Because of its origin and method of manufacture, vanillin derived from sulfite waste liquor neither is considered to be a natural food component nor is labeled in the European countries and U.S. Therefore, there is a growing interest to produce "natural" vanillin from natural substrates by bioconversion. Until now, only the bioconversion of ferulic acid to vanillin had been developed to an economically feasible process (16,17). However, these bioconversions depended on the use of the expensive substrate ferulic acid. One suitable alternative natural feedstock for biotechnological vanillin production could be isoeugenol, which is a commercially available natural raw material with a market price of US\$ 9/Kg. In this study; we describe the isolation and initial characterization of a novel bacterium from soils of spicy plants farms, which was found to transform isoeugenol to vanillin. The yield of vanillin produced from isoeugenol by isolated strain ISPC2 was comparable to those previously reported for

microbial conversions of isoeugenol to vanillin (Table 2). Vanillin production from isoeugenol through this biotechnological route is not very economical as the vanillin level was 1150 mg/l at the 96 h. However, it should be stressed that this molar yield (12.4%) was accomplished in a non optimized process, indicating the great potential of strain ISPC2 for production of vanillin. The isolation and screening procedures used in this study proved to be useful for obtaining transforming strains, and allowed the isolation of a *Pseudomonas* sp. strain ISPC2 capable of transforming isoeugenol to vanillin. Further studies for improving the yield of vanillin using strain ISPC2 by process optimization (by varying the environmental parameters and nutrient condition and also mutation) as well as purification and characterization of the enzymes which catalyzed these reactions need to be performed.

## ACKNOWLEDGMENT

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