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RESEARCH ARTICLE

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Biotransformation of eugenol to vanillin by a novel strain *Bacillus safensis* SMS1003

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ABSTRACT

Due to the extensive applications of vanillin as flavored compound and increasing consumers concern for its natural and environment friendly mode of production, present work was focused on the selection of bacterial isolate capable of producing vanillin using eugenol biotransformation. Bacterial strain SMS1003 is evidenced as the potential strain for vanillin production and identified as *Bacillus safensis* (GeneBank accession no. MG561863) using biochemical tests and molecular phylogenic analysis of its 16S rDNA gene sequence. Molar yield of vanillin reached up to 10.7% (0.055 g/L) at 96 h of biotransformation using growing culture of *B. safensis* SMS1003 in following culture conditions: eugenol concentration 500 mg/L; temperature 37 °C; initial pH 7.0; inoculum volume 4%; volume of culture media 10%; and shaking speed 180 rpm. Vanillin was detected as the single metabolite with a molar yield of 26% (0.12 g/L) at 96 h using resting cells of *B. safensis* SMS1003. Product confirmation was based on spectral scan using photodiode array detector, Fourier-transform infrared spectroscopy, high-performance liquid chromatography, and mass spectroscopy.

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KEYWORDS
Biotransformation; eugenol; vanillin; PDA; molar yield; *Bacillus safensis*

1. Introduction

During the past few years, worldwide increasing market demand for healthy food additives as well as compounds of various cosmetic and pharmaceutical uses, primes the development of safe and ecofriendly industrial processes for their production. Generally, various aromatic compounds impart a significant role in food and chemical industries. Vanillin (4-hydroxy-3-methoxybenzaldehyde) is one such widely used aromatic compound used as a flavor in food, confectionary, beverage production and also has certain applications in medical fields and pharmaceuticals (Priefert et al. 2001). Natural vanillin production was based on the fermentation of cured pods obtained from two different species of vanilla orchids: *Vanilla planifolia* and *Vanilla tahitensis*. Traditionally, a chemical method of synthesis was used for their production leading to the formation of undesirable racemic mixtures, lacking substrate selectivity and reduce process efficiency by increasing the downstream processing cost and cause environmental pollution (Longo and Sanroman 2006; Xu et al. 2007). Microbial transformation that is fermentation (= *de novo*) and biotransformation of appropriate precursor compounds act as a substitute in the biosynthesis of these aromatic compounds. Microbial whole cells, immobilized cells as well as their enzymatic preparations are considered as efficient and environment friendly bioconversion tools (Ashengroph et al. 2012).

Plant derived phenylpropanoids which are a diverse family of the organic compounds synthesized by the plants can act as the potential precursor for microbial biotransformation (Giedraityte and Kalediene 2014). Various plant-derived phenylpropanoids (eugenol, isoeugenol, and ferulic acid) can be used as the natural renewable and environment friendly sources for the production of fine chemicals (Ashengroph et al. 2011). Out of all potential sources, use of ferulic acid has been widely investigated for biotransformation (Giedraityte and Kalediene 2014). However, due to the limited availability of natural ferulic acid and its high price, eugenol was explored as an alternative precursor for enzymatic oxidative transformation to other valuable eugenol derivatives like ferulic acid and vanillin (Rabenhorst 1996).

Eugenol (4-allyl-2-methoxyphenol) is the major aromatic constituent present in the clove oil extracted

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from the clove plant, *Syzygium aromaticum* and has been explored as a potential precursor for the production of various methoxyphenol derivatives. It is an inexpensive substrate with the market price of around US$50/kg and also commercially available. According to the US and European legislation production of flavored compounds using a raw material through biotechnological route is "natural" (Hua et al. 2007). Use of eugenol as a food additive and potential precursor for industrial vanillin production by microorganisms is generally recognized as safe (Gallage and Moller 2015).

Vanillin as well as its associated metabolites formed during eugenol biotransformation have widespread applications and are of great economic importance (Ashengroph et al. 2011). Coniferyl aldehyde exhibits antibacterial, pesticidal and chemoattractant properties. Similarly, various biomedical, pharmaceutical and industrial applications have been associated with the use of ferulic acid. It acts as a potential antioxidant, anti-diabetic and anti-ageing agent and is an effective component of various Chinese medicinal herbs (Graf 1992; Mishra et al. 2013).

Rabenhorst developed a method of eugenol biotransformation to vanillin using *Pseudomonas* sp. strain (Rabenhorst 1996). Various other microorganisms, including *Pseudomonas fluorescens*, *P. putida*, *Corynebacterium* sp., *Fusarium solani*, *Byssochlamys fulva*, and *Penicillium simplicissimum*, were also reported on the basis of their ability to degrade eugenol (Tadasa and Kayahara 1983; Furukawa and Nagasawa 1998; Priefert et al. 2001; Giedraityle and Kalediene 2014). Biodegradation of eugenol to vanillin was also reported using *Bacillus* sp (Kadakol and Kamanavali 2010). Although, various microorganisms have been explored so far for vanillin production using eugenol (Tadasa and Kayahara 1983; Rabenhorst 1996; Furukawa and Nagasawa 1998; Priefert et al. 2001; Kadakol and Kamanavali 2010; Giedraityle and Kalediene 2014). Extensive screening of other potential microorganisms needs to be done with the aim to improvise the yield of vanillin as a single metabolite. This study is focused on the isolation and selection of a bacterial strain with the potential to transform eugenol into value added methoxyphenol derivatives like vanillin.

In the current study, a novel strain of *Bacillus safensis*, labelled as SMS1003, capable of transforming eugenol into derivatives like vanillin and coniferyl aldehyde was identified. Further, in response to optimization of various culture conditions, a significant amount of vanillin (0.12 g/L) was obtained as a single metabolite.

2. Materials and methods

2.1. Chemicals

Standards such as eugenol (99%), coniferyl aldehyde (98%), ferulic acid (99%), vanillin (99%), vanillic acid (98%), and protocatechuic acid (98%) were purchased from Sigma-Aldrich (Bangalore, India). Analytical grade methanol and ethyl acetate as well as high-performance liquid chromatography (HPLC)-grade solvents such as methanol, and Trifluoroacetic acid were procured from Himedia (Mumbai, India). All other chemical used as media components were of analytical grade and purchased from Hi-Media Laboratory India.

2.2. Isolation of bacterial cultures

Eugenol metabolizing bacteria was isolated from soil samples of *Ocimum* and *Petunia* sp. cultivated field of BIT Mesra medicinal garden. Soil suspension was prepared using 10 g of the wet soil sample in 100 ml of sterile saline water, and appropriate dilutions of these suspensions were prepared. From the above prepared dilutions, 100 µl were plated into the sterilized biotransformation (BT) medium (ingredients: gram per liter of distilled water: $(Na_2)HPO_4$, 4; KH_2PO_4, 1; yeast extract, 1; NaCl, 0.2; $MgSO_4.7H_2O$, 0.2 and anhydrous $CaCl_2.2H_2O$, 0.05; agar, 15; pH 7.0 ± 7.2 supplemented with 100 mg/L of eugenol as sole carbon and energy source to obtain strains capable of utilizing eugenol at 37 °C for 24 h (Muheim and Lerch 1999). Morphologically different microorganisms were selected from master plates and transferred on minimal agar media with eugenol (100 mg/L) by quadrant streak plate technique to obtain the cultures which showed the capacity to utilize eugenol as a sole carbon source for growth and energy in pure form. Isolates were further sub cultured and maintained for future use in minimal medium slants at 4 °C with eugenol.

2.3. Screening of transforming strain

The most effective one step screening method was adopted. Cells were cultured in a 100-ml flask containing 25 ml of sterile (BT) medium. Starter cultures of selected bacterial isolates were prepared individually from stock cultures and incubated at 37 °C for 24 h followed by the transfer of 1 ml of inoculum from selected starter cultures individually to sterilized BT

medium supplemented with filter-sterilized eugenol (100 mg/L) as a sole carbon source. These were incubated upto 144 h at 37 °C initially under static condition. After the incubation, each isolate inoculated was processed individually. The supernatant was acidified with conc. HCl to pH 2 and extracted using ethyl acetate in a ratio of 1:1. Phase separation was followed by the collection of upper organic phase which was then concentrated in Rotary Vacuum Evaporator to dryness under vaccum at 30 °C. Dried samples were resuspended in 1 ml of aqueous methanol (50% v/v). Both qualitative and quantitative analysis of the processed sample was performed.

2.4. Phenotypic and genotypic characterization of transforming strains

Based on the HPLC analysis, the strain showing significant yield (mg/L) of desired metabolites was selected and characterized at morphological, biochemical and molecular levels. Genus identification was based on Bergey's Manual of Determinative Bacteriology (Holt et al. 1994). Morphological characterization involved Gram staining, endospore staining, motility, colony shape, size, and color as well as growth on nutrient agar and MacConkey agar plate. Biochemical tests performed for gelatinase, amylase, catalase, and oxidase activities. Other biochemical tests included indole production, Voges-Proskauer, carbohydrate fermentation, methyl red, citrate utilization, hydrogen sulfide production, nitrate reduction, Tween 20 hydrolysis, and Tween 80 hydrolysis. Species identification was done using 16S ribosomal RNA (rRNA) gene sequencing (Xcelris Labs Ltd. Ahmedabad, India). BLASTN (http://www.ncbi.nlm.nih.gov/Blast) program was used for the homology searches of the resulting consensus sequence. Molecular evolutionary genetics analysis (MEGA) software version 6.0 was used for alignment, phylogenetic and molecular evolutionary analyses by using the neighbor-joining method. Reliability of the phylogenetic tree was tested using bootstrap analysis based on 1000 replicates (Saitou and Nei 1987).

2.5. Quantitative analysis using HPLC

Quantification was based on the HPLC profile of methoxyphenol derivatives obtained during eugenol metabolism. A Waters binary HPLC pump (Waters 1525) equipped with Waters photodiode array detector (PDA Waters 2996) was used, Waters 717 plus Autosampler and Waters Empower login software were used for instrument control and data handling. A sample of 25 ml culture broth obtained after optimized days of incubation was centrifuged (1000 rpm, 10 min, 4 °C) and filtered using 0.22 μm syringe filters. Samples were injected on a Kinetex C18 reverse phase HPLC column (250 × 4.6 mm, 5 μm particle size; Phenomenex, Torrance, CA, USA) keeping injection volume 20 μl and a constant flow rate of 1 ml/min. Separation of samples and standard solutions was performed at room temperature. Sample elutions were performed using mobile phases consisting of 1 mM trifluoroacetic acid in water (Solvent A) and methanol (Solvent B). The elution gradient optimized was: t = 0–20 min: 30% B; t = 25 min: 70% B; t = 40–45 min: 100% B followed by the equilibration of the column for 10–20 min. Total run time was 45 min. Ambient column temperature followed by two detection wavelength of 254 and 310 nm was used. Detection wavelength of 310 nm was used for vanillin, coniferyl aldehyde, ferulic acid and that of 254 nm was used for eugenol, vanillic acid, and protocatechuic acid. Comparative analysis of the analytes present in the samples and their respective reference standards were performed based on their retention times and PDA spectra.

2.6. Identification of methoxyphenol derivatives

Metabolites produced by *B. safensis* strain SMS1003 using eugenol were confirmed through a PDA scan, Fourier-transform infrared spectroscopy (FTIR), and mass spectroscopy. Waters HPLC coupled with a photodiode array detector was used for the spectral scan in the region of 200–400 nm. Identification of the compounds was based on the comparison of maximum adsorption of the identified compound with that of its purified standard compounds. FTIR spectra were recorded using a Shimadzu Corporation FT/IR-Prestige 21, Japan in the region 4000–400 cm^{-1} with KBr pellet technique. Mass spectra were recorded using Thermo Scientific LTQ XL ion trap mass spectrometer. Spectra were generated in positive ion mode at 30 eV. Continuum mode across the range from 100 to 1000 m/z was used to record the mass spectra of metabolites obtained during biotransformation and compared with those of its authentic standard compounds as references.

2.7. Optimization of culture conditions for effective eugenol biotransformation

Lab scale experiments were carried out in Erlenmeyer flasks and setup was designed for the optimization of

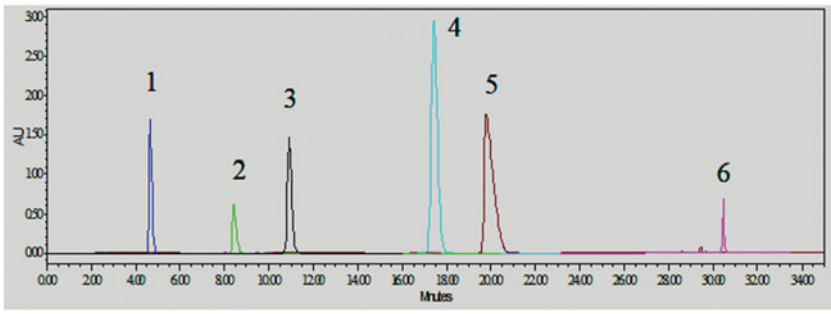

Figure 1. Overlay profile of HPLC indicating authentic standards of protocatechuic acid (Peak 1) at 310 nm vanillic acid (Peak 2) at 254 nm, vanillin (Peak 3) at 310 nm, ferulic acid (Peak 4) at 310 nm, coniferyl aldehyde (Peak 5) at 310 nm, and eugenol (Peak 6) at 254 nm. HPLC, high-performance liquid chromatography.

various process parameters (Environmental and nutritional) using the classical approach of one factor at a time. Different environmental parameters investigated were effect of substrate concentration (100–1000 mg/L), temperature (28, 30, 37, and 45 °C), initial pH (5.0–9.0), inoculum volume (4%–16%), volume of the culture medium (10%–30%), and shaking speed (0–200 rpm). Similarly, the effect of various carbon and nitrogen sources were investigated as the nutritional parameters on the biotransformation of eugenol by *B. safensis* SMS1003 (Tilay et al. 2010). To study the effect of various nutritional parameters, BT media was supplemented with various additional carbon sources like glucose, sucrose, and glycerol at the concentration of 1% and organic nitrogen sources like beef extract and peptone as well as inorganic nitrogen sources like ammonium sulphate and ammonium chloride at the concentration of 0.05%.

2.8. Preparation of resting cells

To enhance the molar yield of vanillin further using eugenol, the resting cell reaction assay was performed. Cells of *B. safensis* SMS1003 were cultured in modified biotransformation (MBT) medium and were centrifuged (10,000 rpm, 10 min, 4 °C) at the late-exponential phase of growth. Harvested cells were then washed twice with cold sodium phosphate buffer (100 mM, pH 7.0) and then pelleted cells were resuspended in the same buffer at a concentration of about 3 g (dry cell weight)/L and used as resting cell culture for all experiments. The biotransformation was conducted in 250 ml flasks with 25 ml of resting cell preparation in the presence of a sterile eugenol solution (500 mg/L) and incubated at 37 °C and 180 rpm for 24–96 h. A Similar experiment was carried out for the control containing 25 ml phosphate buffer without resting cells under the same testing conditions. Experiments were performed in triplicate.

2.9. Biotransformation by cell free extract

Cells were allowed to grow in the presence of eugenol for 24 h (37 °C, 180 rpm, in MBT medium). Harvesting and washing of cells were performed according to the previously reported method (Giedraityle and Kalediene 2014). Washed cells were then disrupted by ultrasonic treatment at 22 kHz in short bursts of 30 s (5 min, titanium probe with tip diameter 13 mm, 35% cycle, 4 °C (VCX 750 Sonics and Materials Inc., Newton, CT, USA). The soluble fraction of the cell free extract was obtained by centrifugation (15,000 rpm, 20 min, and 4 °C). The Resulting supernatant was further concentrated using Amicon Ultra-0.5 CFU (Merck Millipore, Burlington, MA, USA) membrane and used as cell free crude extract for *in vitro* enzyme assays.

In vitro eugenol bioconversion were examined using cell free extract preparation at 37 °C. A reaction mixture of 1.5 ml was prepared to check the *in vitro* conversion of eugenol containing 200 µl of cell extract, 1.8 mM eugenol, and 1.3 mM nicotinamide adenine dinucleotide (NAD^+), 100 mM sodium phosphate buffer pH 7.0. A Similar analysis was carried out to check the *in vitro* conversion of coniferyl aldehyde by supplementing the reaction mixture with 1.3 mM NAD^+ and replacing eugenol with the coniferyl aldehyde. Ferulic acid bioconversion was also tested using an *in vitro* condition reported earlier (Ghosh et al. 2007). All the reaction mixtures were incubated for 12 h and stopped using an equal volume of acetic acid: methanol prepared in the ratio of 1:4.

3. Results

3.1. Isolation and screening of eugenol transforming strain

In the current study total sixteen morphologically different bacterial colonies were isolated using the soil samples collected from an *Ocimum* sp and *Petunia* sp cultivated field, as both the species contains about

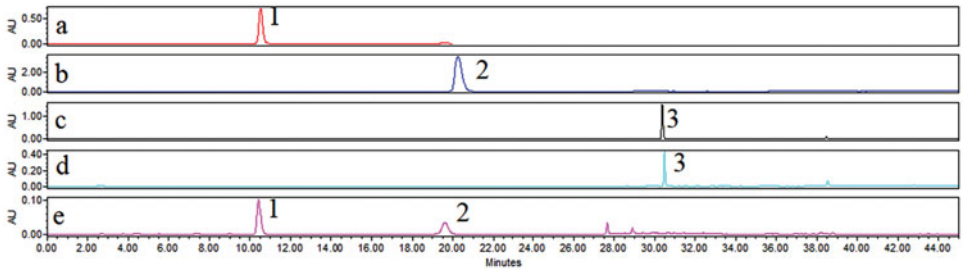

Figure 2. Stack plot of HPLC at 310 nm and 254 nm showing eugenol biotransformation in processed culture filtrate of *Bacillus safensis* SMS1003. Chromatogram a and b represents standard vanillin (1) and coniferyl aldehyde (2) at 310 nm. Chromatogram c represents control containing standard eugenol (3) at 254 nm. Chromatogram d (3) represents eugenol utilization in a filtrate processed at 96 h of eugenol biotransformation at 254 nm. Chromatogram e represents the formation of vanillin (1) and coniferyl aldehyde (2) in a filtrate processed at 96 h of eugenol biotransformation at 310 nm. HPLC, high-performance liquid chromatography.

75% phenylpropanoids including eugenol and isoeugenol (Kasana et al. 2007). The isolates which were found capable of utilizing eugenol at the concentration 100 mg/L as a sole carbon and energy source were further examined for their ability to transform eugenol into other valuable methoxyphenol derivatives using a developed method of RP-HPLC. The retention times of authentic standards obtained in the present developed method were as follows: protocatechuic acid, 4.6 ± 0.1 min; vanillic acid, 8.1 ± 0.1 min; vanillin, 9.9 ± 0.5 min; ferulic acid, 17.3 ± 0.7 min; coniferyl aldehyde, 20.1 ± 0.4 min; eugenol, 31.8 ± 0.1 min; (Figure 1). Various methoxyphenol derivatives produced during eugenol biotransformation were then calibrated with the external standards for quantification. Each experiment was performed in a set of three and results were expressed as the mean ± standard deviation. Methoxyphenol derivatives like coniferyl aldehyde, ferulic acid, vanillin, and protocatechuic acid were detected as the biotransformed products using HPLC (Supporting Information Table S1). Under the given conditions, among the eugenol degrading microorganisms isolated from soil, a bacterial stain designated as SMS1003 produced a significant amount of both coniferyl aldehyde and vanillin after 144 h of incubation (Figure 2).

Products were confirmed using PDA spectral analysis (Figure 3) and mass spectral analysis (Figure 4). In comparison to other isolates, strain SMS1003 was chosen for further optimization studies keeping in view relatively the low productivity of vanillin using eugenol due to its potential oxidation into vanillic acid as well as the toxicity of eugenol (Overhage et al. 1999).

3.2. Characterization of the strain SMS1003

Microbial characteristics of strain SMS1003 as studied are enlisted in Table 1. Phylogenetic analysis was performed for the sequence similarity search of 16S rRNA sequence of strain SMS1003. The sequence showed 99% similarity with *B. safensis* strain NBRC 100820 (Figure 5). Strain SMS1003 was identified as *B. safensis* based on its phenotypic and genotypic characteristics. The 16S rRNA sequence of SMS1003 was submitted to GenBank under accession number, MG561863.

3.3. Biotransformation using growing culture of *B. safensis*

Growing culture of selected bacterial strain SMS1003 was examined to biotransform eugenol into valuable methoxyphenol derivatives initially at the concentration of 100 mg/L. As analyzed by HPLC, coniferyl aldehyde was initially detected in the processed culture supernatant after 24 h of incubation. Vanillin was formed after 48 h of incubation. Both coniferyl aldehyde and vanillin were accumulated in the BT medium at the concentration of 0.07 mg/L (Molar yield 0.06%) and 0.08 mg/L (Molar yield 0.09%) respectively, at 144 h of incubation under un-optimized culture conditions. Eugenol transformation was not observed in control (un-inoculated media) which was incubated under the same conditions.

3.4.1. Effect of substrate concentration

The effects of different concentration of eugenol were investigated on the biotransformation of eugenol as shown in Figure 6. The selected bacterial strain of *B. safensis* SMS1003 was inoculated in 25 ml of nutrient broth to prepare its starter culture and allowed to grow for 24 h. Experimental runs were then carried out by supplementing the BT medium with eugenol at the concentrations of 100, 300, 500, 700, and 1000 mg/L, inoculated with 4% v/v of the starter culture followed by incubation at 37 °C for 24–144 h. It was found that increased eugenol concentration in the range of 100–500 mg/L proximately increased the concentration of both vanillin and coniferyl aldehyde in

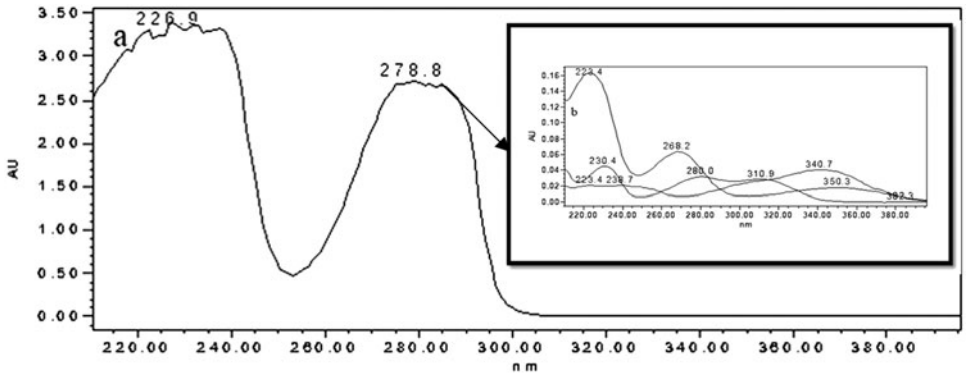

Figure 3. PDA spectral scan in the range of 200–400 nm of a processed culture filtrate obtained using growing culture of *Bacillus safensis* SMS1003 (a) A control containing eugenol at 0 h of incubation (b) Shift in the scan due to eugenol biotransformation into vanillin and coniferyl aldehyde using growing culture of *B. safensis* at 96 h of incubation. PDA, photodiode array detector.

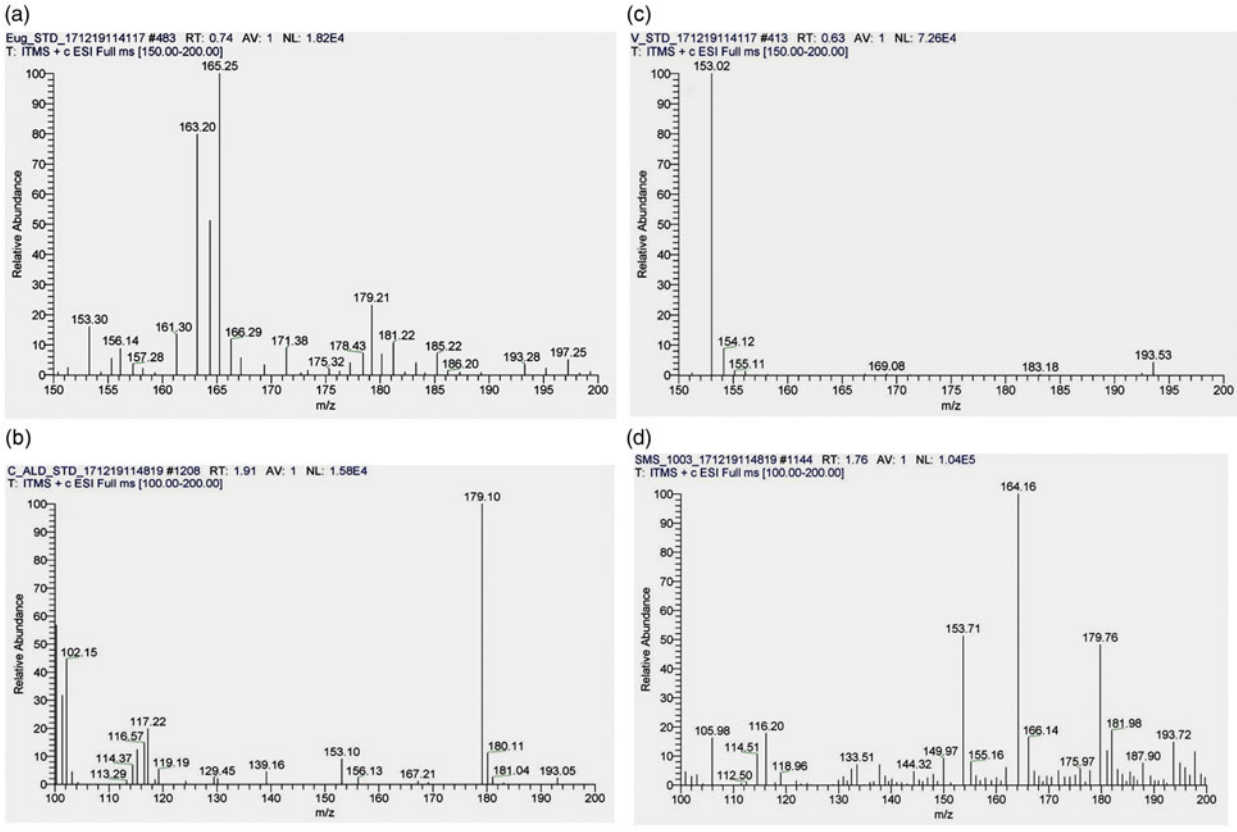

Figure 4. Mass spectra of authentic standards and sample generated in ESI positive ion mode for the detection of eugenol metabolites. (a) Standard eugenol (b) Standard coniferyl aldehyde (c) Standard vanillin (d) Processed sample. CALD, coniferyl aldehyde; ESI, electrospray ionization; EU, eugenol; VAN, vanillin.

the culture medium. However, a further increase in the concentration of eugenol decreased the concentration of both the derivatives, which may be due to the substrate toxicity at the higher concentration or due to the poor solubility of eugenol in water as it is found to be lypophilic in nature (Ashengroph et al. 2011). Moreover, vanillin toxicity was also reported to organisms at high concentrations (Chen et al. 2016). Due to the negative effect of both eugenol and vanillin at higher concentrations on the growth and metabolism of microorganism, eugenol concentration of 500 mg/L was chosen as the optimum substrate concentration to promote growth and biotransformation efficiency of *B. safensis* SMS1003. Under the optimum substrate concentration of 500 mg/L, vanillin and coniferyl aldehyde were accumulated with the molar yield of 0.13% and 0.09%, respectively at 96 h.

Table 1. Morphological and biochemical characteristics of strain SMS1003.

Tests	Observation	Tests	Observation
Colony morphology		Biochemical tests	
Configuration	Round	Growth on MacConkey agar	−
Margin	Irregular margin	Indole	−
Elevation	Undulate	Nitrate test	−
Surface	Smooth	Voges proskauer test	±
Density	Non-luminescent	Citrate utilization	−
Gram reaction	Positive	Starch hydrolysis	+
Shape	Rods	Casein hydrolysis	+
Size	Moderate	Lipid hydrolysis	+
Arrangement	Single	Tween 20 hydrolysis	−
Motility	+	Tween 80 hydrolysis	−
Endospore formation	+	H_2S production	−
Growth at the temperature (°C)		Oxidase	+
4	+	Catalase	+
10	+	Oxidation/fermentation	O
20	+	Gelatin liquefaction	−
30	+	Acid production from	
37	+	D-Glucose	+
45	+	Ribose	+
55	−	Galactose	+
Growth at pH		Fructose	+
4	−	Maltose	+
5	+	Mannitol	+
6	+	Sucrose	+
7	+	Glycerol	+
8	+		
9	+		
Growth on NaCl (%)			
2.5	+		
5	+		
7.5	+		
9	+		
10	+		
15	+		

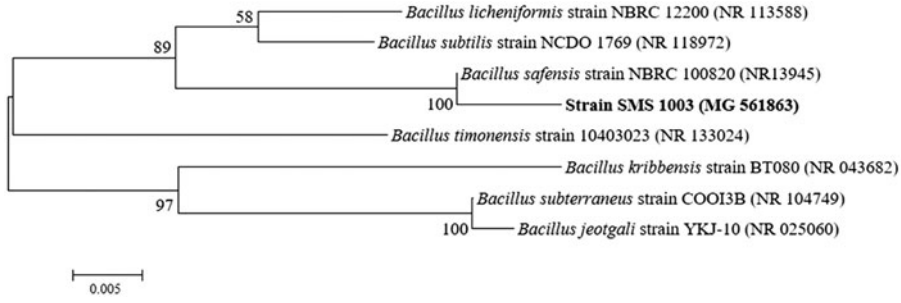

Figure 5. Phylogenetic tree indicating the estimated relationship of strain SMS1003 to other *Bacillus* sp. based on 16S rRNA gene sequence similarity. Bootstrap values (expressed as percentages of 1000 replications) greater than 50% are shown at the branch points. Scale bar, 0.005 substitutions per nucleotide position.

3.4.2. Effect of incubation temperature

Figure 7(a) depicts the effect of four different incubation temperatures (25, 30, 37, and 45 °C) on eugenol biotransformation by *B. safensis* SMS1003. At incubation temperatures 30 and 37 °C, the amount of vanillin and coniferyl aldehyde obtained was 0.59 mg/L and 0.47 mg/L respectively. Whereas the concentration of both the metabolites decreased when the temperature was below 30 °C and beyond 45 °C.

3.4.3. Effect of pH

Since pH has a profound effect on microbial growth as well as regulation of enzymes involved in biotransformation processes (Zhao et al. 2005), the effect of varying pH conditions were studied on the eugenol biotransformation by *B. safensis* and represented in Figure 7(b). A significant increase in the concentration of both vanillin and coniferyl aldehyde was observed when the initial pH of the medium was 7. pH 7 was selected as the optimum pH for the growth and eugenol metabolism by *B. safensis* SMS1003.

3.4.4. Effect of inoculum size

The effect of different inoculum sizes was investigated on the biotransformation of eugenol by *B. safensis*

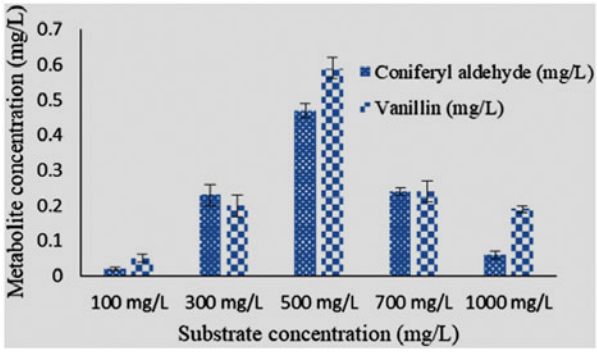

Figure 6. Biotransformation of eugenol by *Bacillus safensis* SMS1003 using different substrate concentration. Results are expressed as means of at least three experiments and the error bars indicate standard deviations.

SMS1003 as shown in Figure 7(c). Inoculum sizes were varied from 4% to 16% and 4% was chosen as the optimum for further optimization studies.

3.4.5. Effect of carbon and nitrogen sources

BT media supplementation with additional carbon sources increased the microbial growth but did not show any biotransformation (Tilay et al. 2010). It can be inferred from the results that the selected strain was not able to utilize eugenol once exposed to different carbon sources in the BT media. As eugenol plays a vital role as an inducer of the enzymatic machinery involved in microbial transformation, no biotransformation was observed in media supplemented with glucose. Presence of beef extract and ammonium sulphate (data not shown) as the additional nitrogen sources in the media increased the vanillin and coniferyl aldehyde concentration up to 5.5 and 4 mg/L, respectively.

3.4.6. Effect of media volume and agitation

One of the most important factors is oxygen concentration which not only affects the growth of the microorganisms but also influences the biotransformation efficiency (Chen et al. 2016). Since oxygen concentration is influenced by the media volume and agitation condition, the effect of varied culture media volume (%) and various shaking speeds were studied on the biotransformation of eugenol by SMS1003 and graphically represented in Figure 7(d) and Figure 7(e). Results imply that vanillin was accumulated at a maximum concentration of 36 mg/L by using 10% volume of culture medium. Further increase in the percentage volume decreased the biotransformation efficiency. It was also observed that incubation of the 10% volume of the inoculated culture media at a shaking speed of 180 rpm significantly increased the concentration of vanillin up to 55 mg/L (Molar yield 10.7%). Therefore, the following optimized parameters were found to show a significant improvement in vanillin production: temperature, 37 °C; initial pH, 7.0; inoculum volume, 4%; eugenol concentration, 0.5 g/L; the volume of culture medium, 10%; and shaking speed, 180 rpm.

3.5. Biotransformation using resting cells of *B. Safensis* SMS1003

Biotransformation of eugenol was also studied using the resting cells of *B. safensis* strain SMS1003 in an optimized conversion condition. In the present study, use of resting cells resulted in a higher production of vanillin and was detected as the single major metabolite in the culture filtrate obtained during eugenol biotransformation shown in Figure 8. Product confirmation was achieved using PDA spectral analysis (Figure 9; Supporting Information Figure S1) and FTIR spectroscopy (Supporting Information Figure S2). No biotransformation was observed in the control culture supplemented with eugenol at the concentration of 500 mg/L, which was incubated under the same testing condition. It was also reported in the previous studies that biomass under non proliferating conditions (resting cells) can be used in a recycled form for at least four times without the loss of bioconversion ability (Torre et al. 2004). The initial eugenol concentration was decreased with an increase in the vanillin concentration and biomass of *B. safensis* SMS1003 as shown in Figure 10. Vanillin concentration reached up to 120 mg/L (Molar yield 26%) at 96 h of incubation. Vanillin was the single metabolite obtained and was found to be stable in the buffer for at least 10 h.

3.6. Metabolic pathway of eugenol in SMS 1003

To confirm the metabolic pathway of eugenol biotransformation by the selected strain of *B. safensis* SMS1003 possible pathway intermediates such as coniferyl alcohol, coniferyl aldehyde, and ferulic acid were added into the BT media at the concentration of 100 mg/L. Bacterial strain was able to grow in the presence of all the mentioned intermediates. No change was observed in the concentration of intermediates prepared as a control. Analyses of the degradation products formed using various intermediates were also carried out after 48–96 h of incubation. Formation of vanillin was observed in a processed culture media containing ferulic acid at 48 h of incubation. Coniferyl aldehyde was found to get biotransformed into ferulic acid after 48 h of incubation. Vanillin degradation product(s) such

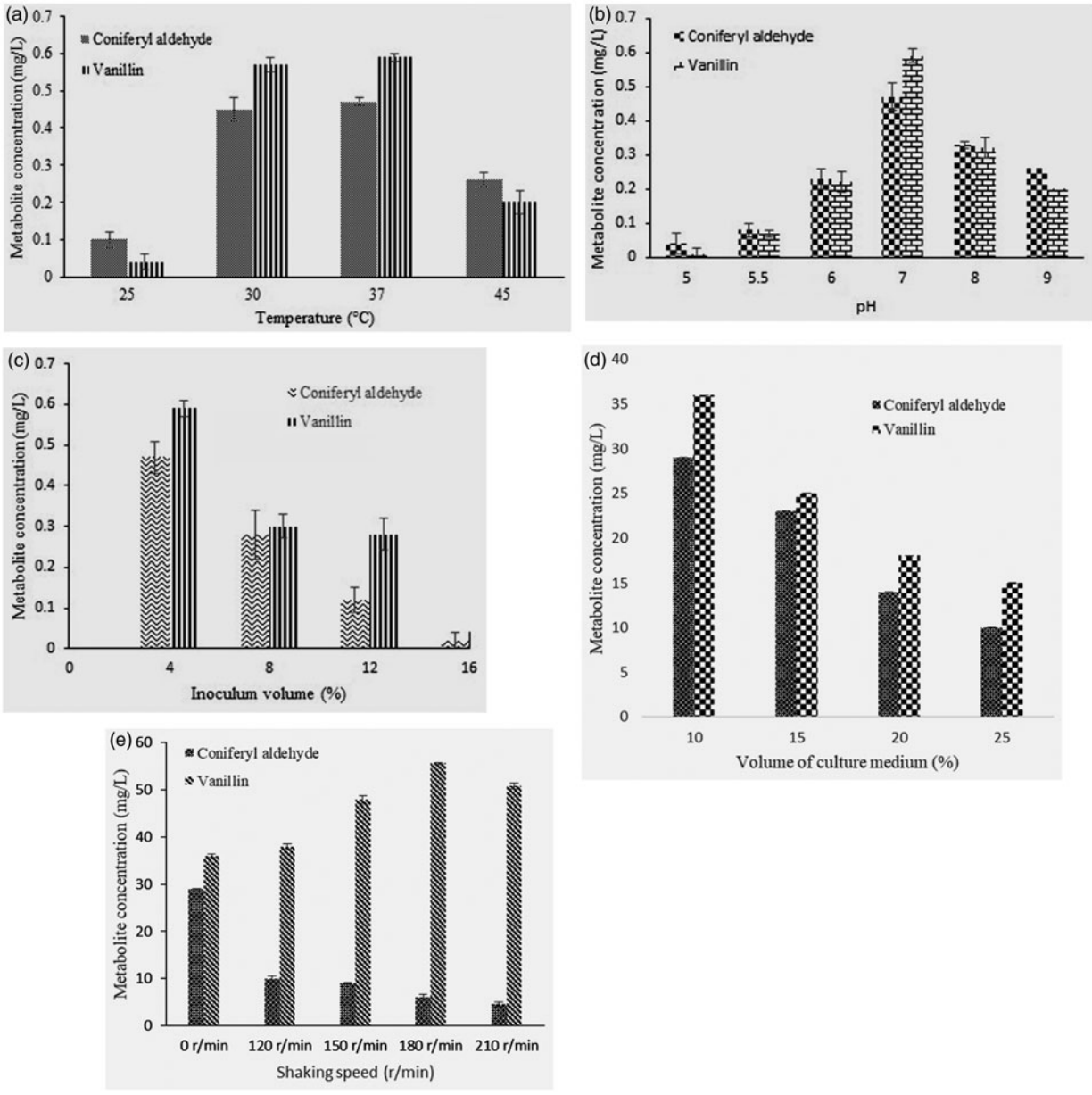

Figure 7. Effect of different culture parameters on biotransformation of eugenol into coniferyl aldehyde and vanillin (mg/L) at 96 h of incubation by *Bacillus safensis* SMS1003. (a) Effect of different incubation temperature on the concentration of coniferyl aldehyde and vanillin (mg/L). (b) Effect of pH on the concentration of coniferyl aldehyde and vanillin (mg/L). (c) Effect of inoculum volume on the biotransformation of eugenol by *B. safensis* SMS1003. (d) Effect of volume of the culture medium on the biotransformation of eugenol by *B. safensis* SMS1003. (e) Effect of shaking speed on the biotransformation of eugenol by *B. safensis* SMS1003. Triplicate assays were performed and the vertical error bars indicate standard deviations.

as vanillic acid, vanillyl alcohol, protocatechuic acid were not detected. These observations suggested vanillin as the end product of eugenol metabolic pathway followed by *B.safensis* SMS1003 via intermediates like coniferyl aldehyde, ferulic acid, and vanillin (Supporting Information Figure S3).

3.7. Biotransformation by cell free extract

Soluble cell free extract preparations were checked for the presence of major enzymes involved in the catalysis of biotransformation process in *B. safensis* SMS1003. Since methoxyphenol derivatives like coniferyl aldehyde and vanillin were detected as the major metabolites, hence, assays were performed for previously reported enzymes of eugenol metabolic pathway such as eugenol dehydrogenase, coniferyl aldehyde dehydrogenase, feruloyl CoA synthetase. Growing cells of *B. safensis* was found to transform eugenol into vanillin via coniferyl aldehyde. Ferulic acid was not detected as the intermediate in the reaction mixture processed after eugenol

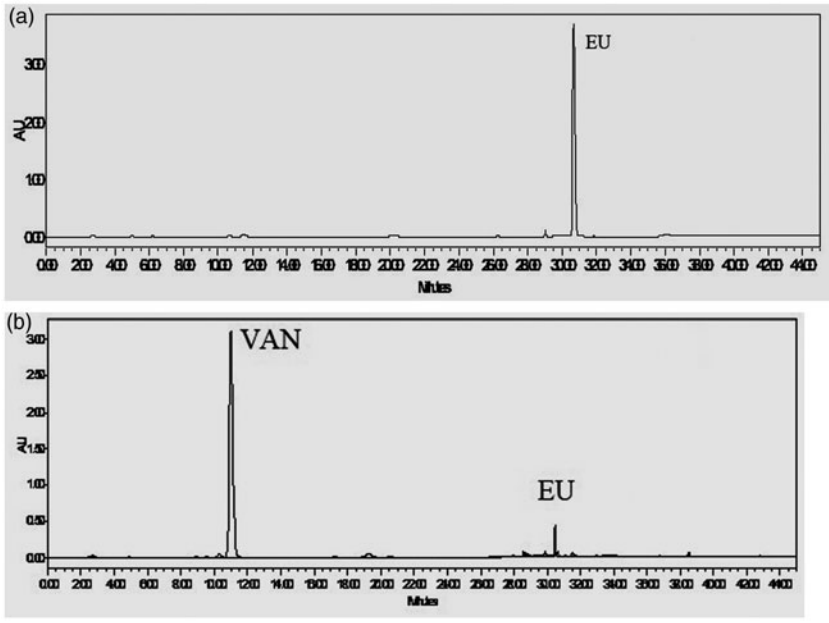

Figure 8. HPLC chromatograms at 254 nm and 310 nm obtained during eugenol (500 mg/L) biotransformation by resting cells of *Bacillus safensis* strain SMS1003. (a) HPLC profile of control at 254 nm. (b) Overlay of HPLC profile at 310 and 254 nm obtained using sample culture filtrate processed at 96 h of incubation. HPLC, high-performance liquid chromatography.

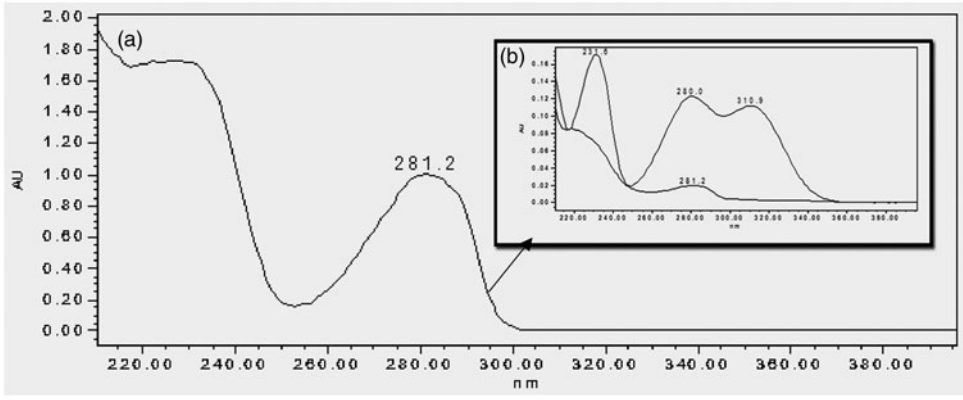

Figure 9. PDA spectral scan in the range of 200–400 nm of a processed culture filtrate obtained using resting cells of *Bacillus safensis* SMS1003. (a) A control containing eugenol at 0 h of incubation. (b) Change in the absorbance of eugenol due to vanillin formation at 96 h of incubation using resting cells of *B. safensis*. PDA, photodiode array detector.

biotransformation using growing culture of *B. safensis*. But cell free extract incubated with eugenol has shown both the consumption of eugenol and its biotransformation into coniferyl alcohol and ferulic acid after 12–14 h. Again when the extract was incubated with coniferyl aldehyde, its biotransformation into vanillin was observed. Results indicated the active presence of both eugenol dehydrogenase as well as coniferyl aldehyde dehydrogenase in the cell free extract. When vanillin was used as the substrate, its major degradation product(s) such as vanillic acid, protocatechuic acid was not detected, only change in the absorbance of vanillin was observed as compared to control. From the above results, it is evident that enzymatic catalysis was present in the obtained cell free extract containing a mixture of crude enzymes involved in the pathway followed by *B. safensis* SMS1003. In the present work, the whole microbial cell was preferred for biotransformation as it is considered as an important and efficient tool for microbial biotransformation for large scale synthesis as compared to enzymatic catalysis (Ruiz-Teran et al. 2001; Serra et al. 2005; Xu et al. 2007).

4. Discussion

Due to the vital role of microorganisms in the production of natural substituted methoxyphenols like

vanillin, sixteen bacterial isolates were isolated and screened on the basis of their ability to utilize and biotransform eugenol as a sole carbon source. The isolate SMS1003 which showed reasonable vanillin production was selected for further optimization studies. Production of several substituted methoxyphenols from eugenol using microbial transformation has been reported and is summarized in Table 2. In many of the above biotransformation processes, vanillin was detected in trace amounts and its accumulation was followed by other intermediate byproducts. It was also reported that bioconversion based vanillin production is unstable, as it is utilized as a sole carbon and energy source by some microorganisms. Due to the toxicity of vanillin at higher concentration, soon after accumulation, it gets oxidized to vanillic acid by the microorganisms (Di et al. 2011). So taking into consideration the ineffective conversion of eugenol into vanillin as well as its instability and reactivity, *B. safensis* SMS1003 was screened as an efficient vanillin producer from eugenol at the initial concentration of 100 mg/L. On the basis of previous reports, selected environmental and nutritional parameters were optimized to improvise the yield (Satomi et al. 2006; Tilay et al. 2010). Eugenol concentration of 500 mg/L was found to be the optimum substrate concentration for the selected bacterial strain. *B. safensis* is a mesophilic organism that can grow over the temperature range of 20–40 °C and pH range of 5–9 (Satomi et al. 2006). In the present study, an incubation temperature of 37 °C and pH 7 was found optimum both for its growth and biotransformation ability. For the optimization of nutritional parameters, earlier studies have revealed that supplementation of the media with co-substrate as additional carbon and nitrogen sources helped in augmenting the product yield (Rabenhorst 1996). Importance of nitrogen sources like yeast extract, tryptone, casein hydrolysate, beef extract, and peptone were demonstrated so far to enhance vanillin production (Torre et al. 2004; Tilay et al. 2010). In the present study, only beef extract and ammonium sulphate as additional nitrogen sources enhanced the concentration of products in the media. Further, media volume of 10% and agitation condition of 180 rpm raised the vanillin concentration rapidly up to 96 mg/L at 96 h of incubation. A Similar effect was highlighted earlier on the bioconversion ability of *Escherichia coli* JM109 (pBB1; Torre et al. 2004). Optimized culture conditions

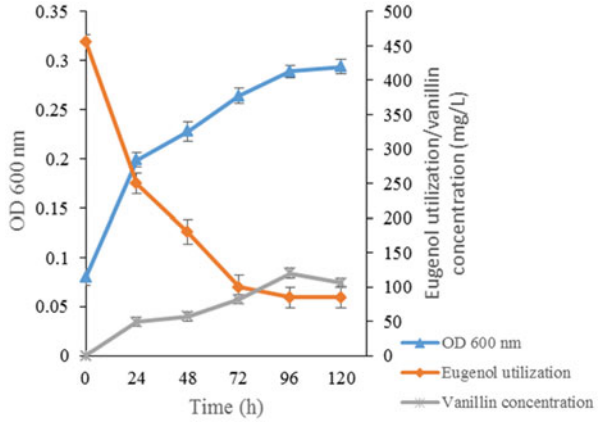

Figure 10. Biotransformation kinetics of eugenol by *Bacillus safensis* resting cells. Experiments were performed in 250-ml flasks containing 25 ml of sodium phosphate buffer (pH 7.0) at 37 °C for 96 h with shaking at 180 rpm. Results expressed as mean ± SD.

Table 2. Biotransformation of eugenol to various methoxyphenol derivatives by microorganisms.

Microorganisms	Metabolites detected	Vanillin concentration (mg/L)	References
Corynebacterium sp.	Ferulic acid, vanillin, vanillic acid, protocatechuic acid, and keto-adipic acid	Trace amount	Tadasa and Kayahara (1983)
Pseudomonas sp.	Coniferyl alcohol, ferulic acid, vanillin, and vanillic acid	280	Washisu et al. (1993)
Pseudomonas sp.	Coniferyl alcohol, ferulic acid, and vanillic acid	Trace amount	Rabenhorst (1996)
P. putida	Ferulic acid, vanillin, and Svanillyl alcohol	Trace amount	Muheim and Lerch (1999)
Pseudomonas sp. HR199 (genetically modified)	Ferulic acid, coniferyl alcohol, coniferyl aldehyde, ferulic acid, vanillin, and vanillic acid	440	Priefert et al. (1999)
Escherichia coli (genetically modified)	Coniferyl alcohol, coniferyl aldehyde, ferulic acid, and vanillin	300	Overhage et al. (2003)
P. nitroreducens Jin1	Coniferyl alcohol, ferulic acid, vanillin, and vanillic acid	Trace amounts	Unno et al. (2007)
Bacillus cereus PN24	4-vinyl guaiacol, vanillin, vanillic acid, protocatechuic acid, and keto-adipic acid	Not detected	Kadakol and Kamanavali (2010)
P. resinovorans SPR1	Ferulic acid, coniferyl alcohol, coniferyl aldehyde, ferulic acid, vanillin, and vanillic acid	240	Ashengroph et al. (2011)
Bacillus sp.	Vanillin	0.32	Sindhwani et al. (2012)

of eugenol metabolism by the growing cultures of *B. safensis* SMS1003 increased vanillin concentration but also displayed residual amounts of coniferyl aldehyde. Hence to achieve vanillin as the exclusive metabolite, resting cells of *B. safensis* SMS1003 were prepared in optimized culture conditions and evaluated for vanillin production. Since the age of inoculum is considered as a decisive factor for biotransformation, cells in the late exponential growth phase were selected as inoculum. Current work also evidenced the role of prior substrate induction to the microbial culture enhanced the product yield (Rivas Torres et al. 2009). Similar experiments were performed using resting cells of *Pseudomonas resinovorans* SPR1 (Ashengroph et al. 2011) and *Bacillus* sp. (Sindhwani et al. 2012) for enhanced vanillin production from eugenol. The present study proposed the efficient and stable vanillin production of 120 mg/L (Molar yield 26%) as a single metabolite at 96 h using the resting cells of nature isolated *B. safensis* SMS1003 in optimized culture conditions.

5. Conclusion

The proposed work aimed at the isolation, screening, and characterization of potential bacterial strain capable of showing the transformation of eugenol into valuable methoxyphenol derivative like vanillin. Attempts were made to enhance the yield of vanillin using controlled and optimized culture conditions. Due to emerging market demand of biotechnological based vanillin production using the natural raw material, eugenol was used as a potential precursor for biotransformation. The prime goal of the current work was to optimize the yield of vanillin using a selected bacterial strain of SMS1003. The strain was identified as *B. safensis* by phenotypic characterization and 16S rRNA analysis. Our results showed that an appreciable amount of vanillin was achieved using a nature isolated novel strain of *B. safensis* SMS1003. In contrast to other microbial species reported so far (as discussed above), vanillin accumulated as the major end product without any other intermediates in the media. By far, current biotransformation study for vanillin production is evidenced for the first time using *B. safensis* SMS1003. An attempt is being made to optimize the feasibility for large-scale production of vanillin using *B. safensis* SMS1003. Identification and characterization of the genes through the proposed metabolic pathway would help in the metabolic engineering of this industrially important bacterium.

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Disclosure statement

The authors declare that they have no conflicts of interest.

Ethical approval

This article does not contain any studies with human participants or animals performed by any of the authors.

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