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ORIGINAL ARTICLE

Bioconversion of ferulic acid to vanillic acid by *Paenibacillus lactis* SAMS-2001

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Abstract Ferulic acid is an abundant cinnamic acid derivative found in the plant world and has been utilized by microorganisms to produce value-added compounds such as vanillin and vanillic acid. The isolate SAMS-2001, giving the highest vanillic acid yield, was selected and identified as *Paenibacillus lactis* based on its 16S rDNA sequence (GenBank accession number KF699133). Vanillic acid was found to accumulate in the minimal medium as the major product along with the transient formation of vanillin by adding yeast (0.05 %) as co-substrate. In vitro conversion of ferulic acid to vanillin and vanillic acid was also studied with the cell extract of *Paenibacillus lactis* SAMS-2001. This study gives the first evidence for production of vanillic acid (57.3 % molar yield) from ferulic acid within 18 h using *Paenibacillus lactis* SAMS-2001.

Keywords Bioconversion · Ferulic acid · *Paenibacillus lactis* · Vanillin · Vanillic acid

Introduction

Flavours and fragrances are regularly exploited in the food, cosmetic, chemical, and pharmaceutical industries. Various value-added compounds are obtained by chemical synthesis or by extraction from plant and animal sources. The negative aspect of chemical synthesis is that the method is not environmentally friendly and that the desired compounds often occur as unwanted racemic mixtures (Longo and Sanroman 2006). In contrast, natural value-added phenolic compounds are often present in animal and plants at low concentrations, making isolation and purification very costly (Walton et al. 2000; Zamzuri et al. 2014). In order to fulfill consumer demand for natural products, a substitute production of natural value-added metabolites by microorganisms is considered. The USA and European legislation have stated that "*natural flavour substances could be prepared by enzymatic or microbial process*" (Serra et al. 2005). Hence, microorganisms are used to produce a variety of value-added compounds through bioconversion. Ferulic acid (FA) or *4*-hydroxy-*3*-methoxy-trans-cinammic acid is an abundant hydroxycinnamic acid in the plant kingdom found in the cell walls of woods, grasses, corn hulls, wheat, maize, and rice bran (Mariod et al. 2010). It is bound by ester or ether linkages to the lignins and/or polysaccharides and is a possible cause for the production of value-added compounds such as vanillin (V) or *4*-hydroxy-*3*-methoxy benzaldehyde and vanillic acid (VA) or *4*-hydroxy-*3*-methoxy benzoic acid (Rosazza et al. 1995; Kaur et al. 2013). Vanillic acid is the derivative of benzoic acid, used as a flavoring agent and starting material in the chemical synthesis of oxygenated aromatic chemicals such as vanillin, one of the most important flavor molecules commonly exploited in foods, beverages, perfumes, and pharmaceutical industries (Rosazza et al. 1995). It is also a possible food preservative (Civolani et al. 2000; Garcia et al. 2005) and may be useful in the treatment of ulcerative colitis and intestinal inflammatory disorders (Kim et al. 2010).

The bioconversion of ferulic acid to vanillic acid as a major product has been studied with a variety of microorganisms such as *Sporotrichum thermophile* (Topakas et al. 2003), *Streptomyces halstedii* (Brunati et al. 2004), *Streptomyces*

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setonii (Longo and Sanroman 2006), *Streptomyces sannanensis* MTCC 6637 (Ghosh et al. 2007), *Halomonas elongata* DSM 2581(Abdelkafi et al. 2008), *Bacillus licheniformis* SHL1 (Ashengroph et al. 2012), and *Halomonas* sp. B15 (Vyrides et al. 2015). This paper reports for the first time on accumulation of vanillic acid in the test medium by an isolated strain of *Paenibacillus lactis* SAMS-2001 upon bioconversion of ferulic acid within 18 h of incubation.

Experimental procedures

Chemicals

Ferulic acid (99 %), vanillic acid (97 %), vanillin (99 %), DTT (99 %), and CoA (96 %) used in this experiment were purchased from Sigma-Aldrich. NAD^+ (95 %), HPLC grade methanol, and formic acid were purchased from Himedia. ATP was purchased from Merck. Other chemicals were of analytical grade.

Medium and culture conditions

For isolation of microorganisms, the serial dilution technique was performed. The soil sample was aseptically collected from the paddy harvested field approximately 5.0 cm below the soil surface. The soil suspension was plated on minimal medium supplemented with ferulic acid (485 mg/l) as sole carbon source. This was followed by 24–84 h incubation at 37 °C, and the colonies obtained were further purified by quadrant streaking on same medium plates. A single colony thus obtained was maintained on minimal medium slants supplemented with ferulic acid as sole carbon source at 4 °C for future work. Minimal medium was prepared by the addition of basal inorganic salts which contained NH_4NO_3 (3.0 g/l) as a nitrogen source, NaCl (0.2 g/l), $MgSO_4.7H_2O$ (0.2 g/l), KH_2PO_4 (1.0 g/l), Na_2PO_4 (1.0 g/l), and $CaCl_2$ (0.05 g/l) (Muheim and Lerch 1999). The pH was adjusted to 7. All the carbon sources were filter sterilized through a 0.20 μm nylon filter (Sartorius, Minisart) before their addition to minimal medium.

Seed culture preparation and screening of isolates transforming ferulic acid to vanillic acid

To screen bacterial cultures with the ability of converting ferulic acid, cells of individual isolates obtained were inoculated into nutrient broth and grown for 24 h at 37 °C for starter culture preparation. Growth was measured as OD at 600 nm. To check the biotransformation capability of the isolate, homogenous starter culture (4 % v/v) of individual isolates was transferred to 25 ml of sterile minimal medium supplemented with 194 mg/l of ferulic acid as sole source carbon and energy. Concurrently, a control experiment was carried out by adding 194 mg/l of ferulic acid in the same medium without bacterial culture. Samples were withdrawn at intervals of 2, 4, and 6 days and were examined by TLC and a UV–vis spectrophotometer (Lambda 25, Perkin Elmer, USA). The isolate with the highest production of vanillic acid was selected for further study.

Analytical procedures

Culture supernatant was acidified (pH 1–2) and extracted with an equal volume of ethyl acetate. The aqueous phase was discarded and the organic phase evaporated by a rotary vacuum evaporator under condensed pressure. The extract was resuspended in 1 ml of methanol (50 % v/v), and spotted to TLC plates. TLC analysis was performed in 2 % aqueous formic acid as the solvent system (Dey et al. 2003). Vanillic acid was directly visualized under a dual wavelength 254/310 nm UV lamp (Uvitech UK). The TLC chromatogram corresponding to standards were detected on the plate and confirmed with overlay of standards by HPLC. Analyses were carried out after 1 and 5 days of incubation for the detection of product using HPLC (Waters, USA). The HPLC conditions were set as follows: column, Symmetry® C_{18} (4.6x150 mm, 5 μm, Waters); mobile phase, 68 % aqueous trifluoroacetic acid (solvent A) and 32 % methanol (solvent B); flow rate, 1 ml/min; injection volume, 20 μl; and detection wavelength, 254 nm (Photodiode Array Detector-2996, Waters). The analyses of samples were performed using the following isocratic method 68 % A and 32 % B, followed by 10 min for equilibration time using a pump (Binary HPLC Pump, Waters). Compounds were identified by comparison of retention time and peak by the standards (Sachan et al. 2004). Vanillic acid was further quantified by a UV–vis spectrophotometer at a wavelength of 260 nm. The concentration values of the samples were extrapolated from the standard curve in the range of 1–15 mg/l.

Identification and characterization of the selected isolate

The culture, giving the highest vanillic acid yield, was selected. Preliminary identification of the isolate was performed by using morphological and biochemical characterizations (Breed et al. 1957). Species identification of the selected isolate was confirmed on the basis of 16S rRNA gene sequencing (Xcelris Labs Pvt. Ltd. Ahmedabad, India). A consensus sequence, thus obtained, was aligned along with the sequence of the type strain by Clustal W (Thompson et al. 1997). The homology of the sequences was studied by BLAST of the National Center for Biotechnology Information (NCBI). Molecular Evolutionary Genetics Analysis (MEGA) software version 5.05 (Hall 2013) was used to construct phylogenetic trees using the neighbor joining method (Saitou and Nei 1987).

Standardization of different culture conditions for enhanced vanillic acid production

The various cultural parameters considered for maximum vanillic acid production are ferulic acid concentration (194, 485, 970 and 1455 mg/l), incubation temperature (28 and 37 °C), pH (4–8), organic nitrogen source (0.05 % w/v yeast extract), agitation (120 rpm), and additional carbon source (0.1 % glucose w/v). To study the effect of each parameter on product formation, samples were withdrawn at fixed intervals and analyzed by TLC and a UV–vis spectrophotometer. Analyses were performed in triplicates. Data are presented as mean±standard deviation. Simultaneously, a control experiment was carried out by adding ferulic acid into the same medium without culture.

Catabolic pathway of ferulic acid degradation

In order to elucidate the metabolic pathway of ferulic acid degradation, the metabolite previously detected in the media of ferulate-grown culture was independently supplied to the medium as sole carbon source (Estrada-Alvarado et al. 2001). Analyses were carried out after 1 and 5 days of incubation for the detection of product by HPLC.

Cell extract preparation

Cells grown on ferulic acid as substrate were harvested during the mid-exponential phase of growth by centrifugation at 10,000 rpm for 20 min in a cooling centrifuge (5804 R, Eppendorf, Hamburg, Germany). The supernatant was removed and the cell pellet washed twice in 50 mM cold Tris–HCl buffer, pH 7.8, and resuspended in the same buffer containing 5 mM dithiothreitol (DTT). All procedures were carried out at 4 °C. The cell suspension was sonicated in a VCX 750 sonicator (Sonics and Materials Inc., USA) with a titanium alloy probe of tip diameter 13 mm and operating at amplitude of 25 %. Sonication was applied in short bursts of 30 s with a total exposure time of 5 min. After sonication, the cell extract was again centrifuged, the resultant supernatant was subsequently collected and concentrated using an Amicon Ultra-15 Centrifugal Filter (Millipore, USA) Membrane. This concentrated elute was used as a crude extract for the in vitro assay of vanillin and vanillic acid (Ghosh et al. 2007).

In vitro conversion of ferulic acid

The ability of cell extracts to convert ferulic acid into vanillate derivatives was examined for 3 h at 37 °C. The assay mixture (1 ml) consisted of 100 mM Tris–HCl buffer, pH 8.5, 0.4 mM ferulic acid, 3 mM ATP, 3 mM $MgCl_2$, 1.3 mM NAD^+, 0.25 mM CoA, and 200 μl of cell extract (Narbad and Gasson 1998). The sample was then analyzed by HPLC for the detection of enzymatic products.

In vitro degradation of vanillin to vanillic acid

Analyses were carried out in the same way as described above except that ferulic acid was replaced by 0.4 mM vanillin in the absence of ATP, $MgCl_2$, and CoA (Narbad and Gasson 1998).

Results

Identification and characterization of the selected isolate

Based on the morphology, different bacterial cultures were obtained and further checked for their ability to produce vanillic acid. Among the isolates, SAMS-2001 was the only microbe which produced the highest amount of vanillic acid. Morphological and biochemical characterizations were performed for this microorganism and compared with a type strain *Paenibacillus lactis* MB 1871^T as described by Scheldeman et al. (2004) in Table 1. Based on the maximum identity score, sequences were selected and aligned using multiple alignment software Clustal W. The phylogenetic tree was constructed using MEGA 5.05 software (Fig. 1). Analysis of the 16S rRNA gene sequence indicated that isolate SAMS-2001 showed a similarity with *Paenibacillus lactis* NR025739 (98 % similarity). The 16S rRNA gene sequence of SAMS-2001 was submitted to GenBank and an accession number was assigned as KF699133 (http://www.ncbi.nlm.nih.gov/nuccore/KF699133).

Bioconversion of ferulic acid by *Paenibacillus lactis* SAMS-2001

In order to examine the capability of *Paenibacillus lactis* SAMS-2001 to biotransform ferulic acid into vanillic acid, a cell suspension (4 % v/v) of screened isolate was inoculated in biotransformation medium containing ferulic acid (194 mg/l) as the sole carbon source. The culture was incubated at 37 °C for a maximum period of 6 days. Thus, the products obtained were further confirmed by HPLC (Fig. 2).

Standardization of culture conditions for enhanced vanillic acid production

The culture of *Paenibacillus lactis* SAMS-2001 was inoculated in minimal medium containing different concentration of ferulic acid (194, 485, 970, and 1455 mg/l) as sole carbon source and energy. Using 485 mg/l of ferulic acid as the sole carbon source, a maximum amount of 157.8 mg/l vanillic acid was observed in the medium on the sixth day of incubation. When 970 and 1455 mg/l of ferulic acid was provided, a very

Table 1 Comparison of morphological and biochemical characteristics of isolated strain *Paenibacillus lactis* SAMS-2001 with a type strain *Paenibacillus lactis* MB 1871^T

Characteristics	*Paenibacillus lactis* SAMS-2001	*Paenibacillus lactis* MB 1871^T
Gram reaction	Gram-positive	Gram-negative or Gram-variable
Shape	Rod	Rod
Endospore	Positive	Positive
Motility	Positive	Positive
Catalase	Positive	Positive
Oxidase	Positive	Positive
Citrate utilization	Negative	Negative
Carbohydrate Fermentation		
D-glucose	Positive/ no gas	Positive/ no gas
D-xylose	Positive	Positive
D-mannitol	Positive	Positive
D-fructose	Positive	Positive
Rhamnose	Negative	Negative
Sucrose	Positive/ no gas	Positive/ no gas
Lactose	Negative	Negative
H_2S production	Negative	Negative
Gelatin liquefaction	Negative	Negative
Methyl Red (MR) test	Negative	Negative
Voges-Proskauer (VP) test	Positive	Negative or weak
Starch hydrolysis	Positive	Positive

little amount of vanillic acid was obtained. Utilization of ferulic acid (485 mg/l) was also examined, and it was estimated that the substrate was completely consumed after 5 days of incubation. It was also investigated that when the pH of the media was maintained at 7 and cells were incubated at 37 °C, these factors favoured the formation of vanillic acid. Further, yeast extract 0.05 % (w/v) was added in minimal medium and supplemented with 485 mg/l of ferulic acid at 37 °C and 120 rpm agitation and incubated for a fixed period of time. There was a significant increase in the amount of vanillic acid (241.3 mg/l), which was obtained at 18 h of incubation.

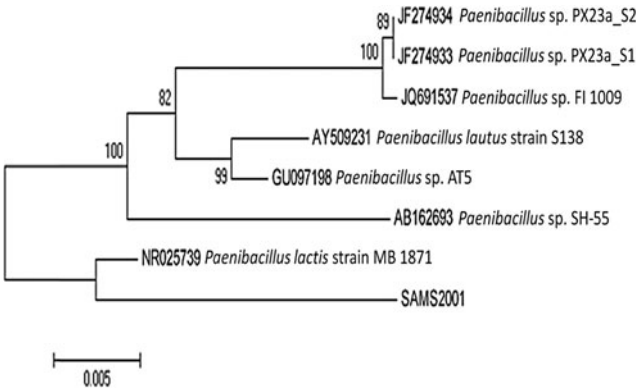

Fig. 1 Phylogenetic tree constructed by MEGA 5.05 software by neighbor joining analysis of the 16S rRNA gene sequence showing the position of the strain SAMS-2001 within the genus *Paenibacillus*. Bootstrap values (expressed as percentages of 1000 replications) greater than 80 % are shown at the branch points. Scale bar represents 0.005 substitutions per nucleotide position

Utilization of ferulic acid was also examined, and it was observed that ferulic acid was fully utilized after 12 h of incubation (Fig. 3). In order to make a high density culture of *Paenibacillus lactis* SAMS-2001, the microorganism was allowed to grow in minimal media supplemented with glucose (0.1 % w/v) as a sole carbon source. When almost all the carbon source was utilized by the microorganism for its growth, ferulic acid (485 mg/l) was added into the minimal medium. In the present study, the additional carbon source did not show a significant increase in product formation (data not shown).

Catabolic pathway of ferulic acid degradation

In order to elucidate the biochemical route of ferulic acid degradation, metabolites detected in the culture medium containing ferulate as the sole carbon source were added to the minimal media as substrates. The microorganism was allowed to grow in minimal medium containing the anticipated degradation products such as vanillin (380 mg/l) and vanillic acid (420 mg/l). Day-basis analysis was carried out for detection of degradation products after the first and fifth day of incubation. The isolated strain transformed vanillin into vanillic acid (Fig. 4a), whereas when vanillic acid (2.5 mM) was used as the sole carbon source, neither reverse conversion to vanillin nor degradation products such as protocatechuic acid or protocatechuic aldehyde were observed (Fig. 4b). This

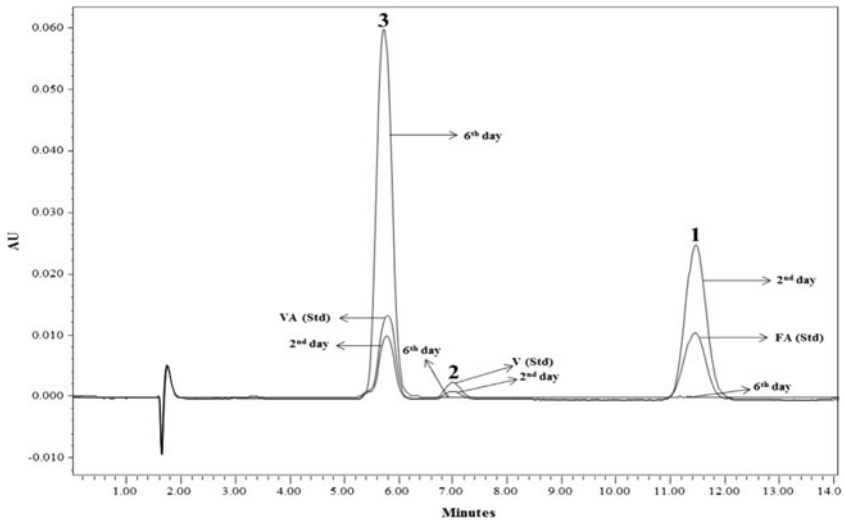

Fig. 2 Overlay of HPLC chromatogram at 254 nm showing degradation of ferulic acid (1) to vanillin (2) and vanillic acid (3) by processed culture filtrate of *Paenibacillus lactis* SAMS-2001 incubated for 2 and 6 days, respectively

observation suggested that vanillic acid is the only other metabolite formed along with vanillin as an intermediate upon consumption of ferulic acid in the medium and does not undergo further degradation to other intermediates. This is only further utilized as the sole carbon source by *Paenibacillus lactis* SAMS-2001 when ferulic acid has been completely utilized.

Biotransformation of ferulic acid by cell extract and cofactor necessities

The ability of ferulate-grown crude cell extract to convert ferulic acid to vanillin and vanillic acid was examined. In the presence of CoA, $MgCl_2$, ATP and NAD^+, ferulic acid was converted to vanillin and vanillic acid within 3 h. In the reaction mixture, vanillic acid formation with a subsequent depletion of ferulic acid and vanillin was observed (Fig. 5). Depletion of ferulic acid was not detected in the absence of CoASH, ATP, and NAD^+ indicating that these three cofactors are necessary for the in vitro conversion of ferulic acid into vanillin and vanillic acid.

Biotransformation of vanillin into vanillic acid by cell extract

Crude cell extracts (of ferulic acid-grown cell) were able to oxidize vanillin into vanillic acid, in the presence of NAD^+, suggestive of a NAD-linked vanillin dehydrogenase function

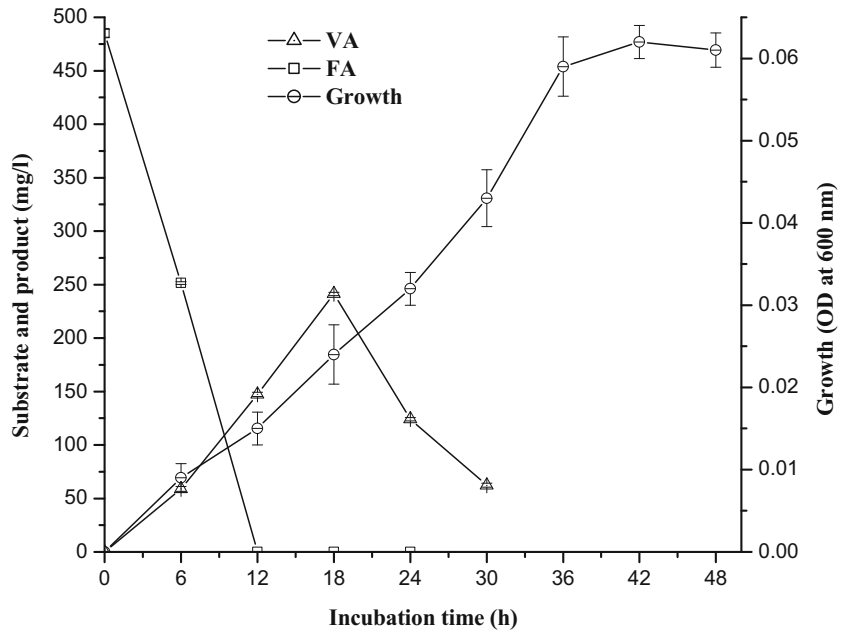

Fig. 3 Degradation of ferulic acid with formation of vanillic acid by *Paenibacillus lactis* SAMS-2001 at 37 °C, after 6–30 h of incubation and an agitation of 120 rpm with 0.05 % of yeast as co-substrate. Growth of the microorganism was also monitored

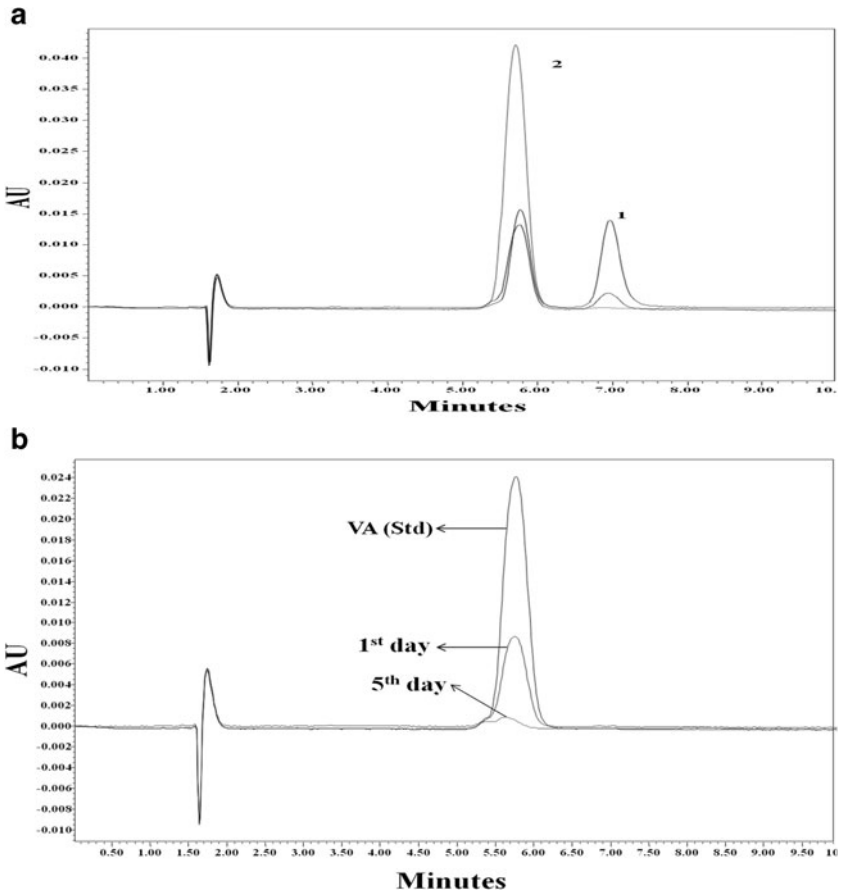

Fig. 4 **a** Overlay of HPLC chromatogram at 310 nm showing catabolic route of vanillin: (1) into vanillic acid and (2) by processed culture filtrate of *Paenibacillus lactis* SAMS-2001. Chromatogram represents decrease in the amount of vanillin (1) with the formation of vanillic (2) when supplemented with the same on the first and fifth days of incubation, respectively **b** Overlay of HPLC chromatogram at 254 nm showing catabolic route of vanillic degradation by processed culture supernatant of *Paenibacillus lactis* SAMS-2001. Chromatogram represents decrease in the amount of vanillic without any product formation when supplemented with the same on the first and fifth days of incubation, respectively

(Fig. 6). Although a small amount of vanillic acid was detected even in the absence of NAD^+.

Discussion

The accessibility of ferulic acid from agricultural by-products such as corn hulls, rice bran, wheat bran, and softwood lignin has created interest in utilizing ferulic acid as a natural renewable resource for the production of vanillin and vanillic acid (Ashengroph et al. 2012). Studies have been conducted on biotransformation of ferulic acid to vanillic acid as a major metabolite by various microorganisms. In case of *Streptomyces setonii*, a 52 % molar yield of vanillic acid was obtained when incubated for 24 h in test medium (Muheim and Lerch

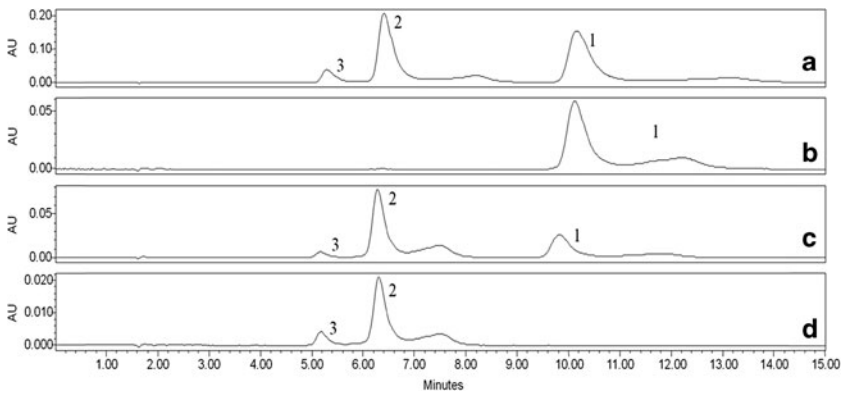

Fig. 5 Stack plot of HPLC chromatogram at 310 nm showing the in vitro conversion of ferulic acid (1) into vanillin (2) and vanillic acid (3) by a cell extract of *Paenibacillus lactis* SAMS-2001. Chromatogram [**a**] represents ferulic acid (1), vanillin (2), and vanillic acid (3) as standards. Chromatogram [**b**], [**c**], and [**d**] represent subsequent depletion of ferulic acid (1) with formation of vanillin (2) and a low level of vanillic acid (3) in the presence of CoA, $MgCl_2$, NAD^+ and ATP in the reaction mixture on 0, first, and third h of incubation, respectively

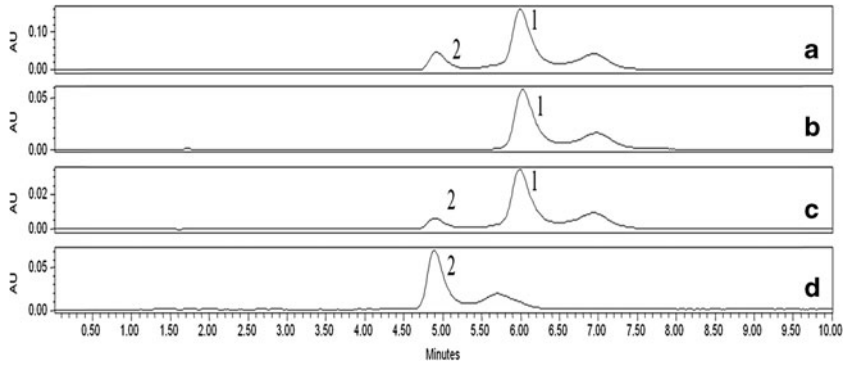

Fig. 6 Stack plot of HPLC chromatogram at 310 nm showing the in vitro conversion of vanillin (1) into vanillic acid (2) by a cell extract of *Paenibacillus lactis* SAMS-2001. Chromatogram [**a**] represents vanillin (1) and vanillic acid (2) as standards. Chromatogram [**b**], [**c**], and [**d**] represent a decrease in the level of vanillin (1) with formation of vanillic acid (2) in the presence of NAD^+ in the reaction mixture on 0, first, and third h of incubation, respectively

1999). In another study with *Sporotrichum thermophile*, vanillic acid was obtained with 35 % molar yield after 3 days of incubation in culture medium (Topakas et al. 2003). In the case of *Streptomyces halstedii*, 80 % molar conversion of vanillic acid was reported after 24 h of incubation (Brunati et al. 2004). In another finding, 47.9 % molar yield of vanillic acid was produced by *Streptomyces sannanensis* in 20 days of incubation (Ghosh et al. 2007). Resting cells of *Halomonas elongata* DSM 2581 T strain resulted in the formation of vanillic acid (82 %) after 10 h of incubation as described by Abdelkafi et al. (2008). A novel strain of *Bacillus licheniformis* SHL1 converted ferulic acid into vanillic acid under resting cells conditions with a molar yield of 60 % within 45 h (Ashengroph et al. 2012). In another study, *Halomonas* sp. B15 produced a 36.5 % molar yield of vanillic acid after 31 h from ferulic acid (Vyrides et al. 2015).

In this investigation, we present for the first time the potential of an isolated strain, *Paenibacillus lactis* SAMS-2001, to biotransform ferulic acid into vanillic acid within 18 h of incubation. Vanillic acid accumulated as the sole product in the culture medium with the transient formation of vanillin. Vanillic acid with 57.3 % of molar yield was accumulated in the minimal medium containing 485 mg/l of ferulic acid as a sole source of carbon and energy with 0.05 % yeast extract (w/v) as an additional organic nitrogen source. The optimum incubation temperature was observed to be 37 °C, and the media pH was maintained at 7. It has been earlier reported that the use of an additional carbon source aided the increase of biomass, which resulted in a reduced time period with increased concentration of product formation (Oddou et al. 1999). But in the current study, although the time period was reduced, an additional carbon source (glucose) did not show any significant increase in the concentration of the product.

In the present study, it was observed that the in vitro conversion of ferulic acid into vanillic acid requires the presence of CoA, ATP, $MgCl_2$, and NAD^+. A similar study was reported for *Pseudomonas fluorescens* AN103, *Paecilomyces variotii* MTCC 6581, and *Streptomyces sannanensis* MTCC 6637 that they metabolized ferulic acid via vanillin using a CoA-dependent pathway (Narbad and Gasson 1998; Ghosh et al. 2006, 2007). Further in vitro conversion of vanillin to vanillic acid was found to be via an inducible NAD-linked vanillin dehydrogenase enzyme. In the absence of NAD^+, extracts of ferulate-grown cells showed a low level of activity converting vanillin to vanillic acid. This was probably due to low amounts of NAD^+ or a non-specific aromatic aldehyde oxidase present in the cell extract as reported earlier in the case of *Bacillus subtilis* (Gurujeyalakshmi and Mahadevan 1987).

Conclusion

In summary, *Paenibacillus lactis* SAMS-2001 is capable of rapid bioconversion of ferulic acid to vanillic acid (57.3 %) and is the only metabolite within 18 h of incubation, which makes it a promising isolate for product formation in the spent medium.

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

Conflict of interest The authors declare that there is no conflict of interest.

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