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ORIGINAL PAPER

Microbial transformation of ferulic acid to vanillic acid by *Streptomyces sannanensis* MTCC 6637

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Abstract *Streptomyces sannanensis* MTCC 6637 was examined for its potentiality to transform ferulic acid into its corresponding hydroxybenzoate-derivatives. Cultures of *S. sannanensis* when grown on minimal medium containing ferulic acid as sole carbon source, vanillic acid accumulation was observed in the medium as the major biotransformed product along with transient formation of vanillin. A maximum amount of 400 mg/l vanillic acid accumulation was observed, when cultures were grown on 5 mM ferulic acid at 28°C. This accumulation of vanillic acid was found to be stable in the culture media for a long period of time, thus facilitating its recovery. Purification of vanillic acid was achieved by gel filtration chromatography using Sephadex™ LH-20 matrix. Catabolic route of ferulic acid biotransformation by *S. sannanensis* has also been demonstrated. The metabolic inhibitor experiment [by supplementation of 3,4 methylenedioxy-cinnamic acid (MDCA), a metabolic inhibitor of phenylpropanoid enzyme 4-hydroxycinnamoyl-CoA ligase (4-CL) along with ferulic acid] suggested that biotransformation of ferulic acid into vanillic acid mainly proceeds via CoA-dependent route. In vitro conversions of ferulic acid to vanillin, vanillic acid and vanillin to vanillic acid were also demonstrated with cell extract of *S. sannanensis*. Further degradation of vanillic acid to other intermediates such as, protocatechuic acid and guaiacol was not observed, which was also confirmed in vitro with cell extract.

Keywords Biotransformation · *Streptomyces* · Ferulic acid · Vanillic acid · Vanillin

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Introduction

Vanillic acid (or 4-hydroxy-3-methoxy benzoic acid) is an important hydroxybenzoic acid (HBA). It is one of the major constituents of 'natural vanilla' flavour [1]. Vanillic acid is also used as the starting material in the chemical synthesis of vanillin, which is one of the most important flavour molecules [2]. Vanillic acid is used in a variety of products, for example, it could be polymerized to oligomers, or used as a monomer in the synthesis of polyester [3]. Derivatives of vanillic acid such as, 5-nitrovanillic acid and 5-aminovanillic acid have antibacterial activity [4].

Microorganisms are known to be capable of producing hydroxybenzoates from several naturally occurring substrates upon biodegradation [5]. Ferulic acid (or, 4-hydroxy-3-methoxycinnamic acid) is one of such phenolic substrates whose distribution is nearly ubiquitous in the plant kingdom. The graminaceous plants in particular, are known to accumulate a high quantity of ferulic acid in their cell wall mostly by ester or ether linkages to the lignins and/or polysaccharides [6]. In principle, vanillic acid can be produced from ferulic acid via microbial transformation, a process that requires a substrate, whose structure is closely related to the anticipated product. Elimination of the phenyl-propenoic C_2-side

chain from ferulic acid (C_6–C_3 compound) can generate vanillic acid (C_6–C_1 compound).

In the past two decades, several species of actinomycetes were explored for generating vanillin and vanillic acid via biotransformation of ferulic acid [7–10]. The rationale for exploiting actinomycetes was based on the fact that, several of this genera, such as *Streptomyces* species seem to be particularly more efficient in utilizing hydroxycinnamic acids that are available in the soil as a result of lignin biodegradation. This might be due to their saprophytic life style and also their proximity to plant in the soil environment [11]. However, problem still remains with the accumulation of the metabolite in the culture medium. In most of the instances as reported earlier, the accumulated vanillic acid undergoes rapid degradation to other intermediates in the pathway [12, 13]. This encounters a serious difficulty in product recovery. This paper reports for the first time on the stable accumulation of vanillic acid in the spent medium of *Streptomyces sannanensis* MTCC 6637 upon transformation of ferulic acid.

Materials and methods

Microorganism

Streptomyces strain S39 was isolated from the agro-waste rich soil as essentially described before [14, 15]. This microorganism was later designated as *Streptomyces sannanensis* MTCC 6637 by the Microbial Type Culture Collection (MTCC; http://www.mtcc.imtech. res.in), Chandigarh, India. Pure cultures were maintained in Arginine Glycerol Salt (AGS) [16] slants at 28°C. Growth of *S. sannanensis* was measured as described earlier by Sachan et al. [15].

Medium and culture conditions

The minimal medium was prepared by the addition of basal inorganic salts, NH_4NO_3 (3.0 g/l) as a nitrogen source, $MgSO_4{\cdot}7H_2O$ (0.2 g/l), NaCl (0.2 g/l), KH_2PO_4 (1.0 g/l), Na_2HPO_4 (4.0 g/l), $CaCl_2$ (0.05 g/l) as essentially described earlier by Muheim and Lerch [8]. The pH of the medium was adjusted to 7.0. All the carbon sources were filter sterilized through 0.2 μm nylon filter, before their addition to minimal medium. After growth on AGS broth for 7 days, 1 ml cell suspension of *S. sannanensis* was aseptically transferred into 100 ml flask containing 25 ml of minimal medium with ferulic acid as a sole carbon source. Microbial transformation experiments were carried out in static flask culture. After a defined period of incubation (at an interval of 96 h) at 28°C, culture media were processed to detect the biotransformed product(s) of ferulic acid. Analyses were performed in triplicates and repeated at least twice. The standard deviations of the analyses were less than 5%.

Analytical procedures

Spent media were processed for thin layer chromatography (TLC) and high performance liquid chromatography (HPLC) analyses as described before [17]. The TLC analysis was performed in cellulose plate as described in the literature [18]. HPLC analyses were carried out according to the method of Sachan et al. [19] using an isocratic mode of separation (aqueous trifluoroacetic acid and methanol, 68:32) at a flow rate of 1.0 ml/min on a reverse phase C_{18} column (Synergi 4 μ Hydro-RP, Phenomenex,Torrance, USA). ESI-MS of LC-purified fractions were carried out as described earlier [20].

Product purification and recovery

Purification of the vanillic acid from the processed culture supernatant was carried out by gel filtration chromatography using ÄKTA Prime™ system (Amersham-Pharmacia Biotech, Hong Kong, China) equipped with Sephadex™ LH-20 matrix (Amersham-Pharmacia Biotech, Hong Kong, China) and monitored at 254 nm using Prime-View™ software (GE-Health Care, Hong Kong, China). Phenolic compounds were extracted as described above and resuspended in 2 ml of 50% (v/v) ethanol. The sample (2.0 ml) was injected into the column and eluted using deionized water as mobile phase. The elute was collected in test tubes using an in-built fraction collector of the ÄKTA Prime™ system. The tubes corresponding to the single peak (as viewed in software) were pooled together and the purity of single peak was checked by comparing the HPLC retention time with that of the authentic standard of vanillic acid. This was followed by UV-spectral overlay using a SPECORD-S-100 Diode-Array Spectrophotometer (AnalytikJena AG, Jena, Germany) to confirm the identity of vanillic acid.

Preparation of cell extract

Cells grown on ferulic acid as substrate were harvested during the late-exponential phase of growth by centrifugation at 12,000 rpm for 20 min. The cell pellet was washed twice in 50 mM cold Tris–HCl buffer, pH 7.8 and resuspended in 50 mM cold Tris–HCl buffer containing 5 mM dithiothreitol (DTT). All subsequent procedures were carried out at 4°C. The cell suspension was sonicated in a VC-130 ultrasonicator (Sonics and Materials Inc., USA) with a titanium probe of tip diameter

9.5 mm and operating at an amplitude of 20 μm. 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 and resultant supernatant was subsequently collected and concentrated (20 times) using Amicon® Ultra-4 CFU (Millipore, USA) membrane. This concentrated elute was used as crude extract for in vitro assay of vanillin and vanillic acid.

In vitro conversion of ferulic acid

The ability of cell extract to convert ferulic acid into vanillate-derivatives was examined at 30°C. The complete reaction mixture (1 ml) contained 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 [21]. The mixture was incubated for 10 h and the reaction was stopped by adding an equal reaction volume of acetic acid: methanol (1:4). The sample was then analyzed by HPLC for the detection of enzymatic products.

In vitro conversion of vanillin to vanillic acid and vice-versa

Analyses were carried out in the same way as described above except that ferulic acid was replaced by 0.4 mM vanillin, and ATP, $MgCl_2$ and CoASH were all omitted. The reverse conversion was also checked by replacing vanillin with vanillic acid, while other conditions remained unchanged.

In vitro degradation of vanillic acid

The ability of cell extract of *S. sannanensis* to convert vanillic acid into protocatechuic acid was examined at 30°C as described by Cartwright and Smith [22]. The reaction mixture contained 100 mM assay buffer, pH 8.5, 2.0 μmol GSH, 1.0 μmol NADPH, 2.0 μmol vanillic acid and cell extract (200 μl). In another set of experiment, decarboxylation of vanillic acid to guaiacol was also checked with cell extract incubated with vanillic acid [23]. Analyses were carried out in the same way as described above.

Results

Transformation of ferulic acid by *S. sannanensis*

In order to examine the capability of *S. sannanensis* to biotransform ferulic acid into vanillic acid, cell suspension (4% v/v) of *S. sannanensis* were inoculated in minimal medium containing ferulic acid (5.0 mM) as sole source of carbon. Cultures were incubated at 28°C for a maximum period of 20 days. Culture media were processed and analyzed by TLC to detect any accumulation of biotransformed product(s) (Fig. 1). A subsequent analysis by HPLC confirmed the presence of vanillic acid as the major biotransformed product of ferulic acid. This accumulated vanillic acid remained stable in the medium for a long period of time. Low amount of vanillin accumulation was also detected. The chemical identity of vanillic acid was further confirmed by ESI-MS (figure not shown).

Standardization of cultural parameters for enhanced vanillic acid accumulation

Cultures of *S. sannanensis* were inoculated in minimal medium containing different concentration of ferulic acid (1.0 mM, 2.5 mM, 5.0 mM, 7.5 mM, 10.0 mM) as sole carbon source and energy (Fig. 2a). Using 5 mM ferulic acid as sole carbon source, a maximum amount of 400 mg/l vanillic acid accumulation was observed in the medium after 16 days of incubation. Consumption of ferulic acid was also monitored and it was observed that ferulic acid was completely consumed after 16 days of incubation (Fig. 2b). It was further observed that an incubation at 28°C favoured vanillic acid formation, whereas *S. sannanensis*, when grown at 37°C, accumulated a very less amount of vanillic acid. Supplementation of trace salts in the ferulic acid-containing minimal

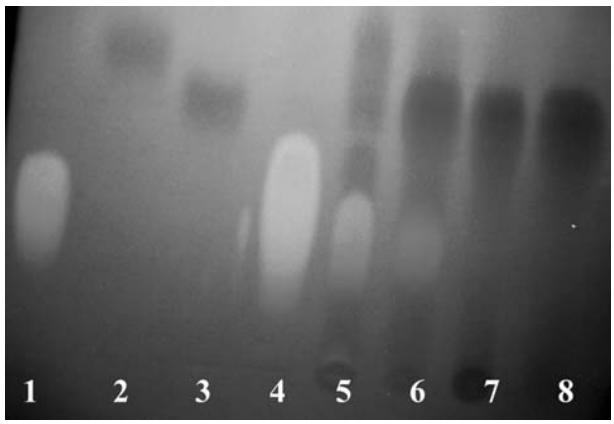

Fig. 1 TLC chromatogram showing separation of phenolic acids standards and processed culture supernatant of *Streptomyces sannanensis*. *Lane 1* ferulic acid; *lane 2* vanillin, *lane 3* vanillic acid, *lanes 4*, *5*, *6*, *7* and 8 processed culture filtrate of *S. sannanensis* incubated for 96, 192, 288, 384 and 480 h, respectively with ferulic acid (5 mM) as sole source of carbon. Vanillin was detected only after 96 h of incubation (*lanes 5* and *6*) but not after 288 h (12 days) of incubation (*lanes 7* and *8*). This observation suggests that vanillin appears as an intermediate for vanillic acid formation

medium showed only a marginal increase (ca. 10%) in vanillic acid accumulation.

Catabolic route of ferulic acid degradation

In order to explore the catabolic route of ferulic acid degradation, vanillin and vanillic acid were independently supplied to the medium as sole carbon source. These experiments were set up essentially according to the strategy adopted by Estrada Alvarado et al. [24]. The aromatic metabolites previously detected in the ferulate-grown *S. sannanensis*, served as independent substrates in the minimal media. Analyses were carried out after 24, 72 and 120 h of incubation for the detection of degradation product(s). Formation of vanillic acid was observed, when *S. sannanensis* was grown on vanillin (5 mM). Surprisingly, when vanillic acid was used as sole carbon source, no degradation product(s) of vanillic acid such as, protocatechuic acid or guaiacol was detected. These observations suggest that vanillic acid might have formed via vanillin, upon consumption of ferulic acid and accumulated in the medium (Fig. 3).

Supplementation of a metabolic inhibitor (3,4 (Methylenedioxy)-cinnamic acid (MDCA)) to *S. sannanensis* culture for resolving the involvement of CoA thioesters-dependent route of ferulic acid catabolism

S. sannanensis was grown on minimal medium containing MDCA (25 μM) and ferulic acid as sole source of carbon. Culture media were analyzed after every 96 h of incubation to detect any changes in the accumulation of biotransformed products as compared to control (i.e. without supplementation of MDCA). In this case, vanillic acid formation was considerably reduced as compared to control, where *S. sannanensis* was grown in minimal media containing same concentration of ferulic acid alone (figure not shown). MDCA is a potential inhibitor of hydroxycinnamate CoA-ligase

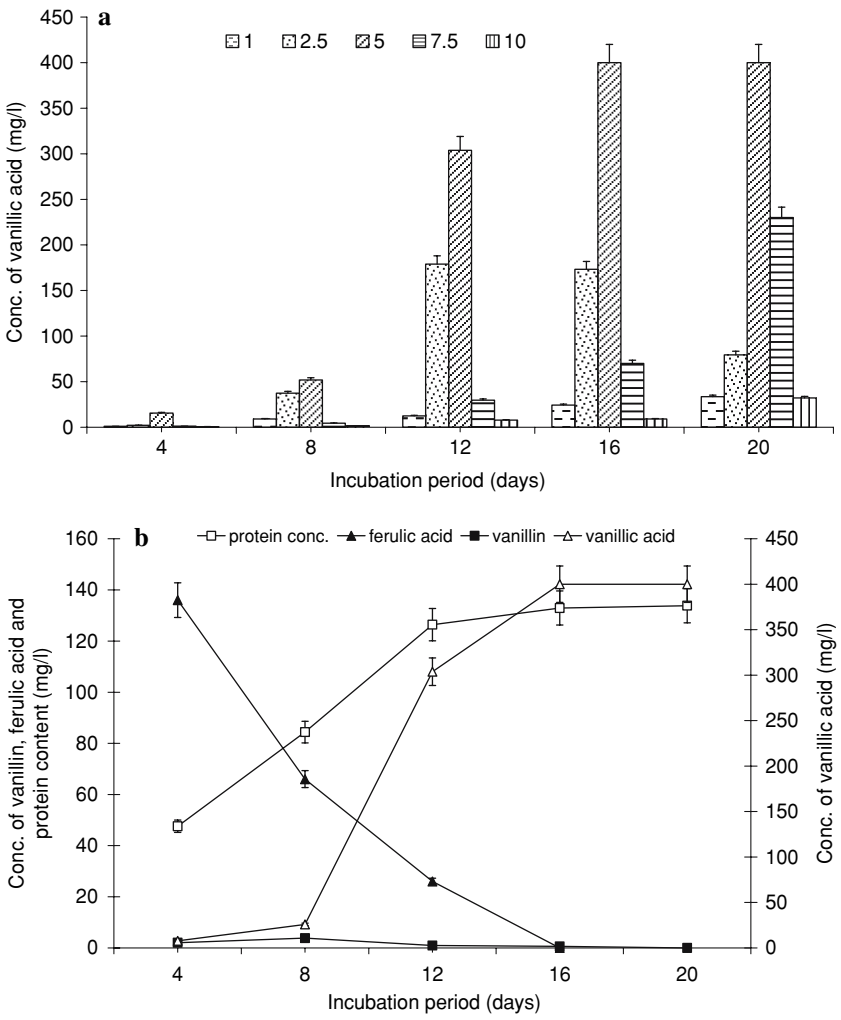

Fig. 2 **a** Time course accumulation of vanillic acid in the culture media of *S. sannanensis* with various concentrations of ferulic acid (1.0, 2.5, 5.0, 7.5 and 10.0 mM). **b** Time course accumulation of vanillin and vanillic acid by *S. sannanensis* grown in minimal medium supplemented with ferulic acid (5.0 mM). Consumption of ferulic acid and growth of the microorganism was also monitored

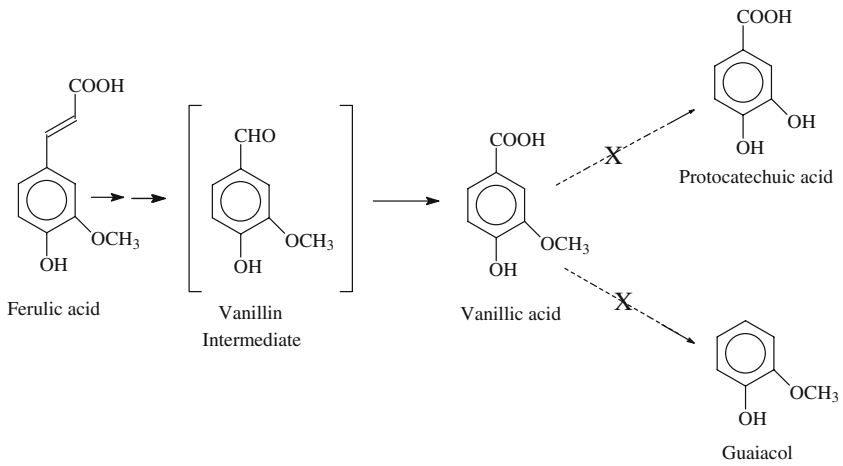

Fig. 3 Proposed pathway of ferulic acid degradation in *Streptomyces sannanensis*

(4CL) [25] that converts hydroxycinnamic acid to hydroxycinnamoyl-CoA thioesters. It appeared that the inhibition of 4CL by MDCA had reduced the metabolic flow towards the formation of downstream products in the CoA-dependent pathway. As a result of this, a subsequent decrease in the vanillic acid formation was seen, thus suggestive of a CoA-dependent catabolic route of ferulic acid transformation.

Biotransformation of ferulic acid by cell extract and cofactor requirements

The ability of ferulate-grown crude cell extract to convert ferulic acid to vanillin and vanillic acid was investigated. In the presence of CoASH, $MgCl_2$, ATP and NAD^+, ferulic acid was converted to vanillin and vanillic acid within 4 h. In another set of experiment, when the extract was incubated with ferulic acid in the presence of CoASH, $MgCl_2$ and ATP, vanillin and very low level of vanillic acid formation was observed after 6 h of incubation. Supplementation of NAD^+ in the reaction mixture increased the amount of vanillic acid formation with a subsequent depletion of ferulic acid and vanillin (Fig. 4). Depletion of ferulic acid was not detected in the absence of CoASH or ATP, indicating that these two cofactors are necessary for the in vitro conversion of ferulic acid into vanillin and vanillic acid.

Biotransformation of vanillin and vanillic acid by cell extract

Crude cell extract (of ferulic acid-grown cells) was found to oxidize vanillin into vanillic acid, in the presence of NAD^+, suggestive of a NAD-linked vanillin dehydrogenase function. Under identical experimental conditions, when vanillin was replaced with the same concentration of vanillic acid, reduction of the latter was not observed. In another set of experiment, when cell extract was incubated with vanillic acid in the presence of GSH and NADPH, no protocatechuic acid formation was observed. We also failed to detect guaiacol formation when cell extract was incubated with

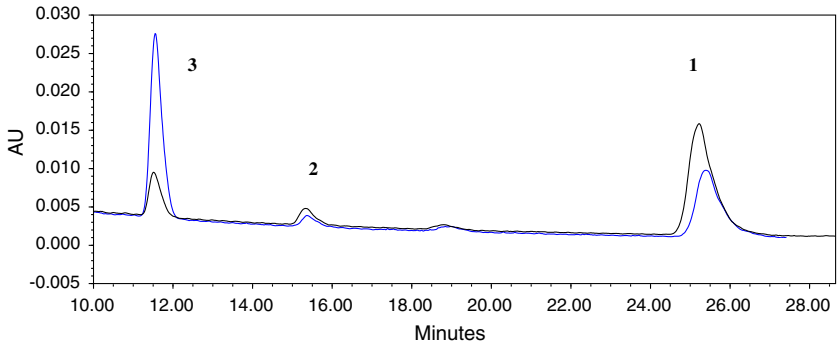

Fig. 4 Overlay of HPLC chromatograms at 310 nm showing the in vitro conversion of ferulic acid (*1*) into vanillin (*2*) and vanillic acid (*3*) by cell extract of *S. sannanensis*. Black chromatogram represents vanillin and very low level of vanillic acid formation in the presence of CoASH, $MgCl_2$ and ATP in the reaction mixture. Blue chromatogram represents an increase in the amount of vanillic acid formation with a subsequent depletion of ferulic acid and vanillin, when NAD^+ was supplemented in the reaction mixture along with CoASH, $MgCl_2$ and ATP. The low level of vanillic acid formation even in the absence of NAD^+ could be either due to low levels of NAD^+ present in the cell extract itself or due to the presence of a non-specific aromatic aldehyde oxidase [20]

vanillic acid. The above results confirmed our idea that vanillic acid was the only stable product obtained upon ferulic acid biotransformation with *S. sannanensis* along with vanillin as an intermediate.

Vanillic acid purification by using Sephadex™ LH20

Purification of the vanillic acid from the culture supernatant was achieved by gel filtration chromatography using Sephadex™ LH-20 matrix. Processed culture filtrate was injected in the column as described in 'Materials and methods'. A single peak was eluted with deionized water. Comparative UV-spectroscopy with authentic standard, and subsequent HPLC analysis, confirmed vanillic acid as the single eluted product (Fig. 5). This was further confirmed by ESI-MS analysis as essentially described elsewhere [20]. No trace of ferulic acid was detected. Crystals of vanillic acid were later harnessed (70% recovery) from this purified aqueous fraction by freeze-drying.

Discussion

This work reports for the first time the stable accumulation of vanillic acid in spent culture medium of *S. sannanensis* MTCC 6637 upon biotransformation of ferulic acid. Actinomycetes are well known to biotransform different hydroxycinnamic acids into value-added products [7]. In our study, a maximum amount of 400 mg/l vanillic acid accumulation was observed in minimal medium containing 5 mM ferulic acid as sole carbon source, along with transient formation of vanillin as an intermediate. With most of the microorganisms studied so far for ferulic acid degradation, it was observed that vanillin was produced as an intermediate, which rapidly oxidized to vanillic acid. This vanillic acid was either *O*-demethylated to protocatechuic acid [26], or decarboxylated to guaiacol [27]. It appears that actinomycetes are capable of utilizing ferulic acid in different ways depending on their main activity towards the substrates. These could be either complete degradation of ferulic acid or slow degradation of ferulic acid via vanillin and vanillic acid as observed with *S. halstedii* [10]. In another study, a completely different metabolic flux was observed with *S. setonii*. During the metabolism of ferulic acid, this strain accumulated vanillic acid only to a level of around 200 mg/l and then started to accumulate vanillin as the principal metabolic overflow product [8]. In our study with *S. sannanensis* upon ferulic acid transformation, a stable accumulation of vanillic acid was observed. No further degradation of vanillic acid was observed even after 20 days of incubation. In fact, vanillic acid was not even utilized as sole carbon source by the microorganism, although total ferulic acid was found utilized by that time.

In order to check if ferulic acid catabolism proceeds via CoA-dependent route, a supplementation experiment was set up with 3,4 (Methylenedioxy)-cinnamic acid (MDCA). Since, MDCA competitively inhibits 4CL enzyme of the phenylpropanoid pathway [25], it was anticipated that upon binding of the 4CL (ferulic acid-inducible) by MDCA, a major amount of ferulic acid would remain unutilized, if ferulic acid catabolism proceeded via this CoA-dependent route (through formation of feruloyl-CoA). The MDCA supplementation experiment in *S. sannanensis* showed that vanillic acid accumulation was considerably reduced as compared to cultures grown on ferulic acid alone. This suggests that in *S. sannanensis*, the biotransformation of ferulic acid into vanillic acid mainly proceeds via CoA-dependent route. Although in the above experiment it was observed that the formation of vanillic acid was notably reduced by supplementation of MDCA, a considerable amount of ferulic acid depletion also took place. This could be due to the fact that this unutilized ferulic acid was used up by the microorganism through some alternative means for their growth and maintenance.

In vitro conversion of ferulic acid to vanillic acid was studied in several bacteria [21, 28], however, such studies were limited in actinomycetes [7]. In our case, it was observed that the in vitro conversion of

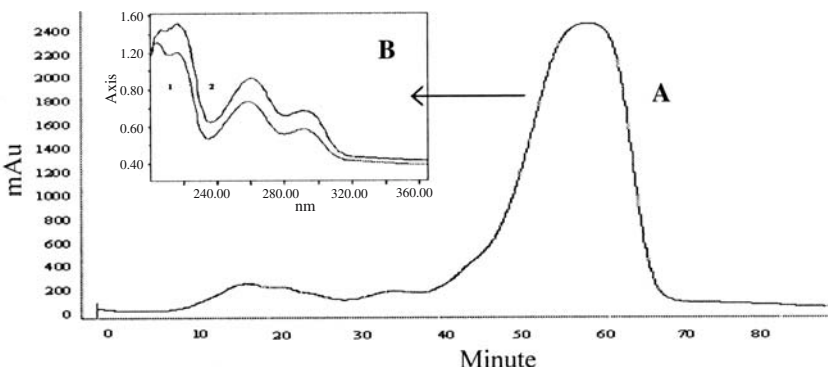

Fig. 5 **a** Elution profile of vanillic acid from Sephadex™ LH-20 column using water as mobile phase. **b** Overlay UV-spectra of standard vanillic acid with LH-20 purified vanillic acid present in sample. *1* Standard vanillic acid *2* sample vanillic acid

ferulic acid into vanillic acid requires the presence of CoA, ATP, $MgCl_2$ and NAD^+. A more or less similar observation was reported in a soil bacterium, *Pseudomonas fluorescens* AN103 that metabolized ferulic acid via vanillin using a novel CoA-dependent pathway [21]. Very recently, a novel CoA-dependent benzoate pathway has been characterized in *S. maritimus* [29]. The ferulic acid degradative pathway in *S. sannanensis* seemed to be tightly but not coordinately regulated, where the structural genes for the enzymes of the pathway appear to be induced only in the presence of the enzyme substrate. A similar inducible system for ferulic acid degradation was reported in *P. fluorescens* [21]. Furthermore, in our experiment, when cell extract of *S. sannanensis* was incubated with vanillic acid in the presence of GSH and NADPH, no protocatechuic acid formation was observed. This suggests an apparent absence of demethylase activity in *S. sannanensis* unlike other species of *Streptomyces*, where demethylation of vanillic acid led to the formation of protocatechuic acid in several species of *Streptomyces* such as *S. albulus* 321, *S. sioyaensis* P5, *S. viridosporus* T7A and *Streptomyces* sp. V7 [26]. Cell extract of *S. sannanensis* also failed to decarboxylate vanillic acid to guaiacol, a mechanism that was demonstrated in another *Streptomyces* sp. D7 [27]. This suggests that the organism was apparently devoid of vanillic acid decarboxylase activity (Fig. 3).

In summary, *S. sannanensis* had shown the ability to bioconvert ferulic acid into vanillic acid without any subsequent degradation. This was an interesting finding with a nature-isolated bacterium, since a similar observation of stable vanillic acid formation was reported only with mutated strains of *Psuedomonas* species [30, 31]. Vanillic acid was not found to be degraded further, possibly because of the apparent absence of vanillic acid demethylase and vanillic acid decarboxylase activities in *S. sannanensis*. As a result of which the product accumulation remained stable for a considerably long period of time. This in turn also led to the attainment of a simpler vanillic acid purification and recovery system. Therefore from the cell physiology point of view, absence of late steps of ferulic acid metabolism in *S. sannanensis* might be of scientific interest to answer why this strain does not further degrade vanillic acid. Whether a lack of genes and enzymes of the lower part of the ferulic acid degradation pathway are responsible for the stable accumulation of vanillic acid, needs a thorough characterization of the pathway at the enzymatic and genetic levels. Work has just been initiated to answer these questions.

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