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Microbial production of 4-vinylguaiacol from ferulic acid by *Bacillus cereus* SAS-3006

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Abstract

Ferulic acid is an abundant cinnamic acid derivative found in the plant kingdom. It is a commercially available substrate utilized to produce flavor compounds such as 4-vinylguaiacol (4-VG), vanillin, and vanillic acid. The isolate *Bacillus cereus* SAS-3006 was screened and selected based on its ability to produce 4-VG upon ferulic acid biotransformation. It was identified based on morphological and physiochemical characteristics and its 16S ribosomal DNA sequence (GenBank accession number: KF699134). A maximum amount (79.4 mg/L) of 4-VG accumulation was observed on the 5th day of incubation when the culture was grown on 2.5 mM ferulic acid as sole carbon source. Further conversion of 4-VG to other intermediates such as vanillin, vanillic acid, protocatechuic acid, acetovanillone, and vanillyl alcohol was not observed. *In-vitro* conversion of ferulic acid to 4-VG was also studied with cell extracts of *B. cereus* SAS-3006. The present study provides the first evidence for production of 4-VG as the sole product using *B. cereus* SAS-3006.

Keywords: *Biotransformation, ferulic acid, 4-vinylguaiacol, metabolite*

Introduction

Flavor and fragrance compounds can be obtained naturally from plant products or can be produced chemically. Although the majority of flavors and fragrances used globally are produced by chemical synthesis (Krings & Berger 1998; Serra et al. 2005; Xu et al. 2007) at low cost, consumers prefer natural compounds because of increasing health and nutrition consciousness (Abraham et al. 1994). Phenolic compounds have antioxidant properties and protect the gastrointestinal tract against damage by reactive species present in foods or produced within the stomach and intestines (Halliwell 2007). The USA and European legislation have stated that "*natural flavor substances could be prepared by enzymatic or microbial process*" (Li et al. 2008). Hence, microorganisms are used to produce various value-added compounds through biotransformation. Ferulic acid is obtainable from grasses, corn hulls (Ishii 1997; Oosterveld et al. 2000), wheat, maize, and rice bran (Walton et al. 2003; Mariod et al. 2010), where it is present as a major component of lignin and could be an economic substrate for a biotransformation process to produce flavor compounds such as 4-vinylguaiacol (4-VG), vanillin, and vanillic acid. 4-VG is a volatile phenolic compound which has an opaque, spicy, and clove-like aroma and is used in the food industry (Baqueiro-Peña et al. 2010). It is mainly found in coffee, beer, wine, chocolate, whiskey, and soy sauce (Mathew et al. 2007). It has a 40-fold higher economic value than ferulic acid and can be further transformed into various value-added compounds such as apocynin (acetovanillone), ethylguaiacol, vanillin, vanillyl alcohol, and vanillic acid (Karmakar et al. 2000; Landete et al. 2010). 4-VG is also used in the ophthalmic field, as a contact lens solution and eye-drop formulation (Salamone & Xia 2006). Besides its commercial value as a flavor and fragrance substance, 4-VG has medicinal value due to its antioxidant activity (Bortolomeazzi et al. 2007; Terpinc et al. 2011) and possible anti-cancer activity (Jeong & Jeong 2010).

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A wide variety of aromatic compounds can be produced using ferulic acid metabolism by four major pathways, namely I) non-oxidative decarboxylation, II) side chain reduction, III) coenzyme-A-dependent deacetylation, and IV) coenzyme-A-independent deacetylation. Of these, 4-VG is obtained by non-oxidative decarboxylation of ferulic acid (Rosazza et al. 1995; Lee et al. 1998; Donaghy et al. 1999). The bioconversion of ferulic acid to 4-VG has been studied using a variety of microorganisms, such as *Bacillus coagulans*, *Debaryomyces hansenii*, white rot fungus, *Aspergillus niger*, *Lactobacillus farciminis*, *Streptomyces setonii*, *Enterobacter soli*, and *E. aerogenes* (Karmakar et al. 2000; Mathew et al. 2007; Mabinya et al. 2010; Baqueiro-Peña et al. 2010; Adamu et al. 2012; Max et al. 2012; Hunter et al. 2012). To the best of our knowledge, there is no report on the biotransformation of ferulic acid into 4-VG using *B. cereus* strains. In this paper, we report the production of 4-VG as sole metabolic product in the medium with no other by-products.

Materials and methods

Chemicals and reagents

Ferulic acid (99%) and 4-VG (99%) used in this experiment were purchased from Sigma-Aldrich (GmbH, UK). High-performance liquid chromatography (HPLC)-grade methanol and formic acid were purchased from Himedia (India). Other chemicals were of analytical grade.

Bacterial isolation and culture medium

For isolation of bacteria, the serial dilution method was used. A soil sample was collected from a harvested paddy field at Mesra, Ranchi, approximately 5.0 cm below the soil surface. The soil suspension was plated on minimal medium supplemented with ferulic acid as the sole carbon source. This was followed by 24–72 h of incubation at 37°C and the colonies obtained were further purified by quadrant streaking on plates of the same medium. A single colony thus obtained was maintained on minimal medium slants supplemented with ferulic acid as the sole carbon source at 4°C for future work. Minimal medium was prepared by the addition of basal inorganic salts, ammonium nitrate (3.0 g/L) as a nitrogen source, sodium chloride (0.2 g/L), hydrated magnesium sulfate (0.2 g/L), potassium dihydrogen phosphate (1.0 g/L), disodium hydrogen phosphate (1.0 g/L), and calcium chloride (0.05 g/L) (Muheim & Lerch 1999). The pH was adjusted to 7.0. All the carbon sources were sterilized and passed through a 0.20-μm nylon filter (Sartorius, Minisart) before their addition to minimal medium.

Seed culture preparation and screening of biotransformation ability

To screen bacterial isolates with the potential for converting ferulic acid, cells of individual isolates were inoculated into 25 mL of nutrient broth and grown for 24 h at 37°C for seed culture preparation. Growth was measured as optical density at 600 nm. To check the biotransformation ability of isolate, 1 mL of homogenous seed culture of individual isolates was transferred to 100-mL flask containing 25 mL of sterile minimal medium supplemented with 1.0 mM ferulic acid as the sole carbon and energy source. Simultaneously, a control experiment was carried out by adding 1.0 mM ferulic acid in the same medium without cultures. Samples were withdrawn at intervals of 2, 4, and 6 days and were analyzed by thin-layer chromatography (TLC) and UV–Vis spectrophotometry (Lambda 25, Perkin Elmer, USA). The culture with the highest production of 4-VG 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 organic phase evaporated by rotary vacuum evaporation under reduced pressure. The extract was resuspended in 1 mL of methanol (50% v/v), and spotted on to a TLC plate. TLC analysis was performed using 2% aqueous formic acid as solvent system (Dey et al. 2003). 4-VG was directly visualized under a dual-wavelength

Table I. Morphological and biochemical characteristics of isolated strain *B. cereus* SAS-3006.

Characteristics	Description
Shape	Rod
Gram reaction	Gram-positive
Catalase	Positive
Oxidase	Positive
Endospore forming	Positive
Motility	Positive
Citrate utilization	Negative
Carbohydrate Fermentation	
Glucose	Positive/no gas
Sucrose	Positive/no gas
Lactose	Negative
H_2S production	Negative
Gelatin liquefaction	Positive
Methyl Red test	Negative
Voges–Proskauer test	Positive
Starch hydrolysis	Positive

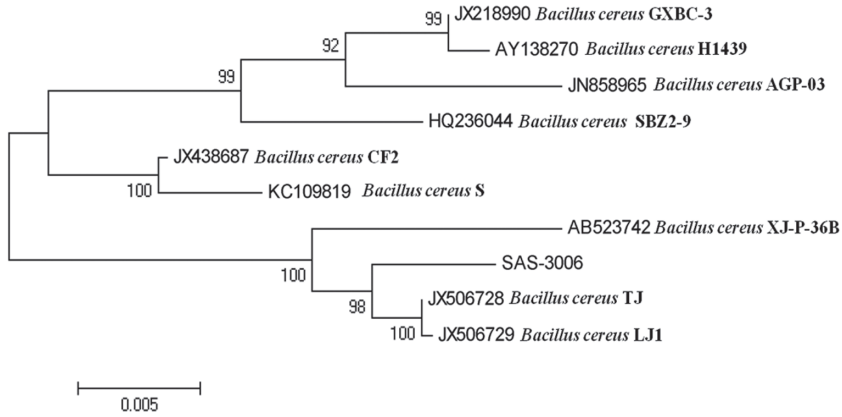

Figure 1. Phylogenetic tree of SAS-3006 as constructed using MEGA 5.05 software by the neighbor-joining method. Numbers at nodes indicate levels of bootstrap values (%) based on the neighbor-joining analysis of 1000 replicates; only values > 50% are given.

(254/310 nm) UV lamp (Uvitec UK). The TLC chromatogram corresponding to the standard was detected on the plate and confirmed by overlay of standards on HPLC (Waters, USA). The HPLC conditions were set as follows: column, Symmetry® C_{18} (4.6 × 150 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). Analysis of the sample was performed using the following gradient: 0–15 min in 68% A and 32% B, and 15–30 min

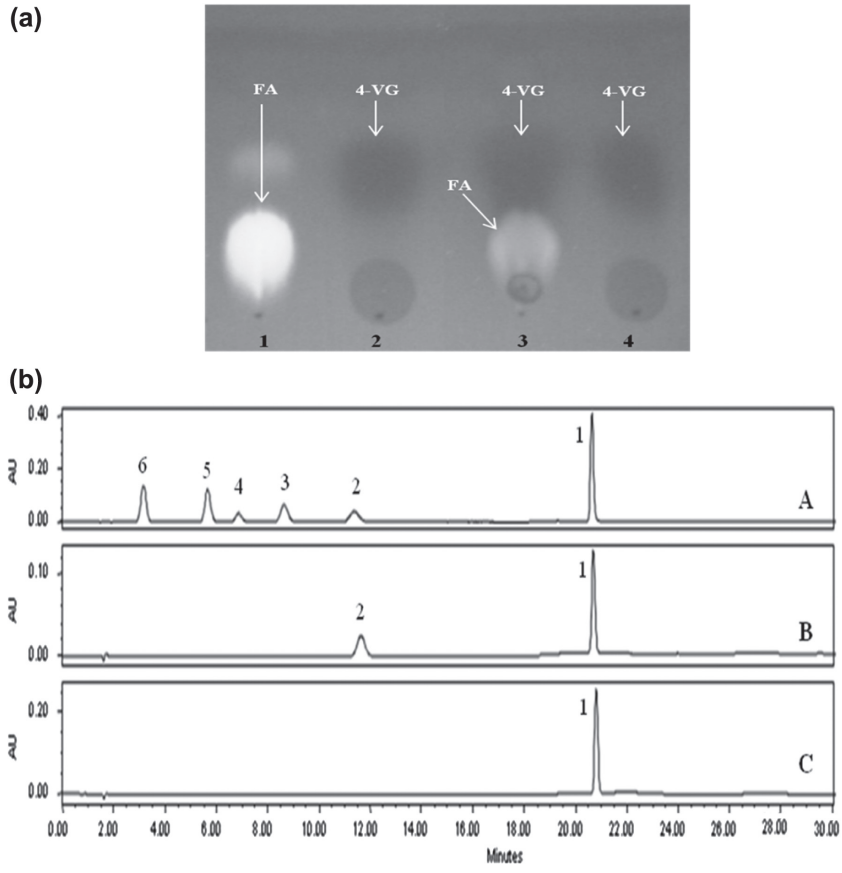

Figure 2. (a) TLC analysis showing separation of standards and processed culture supernatant of *B. cereus* SAS-3006. Lane 1: Ferulic acid; lane 2: 4–VG; lanes 3 and 4: processed culture filtrate of *B. cereus* SAS-3006 incubated for 3 and 5 days, respectively. (b) Stack plot of HPLC chromatogram at 254 nm showing ferulic acid degradation by processed culture filtrate of *B. cereus* SAS-3006. Chromatogram [A] represents 4-VG (1), ferulic acid (2), acetovanillone (3), vanillin (4), vanillic acid (5), and protocatechuic acid (6) as standards. Chromatogram [B] represents conversion of ferulic acid (2) to 4-VG (1) on 3rd day of incubation and [C] represents decrease in the amount of ferulic acid (2) with an increase in formation of 4-VG (1) as the sole metabolite on 5th day of incubation.

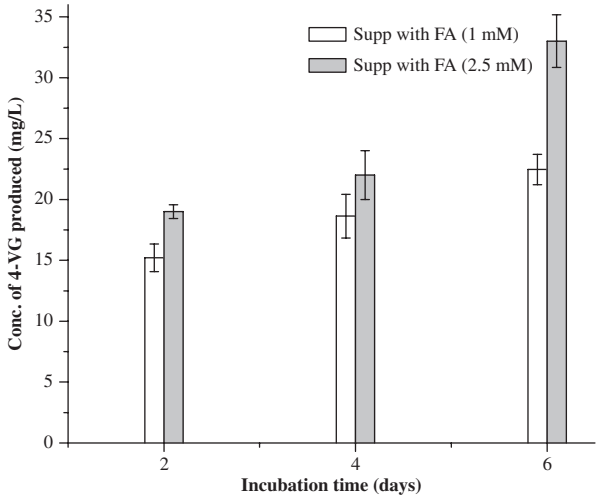

Figure 3. Time course of 4-VG accumulation in the culture media of *B. cereus* SAS-3006 supplemented with 1.0 and 2.5 mM ferulic acid as the sole carbon source after 2, 4, and 6 days of incubation.

in 40% A and 60% B, followed by equilibration time of 10 min using a Binary HPLC Pump (Waters). Compounds were identified by comparison of retention time and peak with the standards (1 mg/mL). 4-VG was further quantified by absorbance at 260 nm. Measurements of concentrations were read from the standard curve in the range of 1–10 µg/mL.

Identification and characterization of selected isolate

The isolate giving the highest 4-VG yield was selected. Preliminary identification of the isolate was done by morphological and biochemical characterizations (Breed et al. 1957). Species identification of the selected bacterial isolate was confirmed on the basis of 16S ribosomal RNA (rRNA) gene sequencing (Xcelris Labs Ltd. Ahmedabad, India). The consensus sequence was aligned with the sequence of the type strain using ClustalW (Thompson et al. 1997). The homology of the sequences was examined using BLAST of National Center for Biotechnology Information (NCBI). Molecular Evolutionary Genetics Analysis (MEGA) software version 5 (Hall 2013) was used to construct phylogenetic trees using the neighbor-joining method (Saitou & Nei 1987).

Optimization of various cultural parameters for maximum 4-VG production

The various cultural parameters considered for maximum 4-VG production were ferulic acid concentration (1.0 and 2.5 mM), incubation temperature (37 and 28°C), pH (6–8), organic nitrogen source (0.05% w/v yeast extract), agitation (120 rpm), and additional carbon source (0.1% w/v glucose). To study the effect of each parameter on product formation, samples were withdrawn at fixed intervals and analyzed using TLC, UV–Vis spectrophotometry, and HPLC. Analyses were performed in triplicate and repeated at least twice.

Degradation of 4-VG

The metabolite (4-VG) previously detected in the media of ferulate-grown *B. cereus* SAS-3006 was independently supplied to the medium as the sole carbon source. Analyses were carried out after 3 and 5 days of incubation for the detection of product using 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 refrigerated centrifuge (5804 R, Eppendorf, Hamburg, Germany). The supernatant was removed and cell pellet washed twice in 50 mM cold sodium phosphate buffer (pH, 6.0), and then resuspended in the same buffer containing 5 mM dithiothreitol. 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 having a tip diameter of 13 mm and operating at an amplitude of 35% with 15000 joule energy. Sonication was applied in short bursts of 20 s with a total exposure time of 6 min. After sonication, the cell extract was again centrifuged and the resultant supernatant was collected and concentrated using an Amicon Ultra-15 Centrifugal Filter (Millipore, USA) membrane. This concentrated eluate was used as a crude extract for *in-vitro* assay of 4-VG.

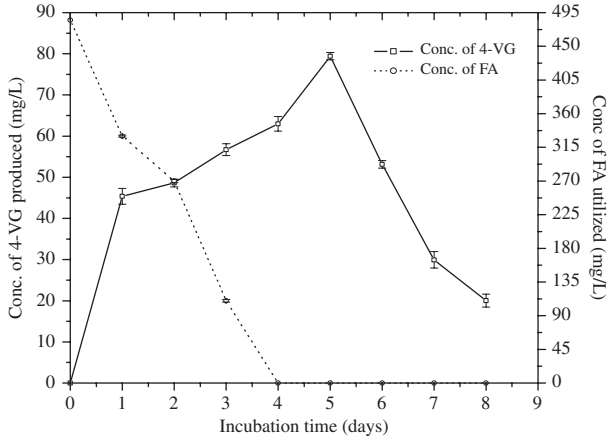

Figure 4. Degradation of ferulic acid with formation of 4-VG by *B. cereus* SAS-3006 at 37°C, after 1–8 days of incubation and agitation of 120 rpm.

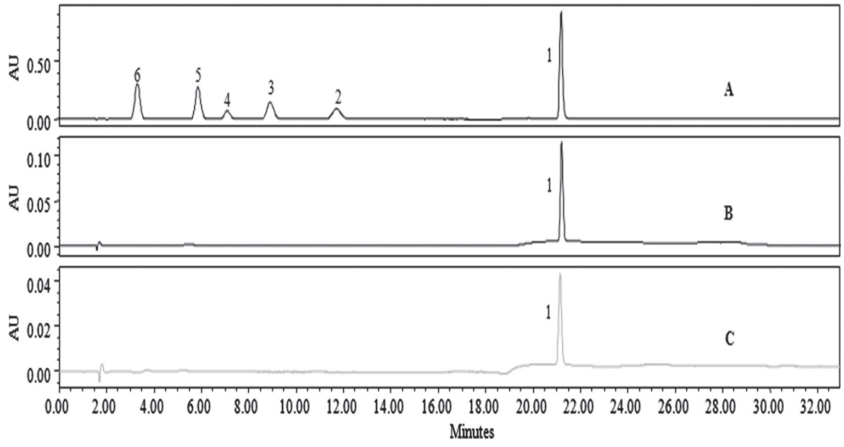

Figure 5. Stack plot of HPLC eluates measured at 254 nm showing potential 4-VG degradation products in processed culture filtrate of *B. cereus* SAS-3006. Chromatogram [A] represents 4-VG (1), ferulic acid (2), acetovanillone (3), vanillin (4), vanillic acid (5), and protocatechuic acid (6) as standards. Chromatogram [B] represents 4-VG (1) used as the sole carbon source on 3rd day of incubation and [C] shows the decrease in the amount of 4-VG (1) without any product formation by the 5th day of incubation.

In-vitro conversion of ferulic acid

The ability of a cell extract to convert ferulic acid into 4-VG was examined. The assay mixture (1 mL) consisted of 50 mM sodium phosphate buffer, pH: 6.0, 5.0 µg/mL of ferulic acid, and 100 µL of cell extract (Degrassi et al. 1995). The sample was then analyzed for ferulic acid decarboxylase activity using UV–Vis spectrophotometry for product detection, by incubating the mixture at 37°C for 120 min. Wavelength ranges were set between 200 and 400 nm to confirm the optimal absorption wavelength.

In-vitro degradation of 4-VG

Analyses were carried out in the same way as described above except that ferulic acid was replaced with 5.0 µg/mL of 4-VG (Degrassi et al. 1995).

Results and discussion

Identification of the bacterial isolate

Based on their morphology, 17 different bacterial isolates were obtained and further checked for their ability to produce 4-VG. Among the cultures obtained, SAS-3006 was the only one which produced 4-VG. Morphological and biochemical characterizations were used for preliminary identification of this microorganism as given in Table I (supplementary file). According to these preliminary investigations, the isolate was identified as *B. cereus*. For further confirmation, sequencing of the 16S rRNA gene was executed (Xcelris Labs Ltd. Ahmedabad, India) and the consensus sequence was used for similarity search using BLAST algorithm with NCBI GenBank nrdatabase. Based on a maximum identity score, sequences were selected and aligned using the multiple alignment software ClustalW. A phylogenetic tree was constructed using MEGA 5 software (Figure 1). Analysis of the 16S rDNA sequence indicated that isolate SAS-3006 showed the closest similarity to *B. cereus* TJ (JX506728, 99% identity according to BLAST, NCBI). The 16S rDNA sequence of SAS-3006 was submitted to DNA Data Bank of Japan (DDBJ)/European Molecular Biology Laboratory (EMBL)/GenBank under accession number, KF699134 (http://www.ncbi.nlm.nih.gov/nuccore/KF699134).

Transformation of ferulic acid by B. cereus SAS-3006

In order to examine the ability of *B. cereus* SAS-3006 to biotransform ferulic acid into 4-VG, a cell suspension (4% v/v) of *B. cereus* SAS-3006 was inoculated into minimal medium containing ferulic acid (1.0 mM) as the sole carbon source. The culture was incubated at 37°C for a maximum period of 6 days. Culture media were processed and analyzed by TLC to detect any formation of metabolite(s) (Figure 2a). The presence of 4-VG as the sole product was further confirmed by HPLC (Figure 2b). The product obtained was quantified using UV–Vis spectrophotometry.

Standardization of culture parameters for enhanced 4-VG production

A culture of *B. cereus* SAS-3006 was inoculated in minimal medium containing different concentrations of ferulic acid (1.0, 2.5, and 5.0 mM) as the sole carbon and energy source. Using 2.5 mM ferulic acid as sole carbon source, a maximum amount of 32.9 mg/L or 0.219 mM 4-VG was observed in the

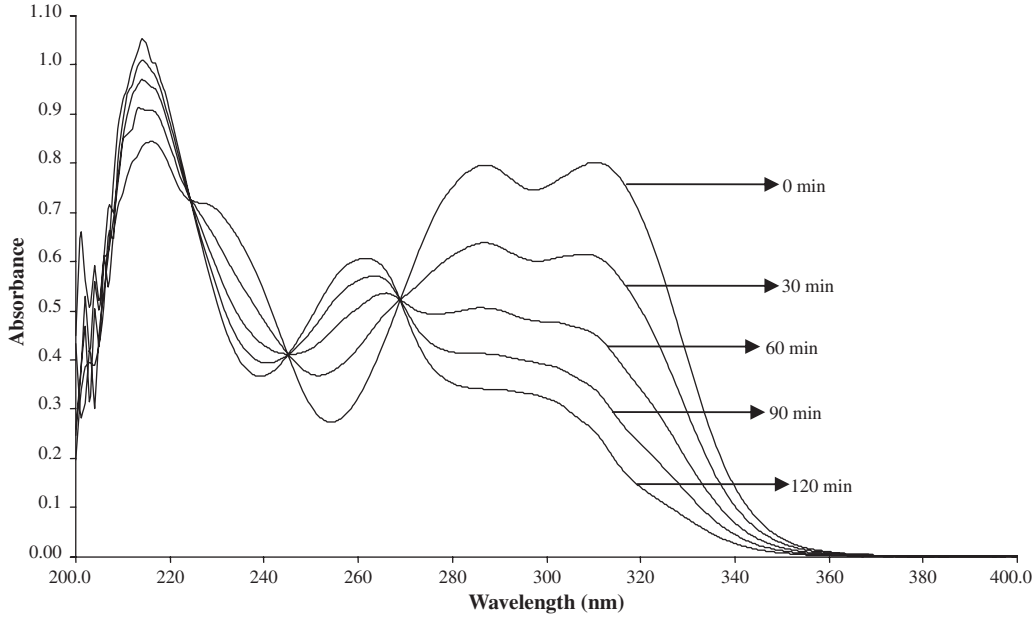

Figure 6. Depletion of ferulic acid in the reaction mixture containing crude cell extract of *B. cereus* SAS-3006 incubated for 0–120 min.

medium on 6th day of incubation (Figure 3). When 5.0 mM ferulic acid was provided, very little utilization of the substrate was observed (data not shown). Consumption of ferulic acid (2.5 mM) was also monitored showing that the substrate was completely consumed after 4 days of incubation. It was also observed that a medium pH of 7.0 and incubation at 37°C favored the formation of 4-VG. Furthermore, yeast extract 0.05% (w/v) was added to the minimal medium and supplemented with 2.5 mM ferulic acid at 37°C and agitation of 120 rpm. There was a significant increase in the amount of 4-VG (79.4 mg/L) obtained on the 5th day of incubation. Consumption of ferulic acid was also examined and it was observed that the ferulic acid was fully utilized after the 4th day of incubation (Figure 4). In order to obtain a high-density culture of *B. cereus* SAS-3006, the microorganism was allowed to grow in minimal media supplemented with glucose (0.1% w/v) as the sole carbon source. It has previously been reported that this approach increased the biomass which reduced the time period of product formation (Oddou et al. 1999). When almost all the carbon source was utilized by the microorganism for its growth, ferulic acid (2.5 mM) was added into the minimal medium. However, the use of an additional carbon source did not show a significant increase in product formation (data not shown).

Catabolic route of ferulic acid degradation

In order to trace the catabolic pathway of ferulic acid degradation, 4-VG was independently supplied to the medium as the sole carbon source. This experiment was performed according to the strategy approved by Estrada Alvarado et al. (2001). The metabolite previously detected in the ferulate-grown *B. cereus* SAS-3006 served as an independent substrate in the medium. Analyses were carried out after 3 and 5 days of incubation for the detection of product. In previous research it was observed that 4-VG accumulated as an intermediate, which was then further degraded to intermediates such as vanillin, vanillic acid, and protocatechuic acid (Karmakar et al. 2000); vanillin, vanillic acid, and acetovanillone (Mabinya et al. 2010); vanillin, vanillyl alcohol, and vanillic acid (Baqueiro-Peña et al. 2010; Hunter et al. 2012); and protocatechuic acid, vanillyl alcohol, vanillic acid, and vanillin (Max et al. 2012). These metabolites resulted from side chain cleavage of ferulic acid (C_2-cleavage) or cleavage of 4-VG (C_1-cleavage) in the culture medium. When 4-VG (2.5 mM) was used as the sole carbon source with *B. cereus* SAS-3006, no degradation products were detected. This observation suggests that 4-VG is the sole metabolite formed upon consumption of ferulic acid in the medium and does not undergo further degradation to other intermediates (Figure 5). This is only further utilized as the sole carbon source by *B. cereus* SAS-3006 when ferulic acid has been completely utilized.

Biotransformation of ferulic acid by cell extract

Ferulic acid decarboxylase was produced by *B. cereus* SAS-3006 as demonstrated by the enzymatic

conversion of ferulic acid to 4-VG using UV–Vis spectrometry, which shows a hypsochromic or blue shift in the peak maxima. The transformation of ferulic acid (λ_{max} 289, 318 nm) to a product with absorption maxima at 210, 260 nm could be attributed to the formation of 4-VG (Figure 6). Ferulic acid was converted to 4-VG within 120 min of incubation. In another set of experiments, when the extract was incubated with 4-VG, no degradation products such as vanillic acid and protocatechuic acid were observed. HPLC analyses showed a similar result for 4-VG formation and ferulic acid degradation (data not shown). It has been reported previously that a ferulic acid decarboxylase, which is responsible for degradation of ferulic acid to 4-VG, showed maximum activity at pH of 7.0 (Faulds & Williamson 1994), but the pH optimum was not examined in the current study.

Conclusion

In this investigation, we provide evidence that a strain of *B. cereus* SAS-3006 transforms ferulic acid into 4-VG as a sole product in the culture medium. Purification and characterization of the enzyme responsible for this conversion is under process.

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Declaration of interest: The authors report no declarations of interest. The authors alone are responsible for the content and writing of the paper.

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