The metabolism of ferulic acid via 4-vinylguaiacol to vanillin by *Enterobacter* sp. Px6-4 isolated from *Vanilla* root

Xuemei Li [a,b,1], Jinkui Yang [a,1], Xian Li [c], Wen Gu [a], Jingwen Huang [a], Ke-Qin Zhang [a,*]

[a] *Laboratory for Conservation and Utilization of Bio-resources, and Key Laboratory for Microbial Resources of the Ministry of Education, Yunnan University, Kunming 650091, PR China*
[b] *Yunnan Academy of Tobacco Science, Kunming 650106, PR China*
[c] *College of Pharmacy, Kunming Medical College, Kunming 650031, PR China*

ABSTRACT

This study reports a bacterium from *Vanilla* roots with the ability to utilize ferulic acid as the sole carbon source to produce vanillin. The strain was identified as *Enterobacter* sp. Px6-4 based on its 16S rDNA sequence and biochemical characteristics. The primary metabolites using ferulic acid as the sole carbon source include 4-vinylguaiacol, vanillin as well as other related chemicals, all of which share the same guaiacol-carbon-skeleton. Based on structural analyses of non-volatile compounds in the culture filtrate of this strain, two putative novel metabolites were isolated and identified. These results allowed us to propose the possible metabolic pathway leading from ferulic acid to vanillin via 4-vinylguaiacol. Our experiments elucidated for the first time of a ferulic acid metabolism via decarboxylation to produce 4-vinylguaiacol and a further conversion to vanillin in *Enterobacter* sp. Px6-4.

© 2008 Elsevier Ltd. All rights reserved.

1. Introduction

During the past several years, biocatalytic productions of fine flavors and fragrance have been expanding rapidly due to the legislations in the US and Europe that stated that "natural flavor substances could be prepared by enzymatic or microbial processes" [1,2]. Among these investigations, the biotechnological production of vanillin has drawn significant attention because vanillin is economically the most significant flavoring substance in terms of consumption level. Specifically, vanillin plays an important role in industries dealing with food flavors, beverages, perfumes and pharmaceuticals [3]. The productions of vanillin based on the biocatalytic transformations of lignin, stilbenes, phenylpropanoids (e.g. isoeugenol, eugenol, and ferulic acid) applying fungi, bacteria, plants or genetically engineered microorganisms have been summarized previously [4–6]. One of the most intensively researched pathways was the biocatalysis of ferulic acid to produce "natural vanillin" [7–11].

Ferulic acid is a modified cinnamic acid and is extremely abundant and distributed widely as esters and amides in higher plants [4]. Several studies have reported the occurrence of vanillin as an intermediate of microbial degradation of ferulic acid [4–6]. An increasing number of microorganisms have recently been reported capable of converting ferulic acid to 4-vinylguaiacol by decarboxylation [12–14]. However, the detailed pathways of ferulic acid metabolism in these microorganisms have not yet been fully elucidated—the only detailed step described so far is the first decarboxylation of ferulic acid to 4-vinylguaiacol.

In the present study, a new vanillin-forming bacterium designated Px6-4 was isolated from a *Vanilla* root by the endophytic-isolation method. It was obtained based on its ability to transform ferulic acid to vanillin. The primary metabolites involved in the transformation included 4-vinylguaiacol, vanillin and other related compounds with the guaiacol-carbon-skeleton. We proposed the putative metabolic pathway of vanillin formation from ferulic acid via 4-vinylguaiacol in this bacterium.

2. Materials and methods

2.1. Chemicals

Ferulic acid (*trans*-, 99%), 4-vinylguaiacol (99%) and vanillin (99%) were all purchased from Sigma–Aldrich (America). All the solvents used for high-performance liquid chromatography (HPLC) analyses were of the HPLC grade. All other chemicals were of the analytic grade.

* Corresponding author. Tel.: +86 871 5034878; fax: +86 871 5034838.
E-mail address: kqzhang111@yahoo.com.cn (K.-Q. Zhang).
[1] These authors contributed equally to this work.

2.2. Isolation of vanillin-producing (VP) bacteria and culture conditions

Bacteria were isolated from the surface-sterilized *Vanilla* roots according to a previously reported method [15] and pure cultures were incubated on LB medium containing (per liter): 10 g tryptone, 10 g NaCl, 5 g yeast extract, and 16 g agar. Vanillin-producing bacteria were selected using the vanillin-producing medium consisting of ferulic acid 1.0 g, yeast extract 1.0 g, KH_2PO_4 0.9 g, Na_2HPO_4 10 g, $MgSO_4·2H_2O$ 0.2 g, $CaCl_2$ 0.1 g, $FeSO_4·2H_2O$ 0.01 g, $MnSO_4$ 0.6 mg and Na_2MoSO_4 0.6 μg in 1 L of distilled water, pH 6.5. During media preparation, the ferulic acid was sterilized using filtration and then supplemented to sterilized VP medium. The isolated strains were cultured in VP medium at 37 °C with 180 rpm of shaking for 5 days. A purified vanillin-forming bacterium was obtained and named Px6-4.

2.3. Identification of strain Px6-4

Morphological and biochemical characteristics of strain Px6-4 were determined following the methods described by Dong and Cai [16]. After the genomic DNA of strain Px6-4 was isolated, its 16S rDNA gene was amplified using the forward primer P1 (5′-ACGGTTACCTTGTTACGACTT-3′) and the reverse primer P2 (5′-AGAGTTTGATCCTGGCTCAG-3′) as described by Weisburg et al. [17]. The partial 16S rDNA sequence has been submitted to the GenBank database with the accession number EF175731. This sequence was compared to known sequences found in the GenBank database using blast (http://www.ncbi.nlm.nih.gov/BLAST/). The neighbor-joining (NJ) method (implemented in MEGA2.0) was used for phylogenetic analysis with the model of Kimura-2-Parameter [18,19]. The robustness of the tree topology was assessed by bootstrap analysis, with 1000 replicates.

2.4. Ferulic acid decarboxylase assays

The cell-free extract was prepared according to a previously described method [20]. The ferulic acid decarboxylase activity was assayed by HPLC. The reaction mixture consisted of 50 mM sodium phosphate buffer (pH 6.0) and 1.0 g of ferulic acid per liter. A cell-free extract (5%, v/v) was added to start the reaction and the mixture was incubated at 37 °C for 1 h. The amount of 4-vinylguaiacol in the reaction mixture was determined using HPLC. One unit of activity was defined as the production of 1 mmol of 4-vinylguaiacol per min.

2.5. Analytical methods

TLC (thin layer chromatography) analyses were carried out on 0.25-mm thick silica gel G and GF_{254} plates (Qingdao, China) and hexane: chloroform: anhydrous ether:acetic acid (4:3:2:1, v/v/v/v) for development. The products were detected under UV at 254 nm and vanillin was specifically stained with 2,4-dinitrophenylhydrazine [21].

GC/MS (gas chromatography/mass spectrum) analysis was performed using HP 6890 GC/5972 MS equipped with a 0.25 mm i.d. × 30 mm glass capillary column. The oven temperature was programmed as follows: initial temperature 50 °C (held for 1 min), 50–260 °C at a rate of increase of 10 °C min^{-1}, and 260 °C for 5 min. Helium was used as the carrier gas at a flow rate of 1 ml min^{-1} and the inlet temperature was set at 250 °C. The MS measurements were made with electron energy of 70 eV. Source temperature was set at 200 °C and mass scan ranges covered 30–450 amu. Peak identifications of the components in the gas chromatograms were carried out by comparing the mass spectral data and the GC retention times with those of authentic known compounds.

The HPLC analysis was performed on Agilent 1100 Series (Hewlett-Packard, USA) equipped with a reverse-phase Waters Symmetry C18 column (3.9 mm × 150 mm). The mobile phase acetic acid (0.5%)–methanol (60:40) was used at a flow rate of 0.8 ml min^{-1}. The absorbance of the separated metabolites was recorded on a Waters 996 detector at 290 nm. The metabolites were quantified by comparing the peak areas and their concentrations were extrapolated from the corresponding standard curve prepared for each known compound of known concentration.

2.6. Analysis of volatile components in the culture

The volatile compounds in the culture (50 mL) were extracted twice with equivalent volumes of ethyl acetate. Subsequently, the ethyl acetate solutions were combined and evaporated in vacuo to remove the solvent. The yellowy condensed extract (5 mL) was dehydrated with anhydrous sodium sulfate and then subjected to GC/MS analysis.

2.7. Analysis of non-volatile intermediate metabolites of ferulic acid in the culture

Column chromatography and semi-preparative reverse-phase HPLC column were used for the isolation of metabolites. The instruments used in the isolation and identification of intermediate metabolites were described as follows. Column chromatography: silica gel (200–300 mesh). TLC: silica gel GF_{254}; optical rotations: SEPA-300 polarimeter. Semi-preparative reverse-phase HPLC was performed on an Agilent 1100 liquid chromatograph with a Zorbax SB-C_{18} column. To elucidate the molecular structures, the isolated metabolites from ferulic acid degradation were analyzed by IR spectra, MS, ^{1}H and ^{13}C NMR spectra.

3. Results

3.1. Isolation and identification of strain Px6-4

Six vanillin-forming bacteria were isolated from *Vanilla* roots. The TLC analysis on the ethyl acetate phase of culture filtrates showed that strain Px6-4 produced more vanillin than other five bacteria. Strain Px6-4 was identified as a Gram-negative rod without spore and was a facultative aerobe. This strain exhibited positive reactions for citrate utilization, gelatin hydrolysis test, and Voges-Proskauer test. Acid and gas were produced from D-glucose. Methyl red test was negative. According to these morphological and biochemical characteristics, strain Px6-4 was identified as belonging to genus *Enterobacter* sp. Px6-4 [22]. This identification was confirmed by its 16S rDNA sequencing (GenBank accession no. EF175731, Fig. 1). Strain Px6-4 has been deposited in the China General Microbiological Culture Collection Center (CGMCC1999).
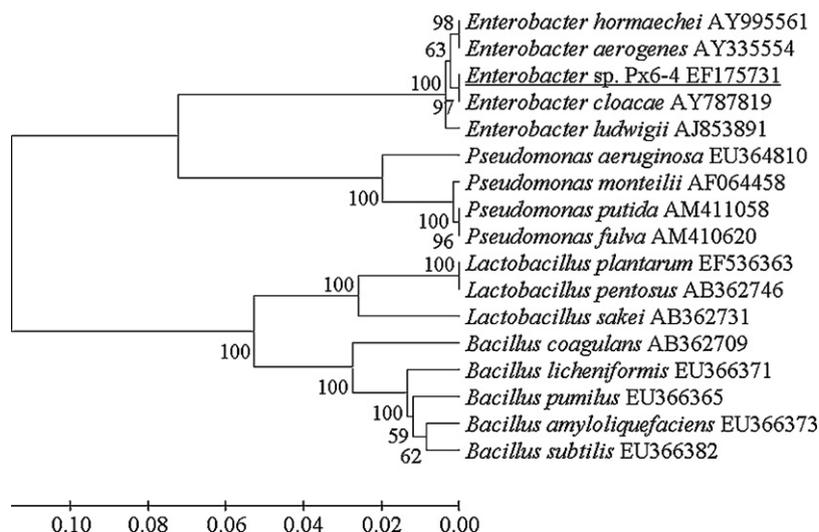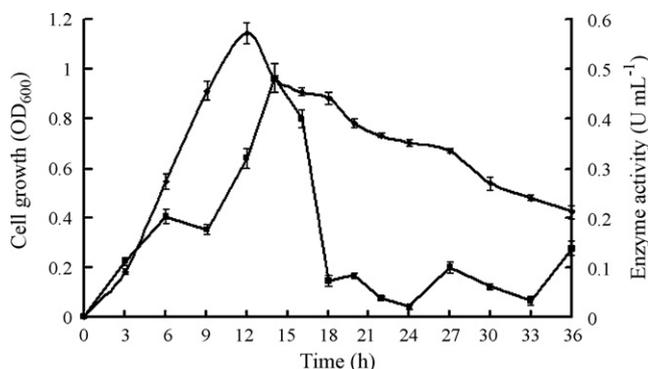

Fig. 1. Phylogenetic analysis of strain Px6-4 and related species by the neighbor-joining approach. Bootstrap values (%) are indicated at the nodes.

Fig. 2. The growth curve and the decarboxylase analysis. (◆) Cell growth (OD$_{600}$); (●) decarboxylase activity.

3.2. Ferulic acid decarboxylase analysis

Enterobacter sp. Px6-4 grew well in the VP medium. It grew very fast between 0 and 12 h, reaching the stationary phase at 12 h and then cell numbers start decreasing gradually (Fig. 2). However, the ferulic acid decarboxylase activity lagged slightly behind the bacterial growth pattern with the highest activity at 14 h after incubation. The activity then dropped rapidly.

3.3. Bioconversion of ferulic acid to vanillin by Enterobacter sp. Px6-4

The bioconversion of substrates and the production of main metabolites in the culture supernatant were quantified by HPLC and the changes of ferulic acid, vanillin, and 4-vinylguaiacol were shown in Fig. 3. A rapid degradation of ferulic acid was observed at a rate of 50.45 mg L^{-1} h^{-1} in the initial 12 h. In contrast, a large quantity of 4-vinylguaiacol (384.3 mg L^{-1}) and a small quantity of vanillin (9.96 mg L^{-1}) were accumulated. As the stationary phase progresses, the amount of vanillin gradually increased, coupled to the obvious decline of 4-vinylguaiacol. Interestingly, the amount of ferulic acid increased gradually after 12 h. The maximum accumulation of vanillin at 17.6 mg L^{-1} was achieved at 108 h. Coincidently, the minimum accumulation of 4-vinylguaiacol (166.2 mg L^{-1}) was detected in the culture at the same time.

3.4. Identification of volatile compounds in the culture by GC/MS

Eight volatile compounds were isolated and identified in the culture. Among them, six metabolites were ferulic acid-derived aroma chemicals, all of which have the same carbon skeleton of guaiacol. These six compounds are vanillin, 4-vinylguaiacol, 4-ethylguaiacol, 4-proylguaiacol, 4-acetylguaiacol and 4,4′-diguaiacol (Table 1).

When we replaced ferulic acid with 4-vinylguaiacol in the VP medium, the strain grew similarly to that in medium containing ferulic acid. TLC and GC/MS analyses (Table 1) demonstrated that the metabolic products of the two different substrates were similar. Our results suggest that ferulic acid was converted into vanillin using 4-vinylguaiacol as an intermediate.

3.5. Isolation and identification of non-volatile compounds in the culture

Three compounds were isolated from the concentrated extract of culture filtrate by silica gel chromatography and semi-preparative RP-HPLC. Compound A, an amorphous powder, was determined as C$_{10}$H$_{10}$O$_5$ by HREIMS ([M]$^+$ at m/z 194.18) and identified as ferulic acid. Compound B was a white amorphous
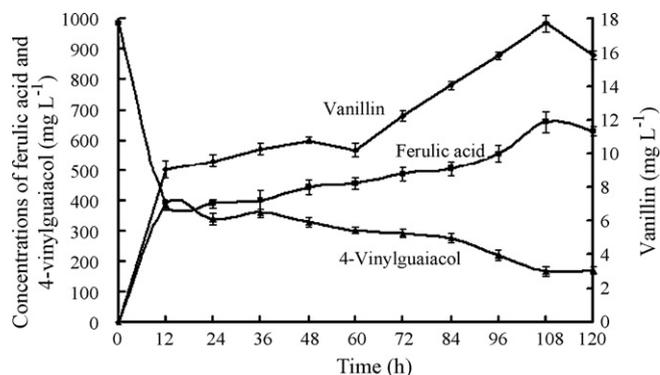
Fig. 3. Bioconversion of ferulic acid to Vanillin by *Enterobacter* sp. Px6-4. Changes in the levels of ferulic acid, 4-vinylguaiacol and vanillin in culture were quantified by HPLC. (◆) Vanillin; (■) ferulic acid; (▲) 4-vinylguaiacol.

powder [α]$_D^{19.1}$ 0 (c 1.374, CHCl$_3$). IR (KBr) ν$_{max}$ cm^{-1}: 3446, 3124, 1602, 1527, 1466, 1454, 1284, 1160, 1035, 824; EIMS (70 eV) m/z (rel. int.): 318 [M]$^+$ (10), 167 (6), 151 (100), 91 (33), 77 (9), 65 (7). The molecular formula for compound B was C$_{18}$H$_{22}$O$_5$ as determined by HREIMS ([M]$^+$ at m/z 318.37). The IR spectrum showed absorptions for a hydroxy group (3446 cm^{-1}) and an aromatic ring (1527, 1602 cm^{-1}). The ^{1}H and ^{13}C NMR data showed the presence of a 1,2,4-trisubstitued benzene ring (Table 2). The ^{1}H NMR spectrum displayed the presence of a Me at δ 1.40 (d, J = 6.40 Hz), a CH at δ 4.73 (q, J = 6.40 Hz), and a MeO at δ 3.74 (s). The fragment ion at ion m/z 151 ([M−167], 100) in the EIMS and the long-range correlation of C-1 (δ 144.5) with H-6′ (δ 6.76, d, J = 8.0 Hz) in the HMBC spectra indicated that C-1 was connected to C-1′ through the O bridge. Thus, compound B was identified as (±)-2-methoxy-4-(8-hydroxyethyl)-phenol-O-2-methoxy-4-(8-hydro-xyethyl)-phenol.

Compound C was obtained as a white amorphous powder [α]$_D^{19.1}$ +41.5 (c 0.189, CHCl$_3$). IR (KBr) ν$_{max}$ cm^{-1}: 3444, 1588, 1524, 1450, 1450, 1284, 1165, 1034, 820; EIMS (70 eV) m/z (rel. int.): 318 [M]$^+$ (7), 167 (10), 151 (100), 91 (35), 77 (10), 65 (7). The molecular formula of compound C was determined as C$_{18}$H$_{22}$O$_5$ by its HREIMS ([M]$^+$ at m/z 318.37). Careful inspection of the data of B and C (Table 2) indicated that the prominent difference between them was the replacement of the C(1)–O–C(1′) in B by a C(8)–O–C(8′) fragment in C. This was also confirmed by the HMBC correlations of H-8 (δ 4.30, d, J = 6.40 Hz) with C(8′) (δ 74.8), and H-8′ (δ 4.30, d, J = 6.40 Hz) with C(8) (δ 74.8), respectively. In combination with the positive optical degree, compound C was identified as (+)2-methoxy-4-(8R-hydroxyethyl)-phenol 8-O-β-2-methoxy-4-(8R-hydroxyethyl)-phenol.

3.6. The putative metabolic pathway of ferulic acid in Enterobacter sp. Px6-4

Based on the identification of volatile and non-volatile compounds in the culture, the proposed pathway for the degradation of ferulic acid in *Enterobacter* sp. Px6-4 was shown in Fig. 4. In this pathway, ferulic acid was decarboxylated with *Enterobacter* sp. Px6-4 to 4-vinylguaiacol, which could be hydrogenated to produce 4-ethylguaiacol, or be hydroxylated to yield a key intermediate 4-(1′-hydroxy) ethylguaiacol. There were four kinds of transformation pathways for 4-(1′-hydroxyl) ethylguaiacol, including the productions of 4-acetylguaiacol by dehydrogenation, vanillin by demethylation, and compounds (B and C) formed through intermolecular dehydration of two intermediates [4-(1′-hydroxyl)ethylguaiacol]. Compound B was formed through dehydration of two hydroxyl groups in an aromatic ring

Table 1
Volatile chemical compounds in the culture of which ferulic acid and 4-vinylguaiacol was the metabolic substrate, respectively

No.	Retention time (min)	Compounds	Molecular structures	Relative content (%)	
				Ferulic acid as substrate	4-Vinylguaiacol as substrate
1	1.92	1-Butenol	$CH_3CH_2(CH)_2OH$	0.34	1.07
2	2.24	3-Hydroxy-2-butenone	$CH_3COCH_2OHCH_3$	0.05	
3	10.25	4-Ethylguaiacol		0.75	0.68
4	11.13	4-Vinylguaiacol		72.31	81.38
5	12.01	Vanillin		0.63	0.69
6	12.74	4-Propylguaiacol		0.09	0.09
7	12.92	Unknown compound		6.34	2.39
8	13.10	4-Acetylguaiacol		0.08	
9	21.26	4,4′-Diguaiacol		1.63	0.25

Table 2
The ^1H and ^{13}C NMR data of compounds B and C*

Compound B   **Compound C**
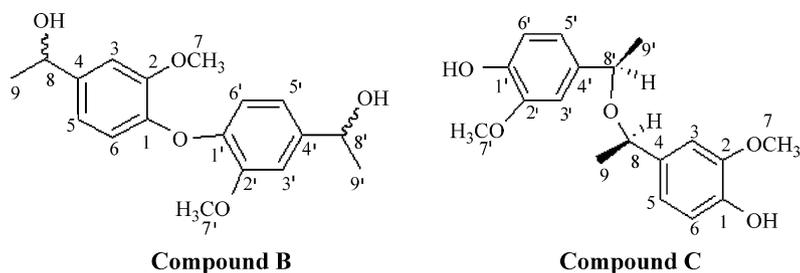
No.	Compound B		Compound C	
	^1H NMR	^{13}C NMR	^1H NMR	^{13}C NMR
1, 1′		144.5 (s)		145.0 (s)
2, 2′		146.5 (s)		146.7 (s)
3, 3′	6.84 (s, each 1H)	108.2 (d)	6.84 (d, J = 1.50 Hz)	108.5 (d)
4, 4′		137.6 (s)		136.1 (s)
5, 5′	6.72 (d, J = 8.06 Hz)	118.0 (d)	6.71 (dd, J = 1.50, 7.95 Hz)	119.5 (d)
6, 6′	6.76 (d, J = 8.06 Hz)	114.2 (d)	6.87 (d, J = 7.95 Hz)	114.1 (d)
7, 7′	3.74 (br s, each 3H)	55.5 (q)	3.90 (s, each 3H)	55.9 (q)
8, 8′	4.73 (q, J = 6.35 Hz)	69.8 (d)	4.17 (q, J = 6.50 Hz)	74.2 (d)
9, 9′	1.40 (d, J = 6.41 Hz)	24.7 (q)	1.36 (d, 6.50 Hz)	24.7 (q)

Data were recorded in C_5D_5N on Bruker AM-400 MHz and Bruker DRX-500 MHz spectrometers (^1H, ^{13}C); chemical shifts (δ) are given in parts per million. Compound B: ($\pm$)-2-methoxy-4-(8-hydroxyethyl)-phenol 1-O-2-methoxy-4-(8-hydroxyethyl)-phenol; Compound C: (+)-2-methoxy-4-(8R-hydroxyethyl)-phenol 8-O-β-2-methoxy-4-(8R-hydro-xyethyl)-phenol.
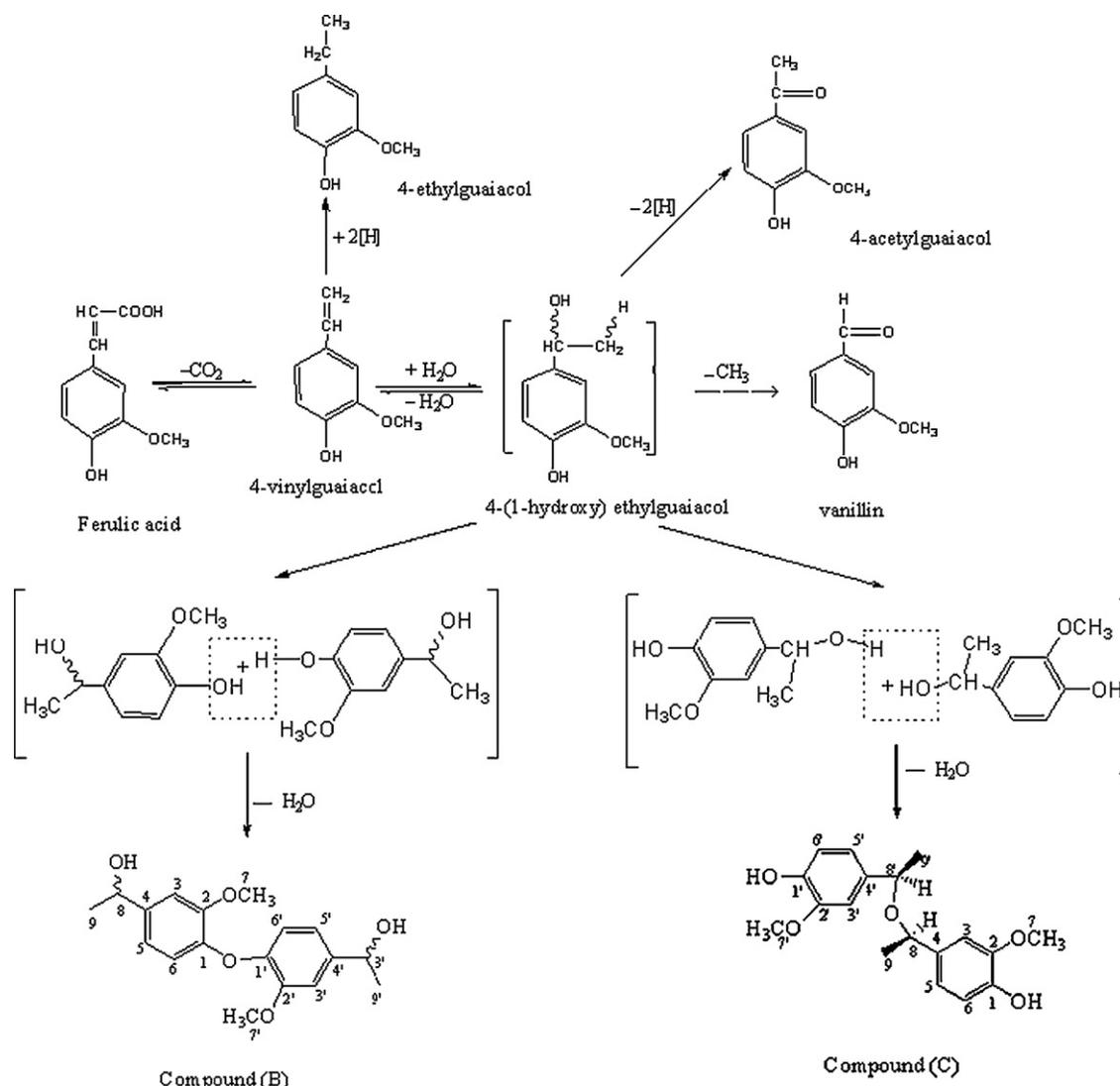
Fig. 4. The proposed pathway of bioconversion from ferulic acid to vanillin by *Enterobacter* sp. Px6-4.

respectively from two molecules of 4-(1′-hydroxyl)ethylguaiacol, while compound C through that of two hydroxyl groups of the phenolic substitute (1-hydroxy)ethyl groups. Compound C was an optical-isomer with reversal optical configurations. The isolation and identification of these compounds suggested that the presence of the key intermediate 4-(1′-hydroxy) ethylguaiacol be derived from the hydroxylation of 4-vinylguaiacol, which would be produced through decarboxylation of ferulic acid.

4. Discussion

In this paper, we described the isolation and characterization of an endophytic bacterium *Enterobacter* sp. Px6-4 capable of converting ferulic acid to vanillin. This appears to be a novel example of *Enterobacter* sp. having the ability to convert ferulic acid into vanillin. *Enterobacter* sp. Px6-4 can also degrade 4-vinylguaiacol and produce metabolites similar to ferulic acid (Table 1). This result suggests that ferulic acid can be converted to 4-vinylguaiacol through decarboxylation and be transformed to vanillin. The initial decarboxylation step is similar to that described in previous reports [13,14,23,24]. However, the downstream steps seem to be different from those of other microorganisms. Previous reports indicated that microorganisms could

convert ferulic acid into vanillin and the substrate was reduced in culture [12,24]. In our present results, *Enterobacter* sp. Px6-4 could produce the maximum amount of 4-vinylguaiacol in 12 h of ferulic acid degradation (Fig. 3), with the maximum activity of ferulic acid decarboxylase at 14 h after culturing (Fig. 2). The gradual increase of vanillin coincided with the increase of ferulic acid during extended culture. This result suggested that the decarboxylation of ferulic acid might be a reversible reaction in *Enterobacter* sp. Px6-4.

Recently, an increasing number of microbial species including those from bacteria, yeasts and filamentous fungi have been reported capable of degrading ferulic acid. Fungi can convert ferulic acid to vanillin by three pathways, including decarboxylation, reduction, and CoA-independent deacetylation [23,25,26]. And four major pathways of ferulic acid degradation have been found in different bacteria [8,13,20,27]. Moreover, certain actinomycetes can also convert ferulic acid to vanillin by three pathways including decarboxylation, CoA-independent deacetylation, and CoA-dependent deacetylation [9,28]. Interestingly, some microorganisms can degrade ferulic acid through multiple pathways. For example, *Pseudomonas fluorescens* can degrade ferulic acid by decarboxylation [23], reduction [27] and CoA-dependent deacetylation [7].

The further conversion of 4-vinylguaiacol has been investigated using $H_2^{18}O$ to demonstrate that bioconversion of 4-vinylguaiacol to 4-(1′-hydroxyethyl)guaiacol involved the addition of water to the vinyl group of vinylguaiacol [4]. Our result also indicated that a key intermediate 4-(1′-hydroxy)ethylguaiacol was formed through the addition of water to the vinyl group of vinylguaiacol. However, the more detailed metabolic process from 4-vinylguaiacol to vanillin needs to be confirmed by isotope analysis. Moreover, some microorganisms can convert ferulic acid to 4-vinylguaiacol, which is immediately converted to vanillin and further oxidized to vanillic acid, protocatechinc acid, and vanillyl alcohol, etc. [21,24,29]. In the bioconversion by *Enterobacter* sp. Px6-4, the above transformed metabolites of vanillin were not detected in the culture filtrate and two novel metabolites containing guaiacol-carbon-skeleton were detected instead. We suggest that the novel pathway described here might be partially responsible for the low yield of vanillin in this strain.

Acknowledgements

References

[1] Krings U, Berger RG. Biotechnological production of flavours and fragrances. Appl Microbiol Biotechnol 1998;49:1–8.

[2] Serra S, Fuganti C, Brenna E. Biocatalytic preparation of natural flavours and fragrances. Trends Biotechnol 2005;23:193–8.

[3] Walton NJ. Molecules of interest-vanillin. Phytochemistry 2003;63:505–15.

[4] Rosazza JPN, Huang Z, Dostal L, Volm T, Rousseau B. Biocatalytic transformations of ferulic acid: an abundant aromatic natural product. J Ind Microbiol 1995;15:457–71.

[5] Priefert H, Rabenhorst J, Steinbüchel A. Biotechnological production of vanillin. Appl Microbiol Biotechnol 2001;56:296–314.

[6] Mathew S, Abraham TE. Bioconversions of ferulic acid, an hydroxycinnamic acid. Crit Rev Microbiol 2006;32:115–25.

[7] Gasson MJ, Kitamura Y, Mclauchlan WR, Narbad A, Parr AJ, Lindsay E, et al. Metabolism of ferulic acid to vanillin. J Biol Chem 1998;273:4163–70.

[8] Overhage J, Priefert H, Steinbüchel A. Biochemical and genetic analyses of ferulic acid catabolism in *Pseudomonas* sp. HR199. Appl Environ Microbiol 1999;65:4837–47.

[9] Achterholt S, Priefert H, Steinbüchel A. Identification of *Amycolatopsis* sp. strain HR167 genes, involved in the bioconversion of ferulic acid to vanillin. Appl Microbiol Biotechnol 2000;54:799–807.

[10] Krings U, Pilawa S, Theobald C, Berger RG. Phenyl propenoic side chain degradation of ferulic acid by *Pycnoporus cinnabarinus*-elucidation of metabolic pathways using [5-^{2}H]-ferulic acid. J Biotechnol 2001;85:305–14.

[11] Plaggenborg R, Overhage J, Loos A, Archer JAC, Lessard P, Sinskey AJ, et al. Potential of *Rhodococcus* strains for biotechnological vanillin production from ferulic acid and eugenol. Appl Microbiol Biotechnol 2006;72:745–55.

[12] Huang ZX, Dostal L, Rosazza JPN. Microbial transformations of ferulic acid by *Saccharomyces cerevisiae* and *Pseudomonas fluorescens*. Appl Environ Microbiol 1993;59:2244–50.

[13] Cavin JF, Barthelmebs L, Guzzo J, Beeumen JV, Samyn B, Travers JF, et al. Purification and characterization of an inducible *p*-coumaric acid decarboxylase from *Lactobacillus plantarum*. FEMS Microbiol Lett 1997;147:291–5.

[14] Cavin JF, Dartois V, Diviès C. Gene cloning, transcriptional analysis, purification and characterization of phenolic acid decarboxylase from *Bacillus subtilis*. Appl Environ Microbiol 1998;64:1466–71.

[15] Coombs JT, Franco CMM. Isolation and identification of actinobacteria from surface-sterilized wheat roots. Appl Environ Microbiol 2003;69:5603–8.

[16] Dong XZ, Cai MY. Manual of identification for general bacteriology (Chinese). Beijing: Publishing Company of Science; 2001.

[17] Weisburg WG, Barns MS, Pelletier AD, Lnae JD. 16S ribosomal DNA amplification for phylogenetic study. J Bacteriol 1991;173:697–703.

[18] Saitou N, Nei M. The neighbor-joining method: a new method for reconstructing phylogenetic trees. Mol Biol Evol 1987;4:406–25.

[19] Kumar B, Tamura K, Jakobsen IB, Nei M. MEGA2: molecular evolutionary genetics analysis software. Tempe, AZ, USA: Arizona State University; 2001.

[20] Degrassi G, De Laureto PP, Bruschi CV. Purification and characterization of ferulate and *p*-coumarate decarboxylase from *Bacillus pumilus*. Appl Environ Microbiol 1995;61:326–32.

[21] Rahouti M, Seigle-Murandi F, Steiman R, Eriksson KE. Metabolism of ferulic acid by *Paecilomyces variotii* and *Pestalotia palmarum*. Appl Environ Microbiol 1989;55:2391–8.

[22] Holt JG, Krieg NR, Sneath PHA, Staley JT, Williams ST. Bergey's manual of determinative bacteriology. Philadelphia, PA: Lippincott Williams & Wilkins; 1994.

[23] Huang ZX, Dostal L, Rosazza JPN. Purification of a ferulic acid decarboxylase from *Pseudomonas fluorescens*. J Bacteriol 1994;176:5912–8.

[24] Karmakar B, Vohra RM, Nandanwar H, Sharma P, Gupta KG, Sobti RC. Rapid degradation of ferulic acid via 4-vinylguaiacol and vanillin by a newly isolated strain of *Bacillus coagulans*. J Biotechnol 2000;80:195–202.

[25] Nazareth S, Mavinkurve S. Degradation of ferulic acid via 4-vinylguaiacol by *Fusarium solani* (Mart) Sacc. Can J Microbiol 1986;32:494–7.

[26] Falconnier B, Lapierre C, Lesage-Meessen L, Yonnet G, Brunerie P, Colonna-Ceccaldi B, et al. Vanillin as a product of ferulic acid biotransformation by the white-rot fungus *Pycnoporus cinnabarinus* I-937: identification of metabolic pathways. J Biotechnol 1994;37:123–32.

[27] Martinez-Cuesta MC, Payne J, Hanniffy SB, Gasson MJ, Narbad A. Functional analysis of the vanillin pathway in a vdh-negative mutant strain of *Pseudomonas fluorescens* AN103. Enzyme Microb Technol 2005;37:131–8.

[28] Muheim A, Lerch K. Towards a high-yield bioconversion of ferulic acid to vanillin. Appl Microbiol Biotechnol 1999;51:456–61.

[29] Priefert H, Rabenhorst J, Steinbüchel A. Molecular characterization of genes of *Pseudomonas* sp. strain HR199 involved in bioconversion of vanillin to protocatechuate. J Bacteriol 1997;179:2595–607.