JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Metabolic Characterization of Newly Isolated *Pseudomonas nitroreducens* Jin1 Growing on Eugenol and Isoeugenol

TATSUYA UNNO,[†] SOO-JIN KIM,[†] ROBERT A. KANALY,[‡] JOONG-HOON AHN,[§] SU-IL KANG,[||] AND HOR-GIL HUR*

Department of Environmental Science and Engineering and International Environmental Research Center, Gwangju Institute of Science and Technology, Gwangju, Korea, Department of Environmental Biosciences, Yokohama City University, Yokohama, Japan, Department of Bioscience and Biotechnology, Bio/Molecular Informatics Center, Konkuk University, Seoul 143-701, Korea, and International Environmental Research Center, Gwangju Institute of Science and Technology, Gwangju, Korea

Newly isolated soil bacterium strain Jin1 was able to grow on both eugenol and isoeugenol each as sole source of carbon and energy. Based on bacterial 16S rDNA analysis, Jin1 belongs to *Pseudomonas nitroreducens* with a similarity of 98.92% (14/1297). *P. nitroreducens* Jin1 was found to biotransform eugenol and isoeugenol to vanillin by different pathways. Eugenol was biotransformed to vanillin through coniferyl alcohol and ferulic acid similarly to the pathway shown previously by *Pseudomonas*sp. HR199 and vanillin produced from eugenol was rapidly metabolized to vanillic acid. Contrastively, *Pseudomonas nitroreducens* Jin1 did not appear to produce metabolic intermediates during the biotransformation of isoeugenol to vanillin which was finally biotransformed to vanillic acid with much slower rate. These results indicate that there seems to be different metabolic regulation systems for the biotransformation of eugenol and isoeugenol by this bacterium. Herein, we report on *Pseudomonas nitroreducens* Jin1, a novel bacterium that produces vanillin from eugenol and isoeugenol by two different metabolic pathways.

KEYWORDS: Biotransformation; eugenol; isoeugenol; vanillin; *Pseudomonas nitroreducens*

INTRODUCTION

Vanillin (4-hydroxy-3-methoxybenzaldehyde) is widely used in foods, beverages, perfumes, and pharmaceuticals, and in various medical industries. Natural vanillin extracted from botanical sources represents approximately only 0.2 % of the global market and costs 3000 USD/kg, whereas chemically synthesized vanillin costs 11 USD/kg (*1*). The demand for natural flavors is growing and production of vanillin from natural raw materials by biotransformation processes is becoming attractive because the product can be regarded as a natural aromatic chemical. One of the methods by which to produce natural vanillin is to biotransform both eugenol and isoeugenol. Eugenol and isoeugenol are thought to be derived from lignin precursors (*2, 3*) and are major constituents of essential oil from clove trees, and they are sold relatively cheaply (*4*). Recently, production of eugenol and isoeugenol by sweet basil and petunia flowers was characterized (*2*), and considering this, it may be possible to engineer plants to produce vanillin from eugenol and isoeugenol with expression of bacterial genes involved in the metabolism of eugenol and isoeugenol.

Many bacteria have been isolated and found to metabolize eugenol. Among them, *Pseudomonas*sp. HR199 was the most intensively studied at the genetic and biochemical levels for its ability to metabolize eugenol to coniferyl alcohol, ferulic acid, vanillin, vanillic acid, and protocatechuate (*5*). However, there has been little specific information available in regard to the metabolism of isoeugenol by bacteria. To date, it has been shown that certain strains of *Bacillus* (*Subtilis* B2 and HS8 and *fusiformis* CGMCC1347) and *Pseudomonas* (*putida* I58 and IE27) metabolize isoeugenol and produce vanillin or vanillic acid (*6–9*), but more research is needed.

Pseudomonas nitroreducens Jin1 isolated from soil was found to metabolize eugenol and isoeugenol each as sole sources of carbon and energy to produce vanillin as a metabolite, and to our knowledge this is the first report of a bacterium that shows this metabolic capability. Vanillin produced from isoeugenol by *P. nitroreducens* Jin1 was stably maintained in the medium, which was not observed in the metabolism of eugenol. We report herein on strain Jin1, a novel strain in terms of its metabolic capability for the biotransformation of eugenol and isoeugenol

* Corresponding author phone: +82-62-970-2437; fax: +82-62-970-2434 e-mail: hghur@gist.ac.kr.
[†] Department of Environmental Science and Engineering.
[‡] Yokohama City University.
[§] Konkuk University.
[||] International Environmental Research Center.

10.1021/jf0715929 CCC: $37.00
Published on Web 09/15/2007

Table 1. Time to Reach $OD_{600} = 0.2$ on the Different Growth Substrates for *P. nitroreducens* Jin1 without or with Induction by Eugenol, Isoeugenol, and Vanillin, Respectively

induction conditions	time (h) to reach $OD_{600} = 0.2$ with growth substrate					
	eugenol	isoeugenol	coniferyl alcohol	ferulic acid	vanillin	vanillic acid
no induction	10.7 (±0.5)[a]	15.2 (±0.3)	5.6	10.0 (±1.0)	7.5 (±0.3)	5.1 (±0.1)
eugenol	4.2 (±0.2)	9.8 (±0.2)	3.5 (±0.2)	8.9 (±0.3)	4.3 (±0.1)	3.4
isoeugenol	6.4 (±0.5)	9.5 (±0.4)	4.5 (±0.1)	10.5 (±0.4)	7.6	3.8 (±0.1)
vanillin	6.1	10.5 (±0.8)	4.6 (±0.4)	10.7 (±0.1)	5.0 (±0.1)	3.8

[a] Mean value ± standard deviation of duplicates.

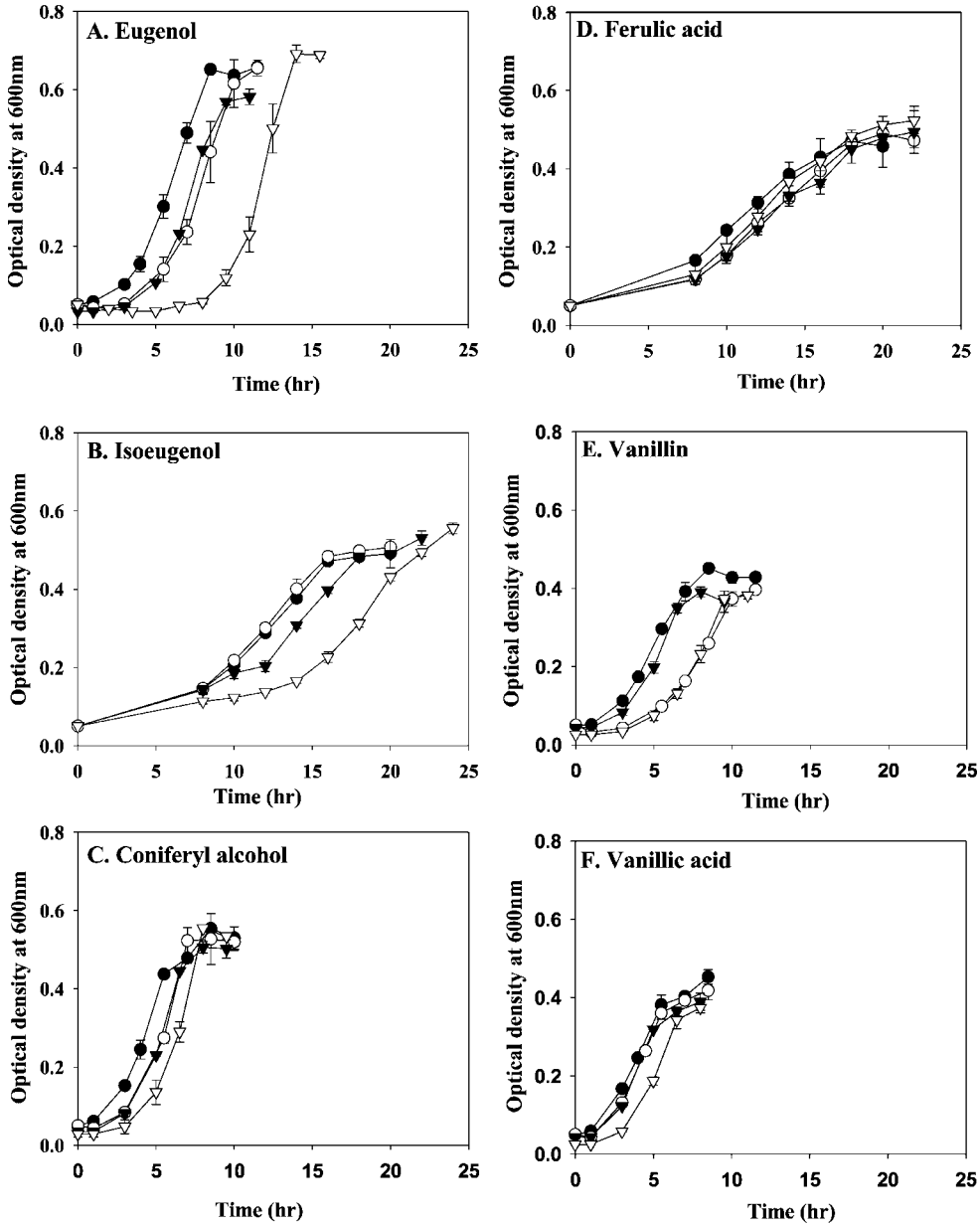


Figure 1. Growth curves in 3 mM of eugenol (**A**), isoeugenol (**B**), coniferyl alcohol (**C**), ferulic acid (**D**), vanillin (**E**), and vanillic acid (**F**) for *P. nitroreducens* Jin1 without (-▽-) or with induction by eugenol (-●-), isoeugenol (-○-), or vanillin (-▼-), respectively.

and discuss the possibility of natural vanillin production by a single bacterial strain.

MATERIALS AND METHODS

Chemicals. Eugenol, coniferyl alcohol, vanillin, and vanillic acid were purchased from Aldrich (Milwaukee, WI). Isoeugenol and ferulic acid were purchased from Sigma (St. Louis, MO). Organic solvents (HPLC grade) were obtained from Fisher Scientific (Fair Lawn, NJ).

Bacterial Isolation and Identification. Soil from a rice field in Jangseong, South Korea, was used to isolate bacteria capable of growing on eugenol and isoeugenol. The soil (5 g) was suspended in 100 mL of Stainer's basal minimal salt buffer (MSB) (*10*) containing 3 mM of eugenol and isoeugenol, respectively, and incubated with shaking at 200 rpm at 27 °C in the dark. After approximately 10 transfers of the bacterial culture using the same growth conditions, the culture solution was spread on nutrient agar plates. Twenty colonies were isolated, and reinoculated into MSB medium containing 1 mM eugenol and iso-

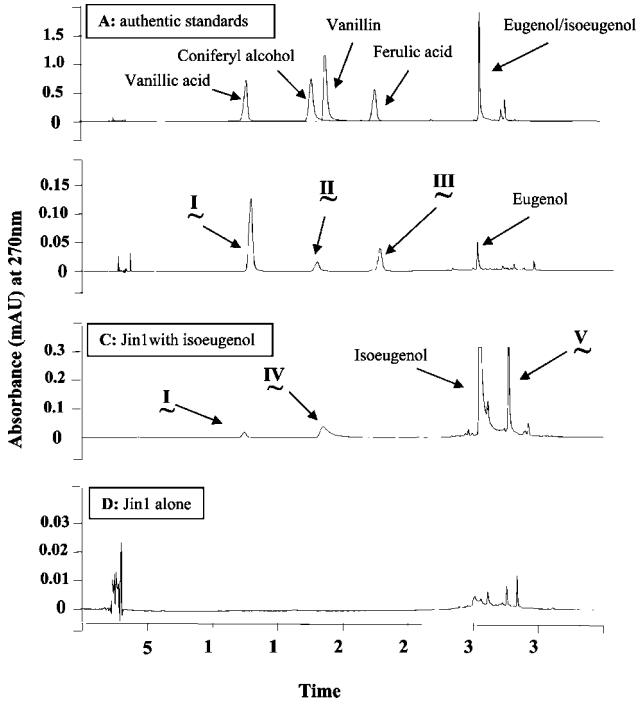


Figure 2. Elution profiles of HPLC chromatograms from eugenol and isoeugenol metabolism analyses. Authentic chemicals (**A**), and cultures of *P .nitroreducens* Jin1 grown with eugenol (**B**), and isoeugenol (**C**), and without eugenol or isoeugenol (**D**) in MSB medium.

eugenol, respectively. One colony capable of utilizing eugenol and isoeugenol as a sole source of carbon and energy was isolated and named Jin1. Chromosomal DNA was extracted from strain Jin1 using the procedure of Pitcher et al. (*11*) as modified by Chun and Goodfellow (*12*). The 16S rDNA was amplified with universal primers; 27F (AGAGTTTGA TCMTGGCTCAG) and 1492R (GGY TACCTTGT-TACGACTT). The PCR program used was as follows: 95 °C for 15 min, followed by 30 cycles of 94 °C for 1 min, 55 °C for 1 min, and 72 °C for 1 min, and a final extension step at 72 °C for 7 min. Amplified 16S rDNA fragments were purified by a PCR fragment purification kit (Bioneer, Daejeon, Korea), and the sequence was determined at Takara Korea Biomedical Inc. (Seoul, Korea). Sequence similarity searches were performed using the web-based program BLAST at http://www.ncbi.nlm.nih.gov/. The sequence information was then imported into the PHYDIT program http://plaza.snu.ac.kr/~jchun/phydit/ (*12*) for further assembly and alignment. The sequence of 16S rDNA for strain Jin1 was then compared to sequences of type strains registered in the GenBank database. The program was also used to calculate nucleotide substitution rates.

High-Performance Liquid Chromatography (HPLC). Analytical HPLC was performed using a Varian ProStar HPLC equipped with a photodiode array (PDA) detector (Varian, Walnut Creek, CA) and a reverse phase C18 column (5 μm particle size, 4.6 mm × 25 cm, Spherisorb, Waters, Milford, MA). The mobile phase consisted of acetonitrile and water containing 0.1% formic acid as follows: 10% acetonitrile at 0 min, 20% acetonitrile at 20 min, 40% acetonitrile at 25 min, 90% acetonitrile at 30 min, 10 min washing with 90% acetonitrile at 40 min. Twenty μL from each sample was injected, the flow rate was 1 mL/min, and UV detection was performed at 270 nm.

Liquid Chromatography–Mass Spectrometry (LC–MS). LC–MS was performed by coupling an Alliance 2695 instrument (Waters, Milford, MA) with a Quattro LC triple quadrupole tandem mass spectrometer (Waters, Milford, MA) in negative electrospray ionization (ESI^-) mode. The elution program was the same as employed for analytical HPLC. The flow rate was set at 0.1 mL/min and UV detection was performed with photodiode array detector 2996 (Waters, Milford, MA). Ten μL was set for injection volume. The source temperature, desolvation temperature, cone voltage, capillary voltage, and electron multiplier voltage were set at 150 °C, 350 °C, 25 V, 3.0 kV, and 700 V, respectively. Nitrogen gas was used as the nebulizer gas and set at 30 L/h and ultrapure nitrogen gas was used as the desolvation gas and set at 500 L/h.

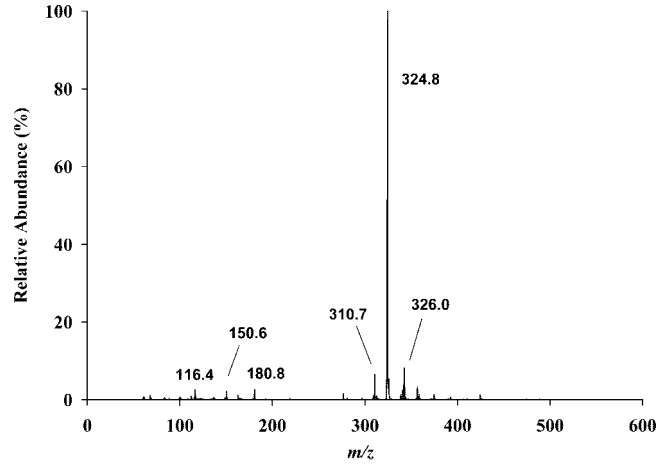


Figure 3. LC–MS spectrum of metabolite V produced from isoeugenol by *P. nitroreducens* Jin1.

Effect of Induction on Bacterial Growth. To investigate the effect of induction on growth of strain Jin1, the strain was cultured in 50 mL of nutrient broth overnight with additions of eugenol, isoeugenol, and vanillin each at final concentrations of 1 mM. The overnight culture was washed twice with the MSB medium, and adjusted to give an OD_{600} = 0.05 in 30 mL of MSB medium. Eugenol, isoeugenol, and possible metabolites such as coniferyl alcohol, ferulic acid, vanillin, and vanillic acid were then added to the MSB medium at final concentrations of 3 mM. The incubations were performed at 27 °C in the dark with shaking at 200 rpm. Bacterial growth was determined by OD_{600} every two hours until the end of the exponential phase.

Whole Cell Reaction for Biotransformation. The whole cell reaction assay was performed to identify metabolites produced from eugenol and isoeugenol by strain Jin1. First, overnight cultures were harvested, washed twice with MSB medium, and adjusted to OD_{600} = 1.0 in MSB medium. Thirty mL of MSB medium containing 3 mM of eugenol and isoeugenol each were incubated at 27 °C in the dark with shaking at 200 rpm. Two mL of the bacterial cultures were extracted with 5 mL of ethyl acetate and evaporated to dryness with an Automated Environmental Speed Vac AES1010 (ThermoProd, Riviera Beach, FL). Dried samples were resuspended in 1 mL of methanol, filtered, and analyzed by HPLC. Concentrations of the metabolites were calculated from the peak area of the HPLC chromatogram using standard curves prepared from each authentic substrate. Thirty mL of MSB medium containing 3 mM eugenol and isoeugenol each were also prepared without bacteria as abiotic controls under both neutral (pH 7) and acidic conditions (pH 4). *Escherichia coli* strain JM109 was also inoculated instead of strain Jin1 as a biological control.

RESULTS AND DISCUSSION

Bacterial Identification. Based on the PHYDIT analysis of 1450 bp of the full 16S rDNA sequence obtained from the gram negative bacterium Jin1, the sequence best matched to *Pseudomonas nitroreducens* with similarity of 98.92% (14/1297), followed by *Pseudomonas citronellolis* (97.41%; 37/1430) and *Pseudomonas alcaligenes* with (96.50%; 50/1429). The sequence was registered to GenBank (GenBank accession no. EF534986).

Effect of Induction and Carbon Sources on Growth Of *P. nitroreducens* Strain Jin 1. Time to reach OD_{600} = 0.2 was used to compare induction effects of eugenol, isoeugenol, and vanillin on the growth of *P. nitroreducens* strain Jin 1 with eugenol, isoeugenol, and their possible metabolites, coniferyl alcohol, ferulic acid, vanillin, and vanillic acid (**Table 1**). The most conceivable induction effect on the growth of strain Jin1

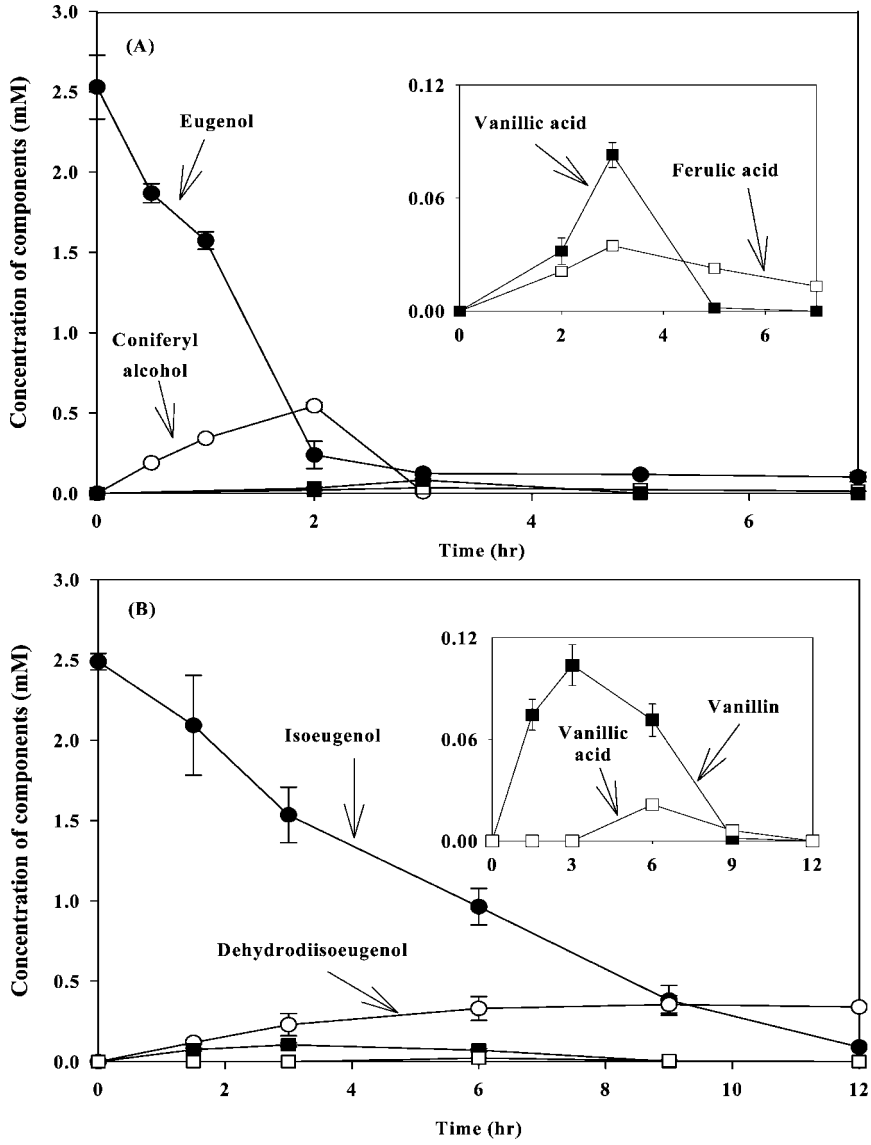


Figure 4. Biotransformation kinetics and metabolites of eugenol (**A**) and isoeugenol (**B**)by whole cell cultures of *P .nitroreducens* Jin1. **A**: eugenol (-●-), coniferyl alcohol (-○-), ferulic acid (-□-), and vanillic acid (-■-). Ferulic acid and vanillic acid are also shown in inset with the same legend; **B**: isoeugenol (-●-), dehydrodiisoeugenol (-○-), vanillin (-■-) and vanillic acid (-□-). Vanillin and vanillic acid are also shown in inset with the same legend.

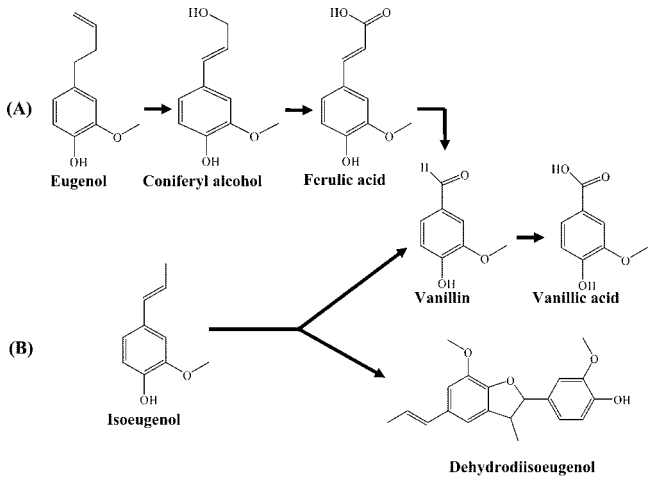
was found with the MSB medium containing eugenol in which time to reach $OD_{600} = 0.2$ was less than half of that of the control experiment. Induction effects of isoeugenol and vanillin were fairly well observed. As found in **Figure 1**, isoeugenol and ferulic acid showed lower growth rate with doubling time about 6 h and the rest of four substrates showed similar growth rate with a doubling time of about 3 h. In all the substrates except for eugenol, exponential growth stopped at approximately $OD_{600} = 0.4 \sim 0.5$. The highest OD_{600} observed was 0.70 in the MSB medium containing eugenol.

Identification of Metabolites Found in the Metabolism of Eugenol and Isoeugenol by *P. nitroreducens* Jin1. Metabolites of eugenol and isoeugenol, which were found in the bacterial cultures, were identified using retention time on the HPLC chromatograms (**Figure 2**), UV–visible spectroscopy (data not shown) and molecular weight detected by LC–MS as compared to those of the corresponding authentic compounds. Metabolites I, II, and III having $[M–H]^-$ ion at *m/z* 167, 179, and 193, respectively, were accumulated in the bacterial culture with eugenol and found to be vanillic acid, coniferyl alchol, and ferulic acid, respectively. Bacterial culture with isoeugenol produced metabolites IV having $[M–H]^-$ ion at *m/z* 150 as well as metabolite I. Metabolite IV was considered to be vanillin. Another metabolite V having $[M–H]^-$ ion at *m/z* 325 was accumulated in the bacterial culture with isoeugenol and appeared to be dehydrodiisoeugenol as suggested in the earlier study (**Figure 3**) (*9*)

Biotransformation Kinetics of Eugenol and Isoeugenol by Whole Cell Culture of *P. nitroreducens* Jin1. Biotransformation kinetics of eugenol, isoeugenol, and metabolites by whole cell culture of strain Jin1 are shown in **Figure 4**. To calculate the concentration of dehydrodiisoeugenol (not commercially available) found during isoeugenol metabolism, we assumed that the molar absorptivity (ε) of dehydrodiisoeugenol is two times that of isoeugenol at 270 nm.

As detected by HPLC, the initial concentration of 2.5 mM eugenol decreased rapidly for the first 2 h while coniferyl alcohol concomitantly increased up to 0.52 mM. Ferulic acid and vanillic acid were then detected in the medium, and the concentrations increased up to 0.035 and 0.083 mM, respectively, by 3 h of incubation. All of the compounds were mostly depleted by 5 h of incubation (**Figure 4A**). However in the case of isoeugenol,

Scheme 1. Proposed Metabolic Pathways of Eugenol (**A**) and Isoeugenol (**B**) by *P. nitroreducens* Jin1.



starting concentration of 2.5 mM, bacterial metabolism to approximately 0.1 mM occurred after 12 h of incubation (**Figure 4B**). As strain Jin1 consumed isoeugenol, vanillin and vanillic acid were detected in the medium with vanillin reaching a maximum concentration of 0.1 mM by 3 h of incubation and vanillic acid reaching a maximum concentration of 0.02 mM by 6 h of incubation. Vanillin and vanillic acid were fully metabolized in the medium by strain Jin1 after 12 h of incubation (**Figure 4**).

Meanwhile, dehydrodiisoeugenol produced by the metabolism of isoeugenol by *P. nitroreducens* Jin1 appeared not to be further metabolized as reported previously (*9*). The increase of dehydrodiisoeugenol was inversely proportional to the concentration of isoeugenol, which consequently caused low yields of vanillin. Production of dehydrodiisoeugenol may have been due to consequences of unknown metabolic activity of strain Jin1 because it was not produced from isoeugenol in abiotic controls under different pH conditions nor in *E. coli* controls.

Metabolic Pathway of Eugenol and Isoeugenol in *P. nitroreducens* Strain Jin1. Pathways of eugenol and isoeugenol biotransformation by strain Jin1 are proposed in **Scheme 1**. The pathway was also confirmed by feeding each postulated metabolite to cultures of strain Jin1. Vanillin was not observed in the bacterial culture with 3 mM of eugenol. However, vanillin was detected when 3 mM of the slow-growth substrate ferulic acid was fed as a single source of carbon and energy (data not shown). On the contrary, vanillin was observed at least for 6 h in the culture of strain Jin1 containing isoeugenol. Moreover, isoeugenol induction effects on vanillin metabolism by strain Jin1 did not result in a shortening of the time to reach $OD_{600} = 0.2$ as compared to the control experiments (**Table 1**). Therefore, different gene induction systems may be involved in the eugenol-vanilln and isoeugenol–vanillin metabolisms for strain Jin1. The pathways shown in **Scheme 1** are the same pathways as found in *Pseudomonas*sp. HR199 (*5*) for eugenol metabolism and also similar to that of *Bacillus subtilis* HS8 (*9*) for isoeugenol metabolism. Although some bacteria have been reported to transform isoeugenol into vanillin, the genes involved in this metabolism have not been identified yet. However, the eugenol metabolic pathway of *Pseudomonas*sp. HR199 has been well studied on the molecular level and was engineered to produce natural vanillin (*13–15*). Contrastively, isoeugenol has not received much attention for the microbial production of natural vanillin due to isoeugenol toxicity and insolubility. In this study, optimization to produce higher amount of vanillin was not particularly developed; however, it may be possible by addition of organic solvents such as DMSO to dissolve sparingly soluble isoeugenol or to supply additional nutrition to enhance metabolism. For example, Yamada et al. (*8*) optimized culture conditions for *P. putida* IE27 by adding 0.1 % (*v/v*) glycerol and 1% (*w/v*) yeast extract and reported that vanillin-producing activity from isoeugenol was increased from 2.01 nmol min^{-1} ml^{-1} to 210 nmol min^{-1} ml^{-1}. They have also reported that the use of 10% (*v/v*) DMSO in culture promoted vanillin-producing activity from isoeugenol to 114%. Other studies have also shown high conversions of isoeugenol. For example, 20 mg of resting cells (as dry matter) of *Pseudomonas putida* I58 converted 10 mM of isoeugenol into vanillic acid through vanillin with 98% yield in 40 min (*6*) and cell free extracts of *Bacillus subtilis* B2 biotransformed 12.8 gL^{-1} (approximately 78 mM) isoeugenol into vanillin with 14% yield in 96 h without optimization (*7*). It was suggested that vanillin production occurs by a pathway from isoeugenol through isoeugenol diol (*9*). Much research has shown the capability of biotransforming eugenol and isoeugenol separately, yet there has not been discussion in regard to the possibility of producing vanillin from both eugenol and isoeugenol simultaneously. In the future, genes involved in the two metabolic pathways need to be identified and characterized for further understanding of the mechanism. The biosynthesis mechanisms of eugenol and isoeugenol by sweet basil and petunia flowers were well characterized (*2*) which implies the possibilities of production of vanillin in plants from eugenol and isoeugenol with expression of genes of strain Jin1 described in this study. Therefore, we conclude that the use of strain Jin1 could provide a biologically economical tool in related industries.

LITERATURE CITED

(1) Priefert, H.; Rabenhorst, J.; Steinbuchel, A. Biotechnological production of vanillin. *Appl. Microbiol. Biotechnol.* **2001**, *56* (3–4), 296–314.

(2) Koeduka, T.; Fridman, E.; Gang, D. R.; Vassao, D. G.; Jackson, B. L.; Kish, C. M.; Orlova, I.; Spassova, S. M.; Lewis, N. G.; Noel, J. P.; Baiga, T. J.; Dudareva, N.; Pichersky, E. Eugenol and isoeugenol, characteristic aromatic constituents of spices, are biosynthesized via reduction of a coniferyl alcohol ester. *Proc. Natl. Acad. Sci. U. S. A.* **2006**, *103* (26), 10128–33.

(3) Wang, J.; Dudareva, N.; Bhakta, S.; Raguso, R. A.; Pichersky, E. Floral scent production in Clarkia breweri (Onagraceae). II. Localization and developmental modulation of the enzyme S-adenosyl-L-methionine:(iso)eugenol O-methyltransferase and phenylpropanoid emission. *Plant Physiol.* **1997**, *114* (1), 213–21.

(4) Walton, N. J.; Narbad, A.; Faulds, C.; Williamson, G. Novel approaches to the biosynthesis of vanillin. *Curr. Opin. Biotechnol.* **2000**, *11* (5), 490–6.

(5) Rabenhorst, J. Production of methoxyphenol-type natural aroma chemicals by biotransformation of eugenol with a new *Pseudomonas*sp. *Appl. Microbiol. Biotechnol.* **1996**, *46*, 470–474.

(6) Furukawa, H.; Morita, H.; Yoshida, T.; Nagasawa, T. Conversion of isoeugenol into vanillic acid by *Pseudomonas putida* I58 cells exhibiting high isoeugenol-degrading activity. *J. Biosci. Bioeng.* **2003**, *96* (4), 401–3.

(7) Shimoni, E.; Ravid, U.; Shoham, Y. Isolation of a *Bacillus*sp. capable of transforming isoeugenol to vanillin. *J Biotechnol* **2000**, *78* (1), 1–9.

(8) Yamada, M.; Okada, Y.; Yoshida, T.; Nagasawa, T. Biotransformation of isoeugenol to vanillin by *Pseudomonas putida* IE27 cells. *Appl. Microbiol. Biotechnol.* **2007**, *73* (5), 1025–30.

(9) Zhang, Y.; Xu, P.; Han, S.; Yan, H.; Ma, C., Metabolism of isoeugenol via isoeugenol-diol by a newly isolated strain of *Bacillus subtilis* HS8. *Appl. Microbiol. Biotechnol.* **2006**.

(10) Stainer, R. Y.; Palleroni, N. J.; Duodoroff, M. The aerobic pseudomonads: a taxonomic study. *J. Gen. Microbiol.* **1966**, *43*, 159–271.

(11) Pitcher, D. G.; Saunder, N. A.; Owen, R. J. Rapid extraction of bacterial genomic DNA with guanidium thiocyanate. *Lett. Appl. Microbiol.* **1989**, *8*, 151–156.

(12) Chun, J.; Goodfellow, M. A phylogenetic analysis of the genus *Nocardia* with 16S rRNA gene sequences. *Int. J. Syst. Bacteriol* **1995**, *45* (2), 240–5.

(13) Overhage, J.; Priefert, H.; Rabenhorst, J.; Steinbuchel, A. Biotransformation of eugenol to vanillin by a mutant of *Pseudomonas*sp. strain HR199 constructed by disruption of the vanillin dehydrogenase (*vdh*) gene. *Appl. Microbiol. Biotechnol* **1999**, *52* (6), 820–8.

(14) Overhage, J.; Priefert, H.; Steinbuchel, A. Biochemical and genetic analyses of ferulic acid catabolism in *Pseudomonas*sp. Strain HR199. *Appl. Environ. Microbiol.* **1999**, *65* (11), 4837–47.

(15) Overhage, J.; Steinbuchel, A.; Priefert, H. Biotransformation of eugenol to ferulic acid by a recombinant strain of *Ralstonia eutropha* H16. *Appl. Environ. Microbiol.* **2002**, *68* (9), 4315–21.

Received for review May 30, 2007. Revised manuscript received August 1, 2007. Accepted August 2, 2007. This study was carried out with the support of "On-Site Cooperative Agriculture Research Project (project no. 20070101080021)", RDA, Republic of Korea and the Korea Research Foundation Grant funded by the Korean Government (MOEHRD) (project no. 420035) to Tatsuya Unno in GIST, Korea.

JF0715929