J Korean Soc Appl Biol Chem (2013) 56, 125–133
DOI 10.1007/s13765-013-3025-9

REVIEW ARTICLE

Bacterial Biotransformation of Phenylpropanoid Compounds for Producing Flavor and Fragrance Compounds

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Received: 30 January 2013 / Accepted: 22 February 2013 / Published Online: 30 April 2013

Abstract Phenylpropanoids are common aromatic compounds synthesized by plants that are often used as starting compounds for the production of various flavor and fragrance compounds. The use of bacterial metabolism as a means to produce value-added compounds from natural resources has been given much attention as an alternative method to replace conventional chemical syntheses. This review describes bacterial metabolisms of the phenylpropanoid compounds *trans*-anethole, isoeugenol, and isosafrole to better understand efficient production of natural fragrance and other value-added compounds.

Keywords biomass · biotransformation · isoeugenol · phenylpropanoid · *trans*-anethole · vanillin

Introduction

The phenylpropanoids are organic compounds synthesized by plants from the precursor amino acid phenylalanine (Humphreys and Chapple, 2002) (Fig. 1). These compounds serve as essential components of a number of structural polymers such as lignin compounds, provide protection from ultraviolet light, defend against herbivores and pathogens, and mediate plant-pollinator interactions as floral pigments and scent compounds. Phenylpropanoid compounds and their metabolic intermediates produced by microorganisms have been used traditionally by the flavor and fragrance industries as starting materials for chemical synthesis of aromatic chemicals (Krings and Berger, 1998; Serra et al., 2005).

Flavors and fragrances widely used by the food, beverage, and cosmetic industries show worldwide market of $22 billion dollars per year in 2010 and is growing steadily. Due to the high cost and unavailability of natural flavor extracts, most commercial flavors and fragrances are nature-identical, which means they are the chemical equivalent of natural flavors but chemically synthesized rather than being extracted from the source materials. Natural flavor and fragrance compounds have been mostly obtained from plants. Since the characterization and identification of their chemical structures as well as effectiveness, various chemical synthesis methods targeting the compounds have been developed to substitute for natural compounds. Thus, currently most of the flavor aromatic compounds are produced via chemical synthesis and has occupied up to 80% of total production of the flavors and fragrances (Krings and Berger, 1998). However, the chemical synthesis often produces unexpected environmentally unfriendly by-products during the synthetic processes.

As society develops, consumers prefer to use food additives that are labeled as a “natural” (Longo and Sanroman, 2006) and natural food flavors were used in Germany as much as 70% in 1990 (Krings and Berger, 1998). Although the price difference between synthetic flavors and natural flavors is remarkable, consumers prefer to use natural and healthy flavors. As an example, the prices of the chemically synthesized natural-identical vanillin and natural vanillin extracted from vanillin pods is about $12 kg^{-1} and $4,000 kg^{-1} respectively. For this, the conventional method using plants to produce the natural compounds is still effective but has some disadvantages such as low concentration of the desired compounds in plants, high dependent productivity on irresistible factors such as weather conditions and plant disease (Longo and Sanroman, 2006).

To overcome the problems mentioned above, the biotechnological

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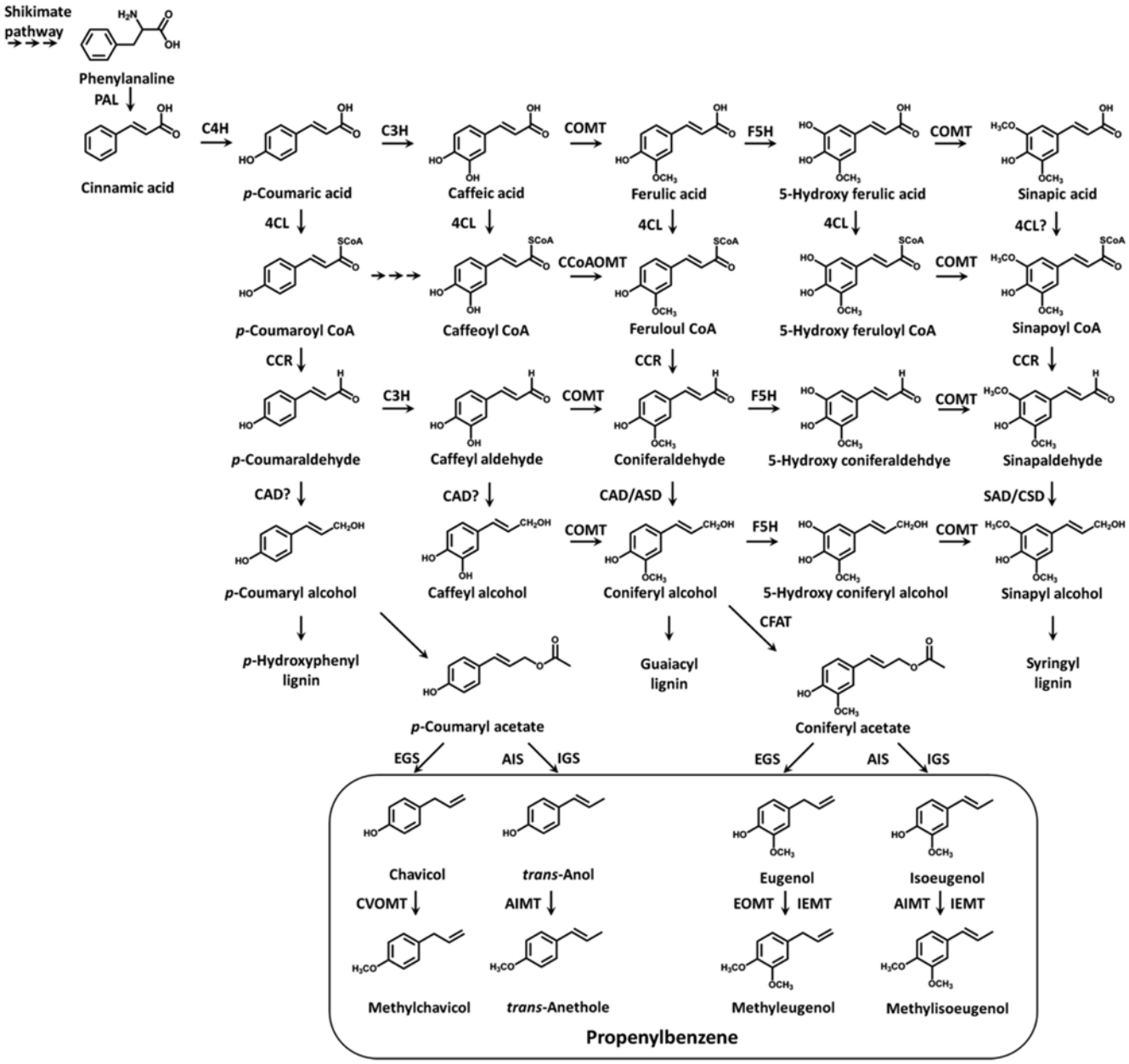

Fig. 1 Expanded phenylpropanoid pathway in plants. 4CL, 4-hydroxycinnamoyl CoA ligase; C3H, *p*-coumarate 3-hydroxylase; C4H, cinnamate 4-hydroxylase; CAD, cinnamyl alcohol dehydrogenase; CCoAOMT, caffeoyl CoA *O*-methyltransferase; CCR, cinnamoyl CoA reductase; COMT, caffeic acid/5-hydroxyferulic acid *O*-methyltransferase; F5H, ferulate 5-hydroxylase; PAL, phenylalanine ammonia lyase; pCCoA3H, *p*-coumaryl CoA 3-hydroxylase; SAD, sinapyl alcohol dehydrogenase; CFAT, coniferyl acetyltransferase; EGS, eugenol synthase; IGS, isoeugenol synthase; AIS, *trans*-anol/isoeugenol synthase; CVOMT, chavicol *O*-methyltransferase; AIMT, *trans*-anol/isoeugenol *O*-methyltransferase; EOMT, eugenol *O*-methyltransferase; IEMT, isoeugenol/eugenol *O*-methyltransferase.

production of natural compounds has recently drawn more attention to the researchers. The methods of biotechnological production for flavor aromatic compounds has been classified as a "natural" by the European and US food legislation (Krings and Berger, 1998). Apart from agricultural farming, scaled up and industrial-scale production using biotechnological tools such as enzyme engineering or up-regulation of metabolisms are applicable (Krings and Berger, 1998). In fact, advantages in biotransformation and bioconversion are expected to be inexpensive and readily available products from renewable natural precursors, such as phenylpropanoids, fatty acids, and amino acids. In addition, biotransformation and bioconversion can introduce chirality and functionalization into chemically inert carbons, selective modifications, and resolution of racemates (Krings and Berger,

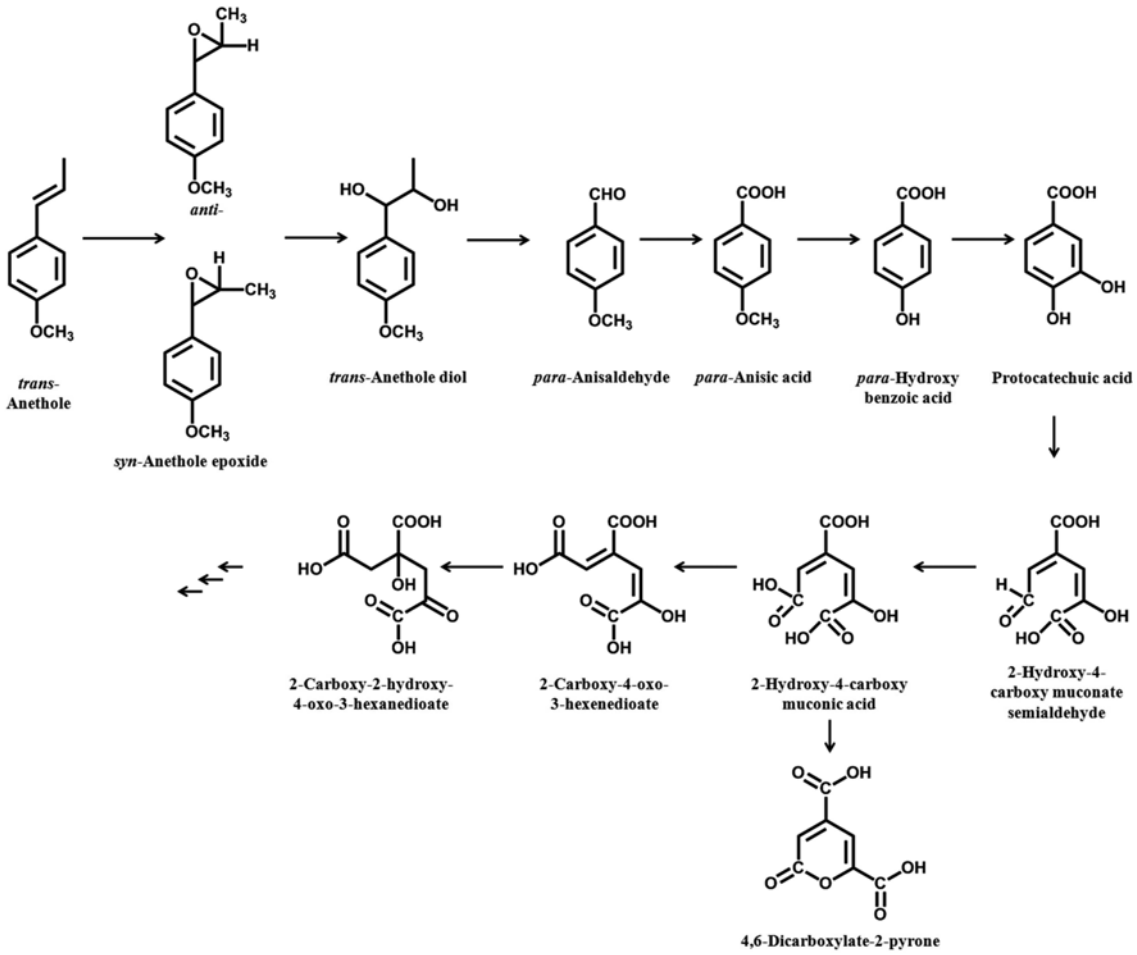

Fig. 2 Biotransformation pathway of *trans*-anethole combined from strains *Pseudomonas putida* JYR-1 and *Arthrobacter* TA13.

1998), which cannot be easily performed by the conventional chemical methods. In this review, bacterial metabolisms of the phenylpropanoid compounds *trans*-anethole, isoeugenol, eugenol, vanillin, and isosafrole are introduced for a better understanding of the efficient production process of natural fragrance and other value-added compounds.

Bacterial Biotransformation of Phenylpropanoid Compounds

Biotransformation of *trans*-anethole. *trans*-Anethole (*p*-methoxy propenylbenzene), the essential oils of anise, fennel, and star anise, is also a type of phenylpropanoid compound formed by terpene synthesis in plants (Newberne et al., 1999). *trans*-Anethole is used commercially as a flavor substance in baked goods, candy, ice cream, chewing gum, and alcoholic beverages (Shimoni et al., 2003). However, there have been few research reports regarding its metabolism (Shimoni et al., 2002; 2003; Passreiter et al., 2004; Kurlemann et al., 2009; Wohlgemuth, 2010).

To date, two bacterial strains, *Arthrobacter* sp. TA13 and *Pseudomonas putida* JYR-1 have been known to use *trans*-anethole as a sole carbon source (Shimoni et al., 2002; Ryu et al., 2005) (Fig. 2). *Arthrobacter* sp. TA13, which is pre-induced by *trans*-Anethol, was capable of growing on anisic alcohol, *p*-anisaldehyde, *p*-anisic acid, *p*-hydroxybenzoic acid, and protocatechuic acid as sole carbon sources (Shimoni et al., 2002). In addition, mutants of *Arthrobacter* sp. TA13 have been introduced and found to accumulate three metabolic intermediates, *trans*-anethole-diol, anisic acid, and 4,6-dicarboxylate-2-pyrone in high amounts, among which four mutants can accumulate trace amounts of anisic alcohol and anisaldehyde. From these results, metabolic pathway of *trans*-anethole by *Arthrobacter* sp. TA13 was postulated that *trans*-anethole was first to be biotransformed into anisic alcohol via an epoxide and diol. The alcohol was further biotransformed to the corresponding anisaldehyde compound or directly biotransformed to the diol, then to anisaldehyde. In addition, during biotransformation of *trans*-anethole by *P. putida* JYR-1, two stereoisomeric epoxides, *syn*- and *anti*-anethole-2,3-epoxides, were identified as metabolic intermediates (Ryu et al.,

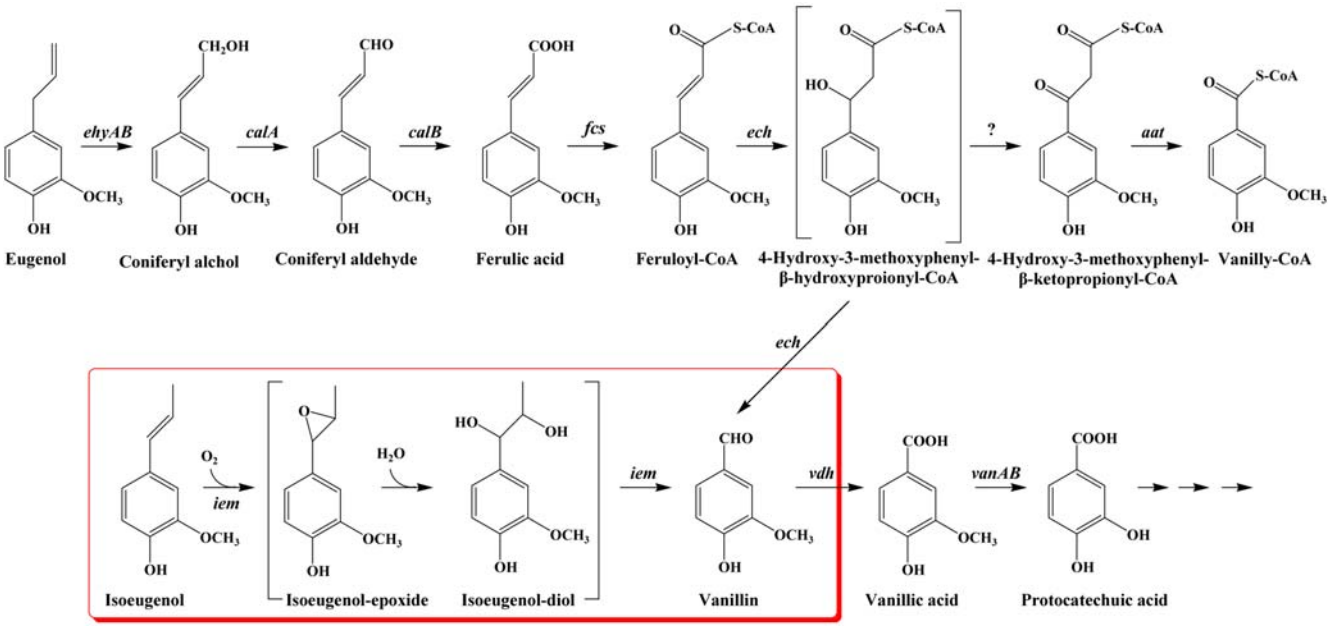

Fig. 3 Bacterial biotransformation pathways of eugenol and isoeugenol. The *ehyAB, calA, calB, fcs, ech, vdh*, *vdhAB, aat,* and *iem* encode eugenol hydroxylase, coniferyl alcohol dehydrogenase, coniferyl aldehyde dehydrogenase, feruloyl-CoA-synthetase, enoyl-CoA-hydratase/aldolase, vanillin dehydrogenase, vanillate-*O*-demethylase, β-ketothiolase, and isoeugenol monooxygenase, respectively (adapted from Ryu et al., 2010).

2005). *p*-Anisic acid and *p*-hydroxybenzoic acid were also detected in the culture medium of *P. putida* JYR-1(Ryu et al., 2005).

Two bacterial strains, *Arthrobacter* sp. TA13 and *P. putida* JYR-1 can also convert various aromatic compounds, whose structures are closely related to that of *trans*-anethole. *Arthrobacter* sp. TA13 was capable of utilizing estragole as the sole carbon source (Shimoni et al., 2003). On the other hand, *Arthrobacter* sp. TA13 failed to utilize eugenol, isoeugenol, safrole or isosafrole as the sole carbon source (Shimoni et al., 2003), *Arthrobacter* sp. TA13 pre-grown on glucose in the presence of isoeugenol, eugenol, isosafrole, and safrole can convert each substrate into vanillin and vanillic acid, ferulic acid, piperonylic acid, and hydroxychavicol (Shimoni et al., 2003). Metabolites of each compounds (except safrole) indicated that strain TA13 could initiate the oxidation of the side chains of propenylbenzene compounds (Shimoni et al., 2003). The absence of specific demethylase to remove the methoxy group at position 3 of the aromatic ring, *Arthrobacter* sp.TA13 probably cannot make cleavage of the benzene ring, resulting in no utilization of the aromatic compounds (Shimoni et al., 2003). In contrasts, *P. putida* JYR-1 was able to utilize not only caffeic acid and *p*-coumaric acid but also isoeugenol and ferulic acid, both of which contain methoxy group at the 3-position of benzene ring as the sole carbon source. *P. putida* JYR-1, however, could not metabolize eugenol by resting cells grown on *trans*-anethole. For understanding the mechanism, genes involved in *trans*-anethole biotransformation were characterized (Han et al., 2012a; 2012b). The genes responsible for the metabolism of *trans*-anethole to protocatechuic acid were cloned and placed on a plasmid, pTA163, approximately 34-kb sized gene fragment from *P. putida* JYR-1 in *Escherichia coli* was obtained (Fig. 4). When the open reading frame (ORF) 19 of the plasmid pTA163 was mutated with Tn5, the ability to catalyze *trans*-anethole was lost. Once ORF 10 (1047 nt) under a T7 promoter in *E. coli* was heterologously expressed, oxidative cleavage of a propenyl group of *trans*-anethole to an aldehyde group was catalyzed, resulting in the production of *para*-anisaldehyde; this gene was designated as *trans*-anethole oxygenase (*tao*). The deduced amino acid sequence of TAO had the highest identity (34%) to a hypothetical protein of *Agrobacterium vitis* S4 and likely contained a flavin-binding site. With ^{18}O-labeled H_2O and O_2, TAO showed preferred incorporation of an oxygen molecule from water into *p*-anisaldehyde, indicating stereopreference of TAO for hydrolysis of the epoxide group (Fig. 5). In addition, TAO from *P. putida* JYR-1 showed relaxed substrate range covering isoeugenol, *O*-methyl isoeugenol, and isosafrole, all of which contain the 2-propenyl functional group on the aromatic ring structure, unlike the extremely narrow substrate range of isoeugenol monooxygenase from *P. putida* IE27 and *Pseudomonas nitroreducens* Jin1. The activity of TAO increased with addition of NAD(P)H to the ultrafiltered cell extracts of *E. coli* (pTA163), suggesting the presence of NADH-dependent oxygenase enzyme. With the relaxed substrate range and single component oxygenase of TAO, the system could have strong possibility for the production of various fragrance compounds from plant phenylpropanoids in the future (Han et al., 2012b).

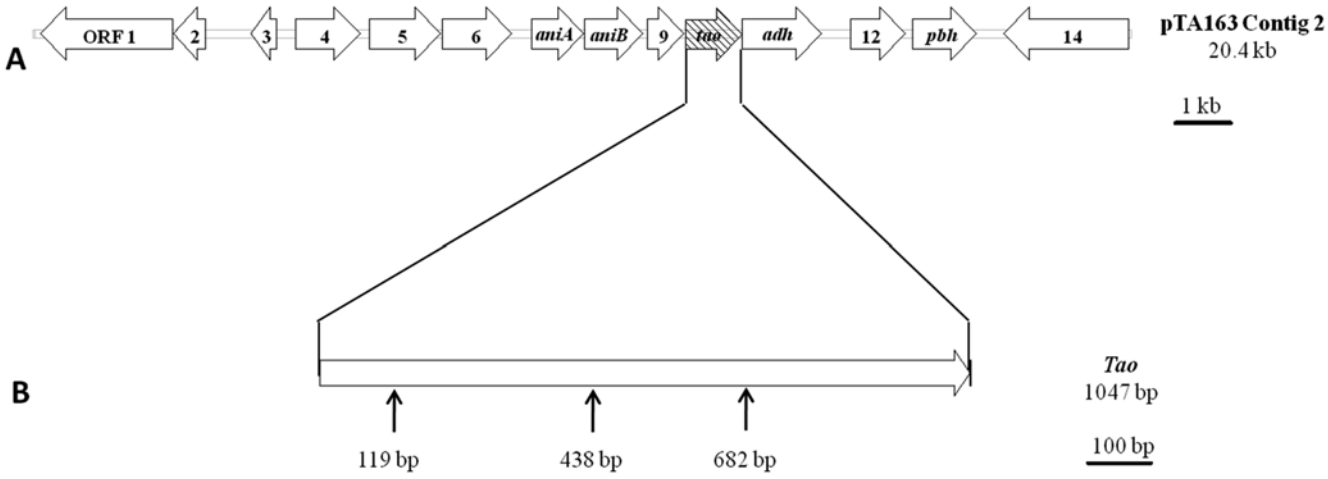

Fig. 4 Contig 2 of fosmid clone pTA163, which contains *P. putida* JYR-1 genomic DNA fragment (A), and *tao* gene with arrow marks on the sites of *Tn5* transposon insertions (B) (adapted from Han et al. 2012).

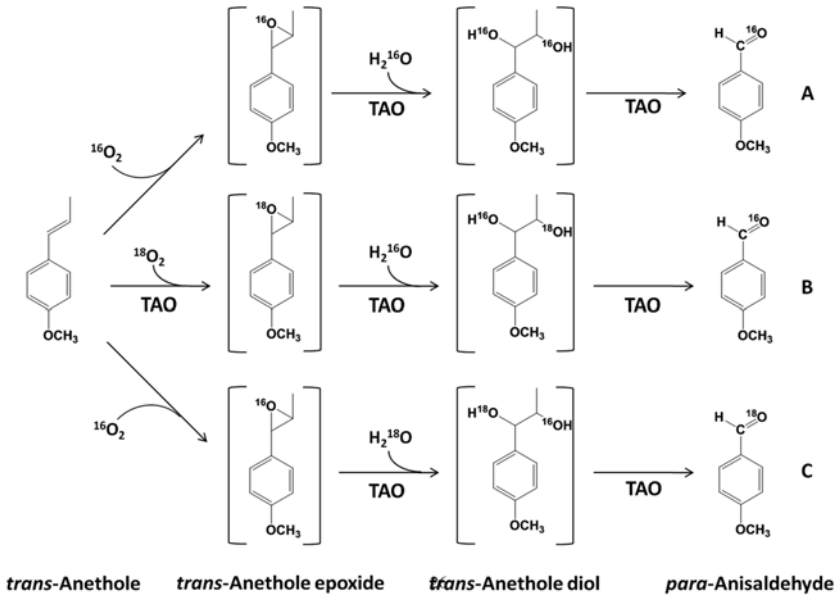

Fig. 5 *trans*-Anethole side chain cleavage reaction by TAO of *P. putida* JYR-1 (adapted from Han et al., 2012).

Biotransformation of isoeugenol. *Pseudomonas*, *Bacillus*, *Brevibacillus*, *Psychrobacter*, and *Nocardia* (Zhao et al., 2005; Zhang et al., 2006; Kasana et al., 2007; Unno et al., 2007; Yamada et al., 2007; Seshadri et al., 2008; Ryu et al., 2010; Ashengroph et al., 2012; Wangrangsimagul et al., 2012), and yeast *Candida* (Ashengroph et al., 2011) can biotransform 1-propenylbenzene such as isoeugenol (Fig. 3). Although Seshadri et al. (2008) proposed several isoeugenol degradation pathways, only one pathway, in which isoeugenol was converted to vanillin via epoxide and diol intermediates by resting cell of *Nocardia iowensis* DSM 45197, was substantially proven. *Bacillus pumilus* and *Bacillus subtilis* HS8 can also produce two intermediates, isoeugenol epoxide and isoeugenol diol during biotransformation reaction from isoeugenol to vanillin (Zhang et al., 2006, Hua et al., 2007). In our laboratory, a soil bacterium *Pseudomonas nitroreducens* strain Jin1 was isolated and was able to grow on both eugenol and isoeugenol as sole sources of carbon and energy. Metabolites from the two compounds suggest different pathways may be involved in the biotransformation of eugenol and isoeugenol to vanillin by *P. nitroreducens* Jin1 (Fig. 3). Eugenol was biotransformed to vanillin through coniferyl alcohol and ferulic acid similarly to the pathway suggested previously by *Pseudomonas* sp. HR199, and vanillin produced from eugenol was rapidly metabolized to vanillic acid. However, during the biotransformation of isoeugenol to vanillin by *P. nitroreducens* Jin1, metabolic intermediates were not present. In addition, biotransformation of vanillin to vanillic

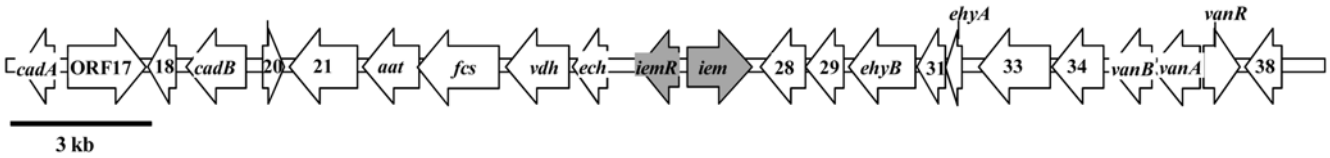

Fig. 6 Gene localization for *iem* in *Pseudomonas nitroreducens* Jin1. ORF 27 encodes isoeugenol monooxygenase (Iem). ORF17, 18, 20, 21, 28, 29, 31, 33, 34, and 38 are annotated as transcriptions-activator-protein, TetR family transcriptional regulator, transcriptions-regulator-protein, methyl-accepting chemotaxis protein, alcohol dehydrogenase, predicted nucleoside-diphosphate-sugar epimerase 1, hypothetical protein, putative gamma-glutamylcysteine synthetase, putative formaldehyde dehydrogenase, glutathione-dependent, and predicted nucleoside-diphosphate-sugar epimerase 2, respectively. ORF26 is annotated as isoeugenol monooxygenase regulator (*iemR*) (adapted from Ryu et al., 2010).

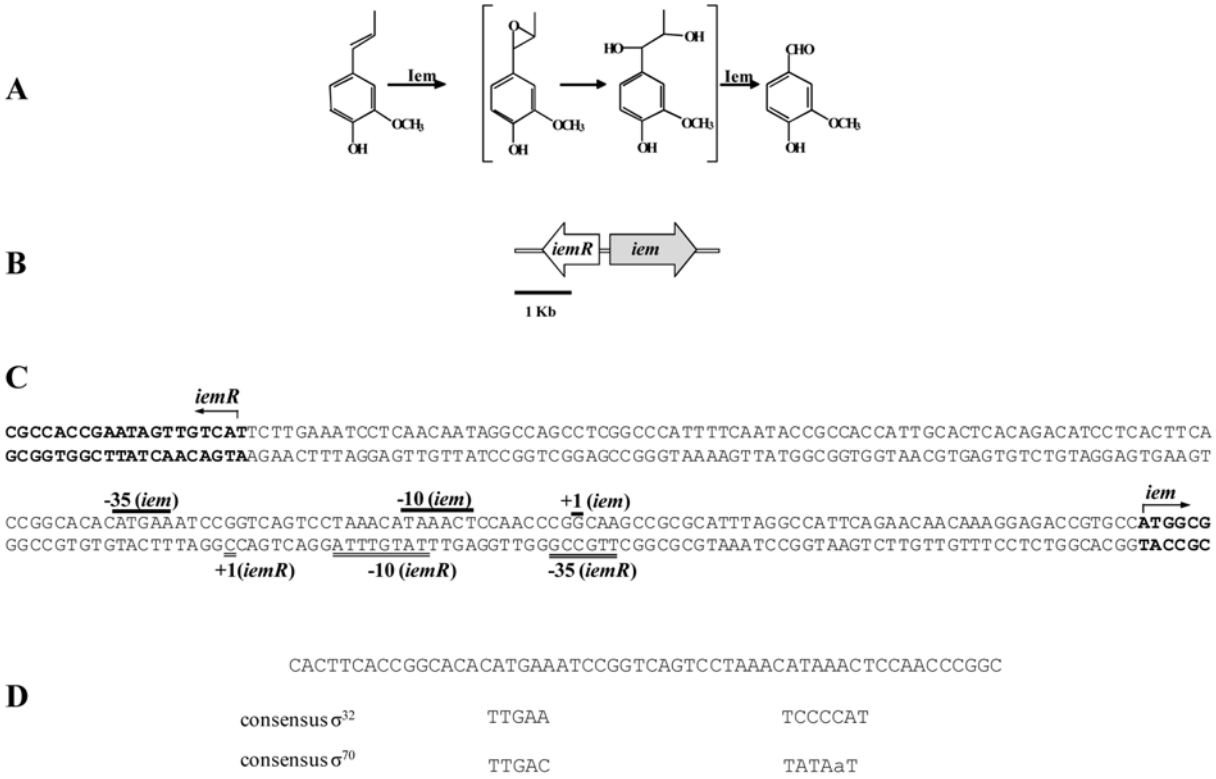

Fig. 7 Biotransformation of and gene arrangement for isoeugenol to vanillin by isoeugenol monooxygenase of *P. nitroreducens* Jin1. Biotransformation pathway of isoeugenol to vanillin by isoeugenol monooxygenase (Iem) (A), the gene arrangement (B) and predicted promoter sequence (C) of isoeugenol monooxygenase regulator (*iemR*) and *iem* genes (B) (adapted from Ryu et al., 2012).

acid occurred in much slower rate (Unno et al., 2007). The genes involved in the metabolism of eugenol and isoeugenol were cloned into plasmid p1500 in *E. coli*, which were clustered in the region of about 30 kb of *P. nitroreducens* Jin1 (Fig. 6). Two ORFs 26 (*iemR*) and 27 (*iem*) out of 23 ORFs in this region were predicted to be involved in the conversion of isoeugenol to vanillin. The deduced amino acid sequence of isoeugenol monooxygenase (Iem) of *P. nitroreducens* strain Jin1 had 81.4% identity to isoeugenol monooxygenase from *P. putida* IE27, which also transforms isoeugenol to vanillin, was heterologously expressed in *E. coli* BL21(DE3), and was found to biotransform isoeugenol to vanillin. Deletion and sub-cloning analyses indicated that expression of *iem* in the presence of isoeugenol required the gene *iemR*, located upstream of *iem,* suggesting protein of *iemR* could be the *iem* positive regulatory gene. IemR contained a putative helix-turn-helix motif in its C-terminal part, representative for members of the AraC/XylS family transcriptional regulators (Tropel and van der Meer, 2004) with about 22.4% amino acid sequence identity to *cad* gene transcriptional regulator from *Bradyrhizobium* sp. HW13. The genes involved in the metabolism of eugenol and isoeugenol were differently induced by isoeugenol, eugenol, and vanillin, based on the reverse transcription, real-time polymerase chain reaction (PCR) analyses (Ryu et al., 2010).

We investigated factors potentially affecting the expression of *iem* in a heterologous expression system, which are the types of chemical inducers,and the transcriptional regulator IemR. The

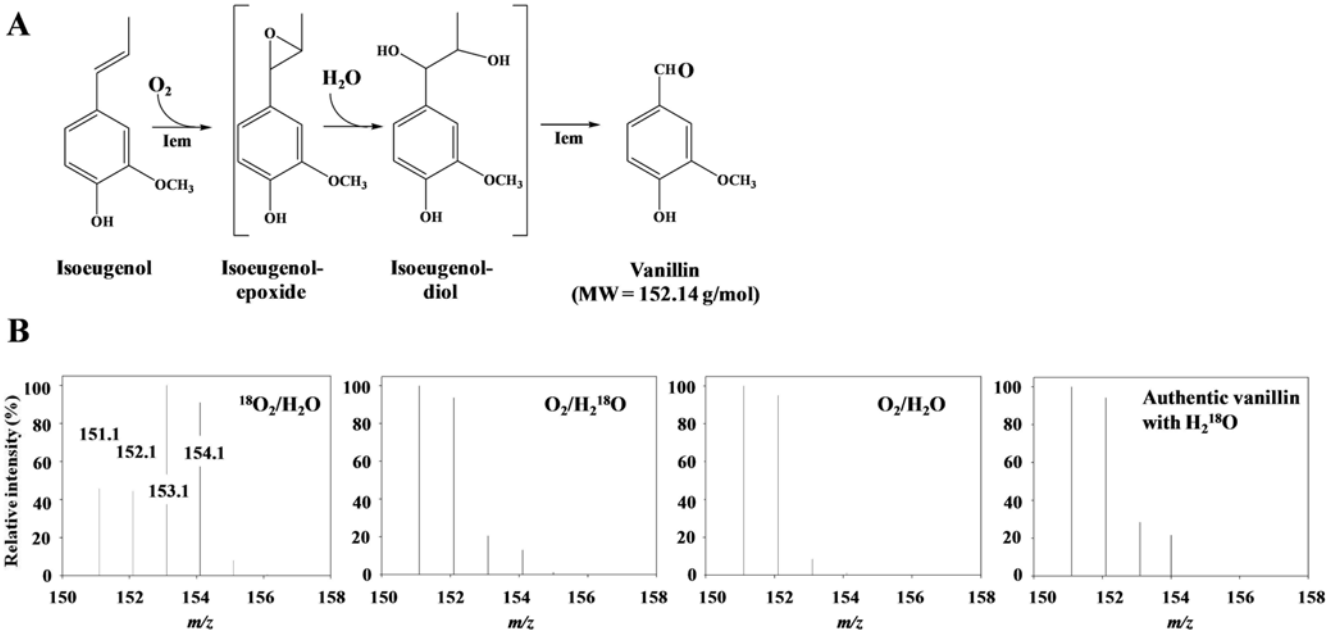

Fig. 8 Oxidative cleavage of isoeugenol to vanillin by Iem (A) and determination of the origin of oxygen located in vanillin by GC/MS of vanillin (B) (adapted from Ryu et al., 2013).

positive regulatory protein IemR of *P. nitroreducens* strain Jin1 is divergently expressed from Iem, and the promoter region is located between the genes (Fig. 7). The levels of isoeugenol and IemR affected transcription of *iem,* and isoeugenol was found to be the best inducer for expressing *iem*, followed by *trans*-anethole, which induced *iem* to 58% of the transcription level observed for isoeugenol. The transcription of *iem* was significantly increased with overproduction of IemR in *E. coli* , up to 96-fold, even in the absence of isoeugenol, as compared to basally expressed IemR, an indication that the transcription of *iem* is dependent on the type of inducers and on IemR. This basic research suggest that the development of bioengineering strategies for increased production of vanillin through high-level expression of the isoeugenol monooxygenase gene in microorganisms (Ryu et al., 2012) could be possible. Furthermore, isoeugenol monooxygenase (Iem) was overexpressed in *E. coli* and purified. Based on the ^{18}Oxygen-labeling experiment, Iem catalyzed the oxidative cleavage of isoeugenol via a monooxygenation reaction and incorporated oxygen atom from O_2 into vanillin, which is preferable to the one from water (Fig. 8). The Iem did not require addition of any metal cofactors. When four conserved histidines (His^{167}, His^{218}, His^{282}, and His^{471}) of Iem, which are known to be associated with Fe^{2+} ligands in the apocarotenoid-15,15'-oxygenase (ACO) of *Synechocystis* sp. PCC 6803, were replaced with alanine by site-directed mutagenesis, Iem lost the activity. We have found Iem activity was inhibited by preincubation with high concentrations of chelators, and addition of iron ions did not affect the activity, indicating tight binding of iron to the Iem. This was proven with inductively coupled plasma mass spectroscopy (ICP-MS) analysis, which showed 0.7 mol of iron per mol of Iem. These results implied that Iem is an iron-containing oxygenase and iron metal was involved in the Iem reaction (Ryu et al., 2013). Due to the advantage of catalytic property, production of vanillin from isoeugenol by utilizing isoeugenol monooxygenase expressed in *E. coli* has been well studied (Priefert et al., 2001; Barghini et al., 2007; Yamada et al., 2008; Kaur and Chakraborty, 2013).

Biotransformation of eugenol. *Pseudomonas* and *Rhodococcus* can biotransform 2-propenylbenzene, eugenol, to vanillin through a different pathway from isoeugenol (Overhage et al., 1999; Plaggenborg et al., 2006; Jin et al., 2007) (Fig. 3). The bacteria first initiate hydroxylation reaction on the 2-propenyl side chain of eugenol to produce coniferyl alcohol by eugenol hydroxylase (EhyAB), which is then oxidized to coniferyl aldehyde by coniferyl alcohol dehydrogenase (CalA), and further oxidized to ferulic acid by coniferyl aldehyde dehydrogenase (CalB). Finally, ferulic acid was transformed to vanillin through coenzyme A acylation and carbon bond cleavage catalyzed by feruloyl-CoA synthetase (FCS) and enoyl-CoA hydratase/aldolase (ECH) (Overhage et al., 1999).

Biotransformation of vanillin. Vanillin dehydrogenase (VDH) of *Pseudomonas* sp. strain HR199 was reported to oxidize vanillin to vanillic acid (Priefert et al., 1997). Vanillic acid was further converted to protocatechuate by the two subunits vanillate *O*-demethylase, VanA and VanB, identied in *Pseudomonas* sp. strain HR199 and *Rhodococcus jostii* RHA1 (Chen et al., 2012).

Biotransformation of isosafrole. Another 1-propenylbenzene, isosafrole, which have the same 1-propenyl side chain as *trans*-anethole and isoeugenol, are used in fragrance industry and are

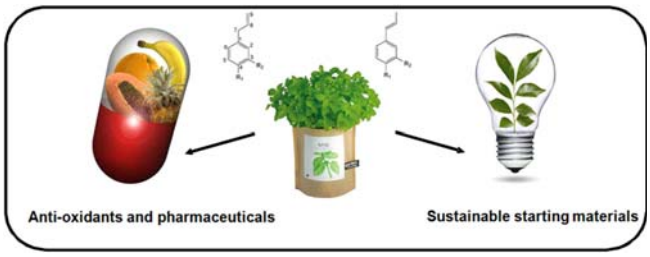

Fig. 9 Bacterial biocatalytic tools for producing value-added compounds of natural flavors and fragrances, and chemical synthons from inexpensive plant-derived biomass.

found in small amounts in various essential oils. It can be commonly obtained by isomerizing the plant oil safrole. Isosafrole is a precursor to the important fragrance piperonal and can also be converted to into the psychoactive drug, 3,4-methylenedioxy-*N*-methamphetamine (MDMA) (ecstasy). Piperonal, also known as heliotropin and structurally related to benzaldehyde and vanillin, is commonly found in fragrances and flavors, and has a floral odor similar to that of vanillin and cherry. There are no genes or enzymes that could transform isosafrole to piperonal. However, the yeast strain *Cladosporium sphaerospermum* was found to yield piperonal from isosafrole (Santos et al., 2003). Shimoni et al. reported that a mutant of *Arthrobacter* sp. TA13 could transform isosafrole to piperonal and piperonylic acid (2003). Thus, the TAO of *P. putida* JYR-1 has potential for the production of piperonal, which is a compound of great commercial importance in the flavor and fragrance industries (Han et al., 2012a; 2012b). *O*-Methyl isoeugenol, derivative of isoeugenol methylation, is also a component of essential oil. Veratraldehyde is widely used as flavor and odorant compounds due to its pleasant woody fragrance in fragrance industry and an intermediate in the synthesis of pharmaceuticals (Surburg et al., 2006).

Conclusion

In this report, we described various bacterial metabolisms of compounds produced by phenylpropanoid pathways in plants such as *trans*-anethole, isoeugenol, and eugenol, with seeking possible applications for industrial production of natural flavors and fragrances. These bacterial metabolisms can provide various biocatalytic tools for producing the value-added compounds of natural flavors and fragrances, and chemical synthons from inexpensive plant-derived biomass (Fig. 9). However, many problems, such as toxicity of the parent compounds and products, efficient expression of the targeted genes, and physiological stability of the recombinant bacterial strains, should be overcome in order to produce expensive "natural" flavors and fragrances by engineered microorganisms.

Acknowledgment This work was supported by the National Research Foundation of Korea (NRF: 2012-0008725) grant, Ministry of Education, Science & Technology, Republic of Korea.

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