

Highly Efficient Biotransformation of Eugenol to Ferulic Acid and Further Conversion to Vanillin in Recombinant Strains of *Escherichia coli*

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The *vaoA* gene from *Penicillium simplicissimum* CBS 170.90, encoding vanillyl alcohol oxidase, which also catalyzes the conversion of eugenol to coniferyl alcohol, was expressed in *Escherichia coli* XL1-Blue under the control of the *lac* promoter, together with the genes *calA* and *calB*, encoding coniferyl alcohol dehydrogenase and coniferyl aldehyde dehydrogenase of *Pseudomonas* sp. strain HR199, respectively. Resting cells of the corresponding recombinant strain *E. coli* XL1-Blue(pSK*vaoA*mP*calA*m*calB*) converted eugenol to ferulic acid with a molar yield of 91% within 15 h on a 50-ml scale, reaching a ferulic acid concentration of 8.6 g liter⁻¹. This biotransformation was scaled up to a 30-liter fermentation volume. The maximum production rate for ferulic acid at that scale was 14.4 mmol per h per liter of culture. The maximum concentration of ferulic acid obtained was 14.7 g liter⁻¹ after a total fermentation time of 30 h, which corresponded to a molar yield of 93.3% with respect to the added amount of eugenol. In a two-step biotransformation, *E. coli* XL1-Blue(pSK*vaoA*mP*calA*m*calB*) was used to produce ferulic acid from eugenol and, subsequently, *E. coli* (pSK*echE*/H*fcs*) was used to convert ferulic acid to vanillin (J. Overhage, H. Priefert, and A. Steinbüchel, Appl. Environ. Microbiol. 65:4837–4847, 1999). This process led to 0.3 g of vanillin liter⁻¹, besides 0.1 g of vanillyl alcohol and 4.6 g of ferulic acid liter⁻¹. The genes *ehyAB*, encoding eugenol hydroxylase of *Pseudomonas* sp. strain HR199, and *azu*, encoding the potential physiological electron acceptor of this enzyme, were shown to be unsuitable for establishing eugenol bioconversion in *E. coli* XL1-Blue.

Vanillin is a frequently used aromatic flavor compound in the food and cosmetics industries. The most intensively studied process for producing vanillin by biotransformation, which then can be designated “natural,” is based on the substrate ferulic acid (22, 24). Previously, the gram-positive microorganisms *Amycolatopsis* sp. strain HR167 and *Streptomyces setonii* were identified as being able to convert ferulic acid to vanillin and which exhibited a very high tolerance towards this highly reactive aromatic aldehyde (2, 14; B. Müller, T. Münch, A. Muheim, and M. Wetli, European Patent Office patent application EP0885968, 1998; J. Rabenhorst and R. Hopp, European Patent Office patent application EP0761817, 1997). When these bacteria were used in ferulic acid bioconversions, vanillin concentrations of more than 10 g liter⁻¹ were obtained (Müller et al., patent application; Rabenhorst and Hopp, patent application). The major drawback of the *Amycolatopsis*- and *S. setonii*-based microbial production of vanillin is the relatively high price of the substrate ferulic acid.

A new strategy for producing ferulic acid and vanillin is the enzymatic oxidative conversion of eugenol (Fig. 1). Eugenol is a cheap “natural” substrate that can be isolated from the essential oil of the clove tree *Syzygium aromaticum* on an industrial scale. The genes encoding proteins of the oxidative eugenol catabolism pathway have been investigated in detail in

different *Pseudomonas* strains (1, 5, 13, 15, 16, 17, 18, 20, 21). The genes *ech* and *fcs* of *Pseudomonas* sp. strain HR199, encoding feruloyl-coenzyme A (CoA) synthetase and enoyl-CoA hydratase/aldolase, respectively, were used to engineer *Escherichia coli* strains able to catalyze the conversion of ferulic acid to vanillin (18). The genes *ehyAB*, encoding the eugenol hydroxylase of *Pseudomonas* sp. strain HR199 (12, 20), were expressed in *Ralstonia eutropha* H16 to establish the key steps of eugenol biotransformation, including the initial conversion of eugenol to coniferyl alcohol, in this recombinant strain (19).

In this study we expressed the genes *ehyAB*, *calA*, and *calB* of *Pseudomonas* sp. strain HR199 in *E. coli* to establish eugenol biotransformation to ferulic acid in this strain. Since no bioconversion was achieved by this strategy, the genes *ehyAB* were replaced by *vaoA* from *Penicillium simplicissimum* CBS 170.90, encoding vanillyl alcohol oxidase (8). This enzyme also catalyzes the conversion of eugenol to coniferyl alcohol (3, 9, 10), and its primary structure shares extensive regions of homology with the amino acid sequence of the flavoprotein subunit of eugenol hydroxylases (5, 20). However, it lacks a cytochrome *c* subunit. By using this gene, a highly efficient biotransformation of eugenol to ferulic acid was established with recombinant strains of *E. coli* on a technical scale.

MATERIALS AND METHODS

Strains, plasmids, and cultivation conditions. The bacterial strains and plasmids used in this study are listed in Table 1. *E. coli* was cultivated in complex media (Luria-Bertani [LB] or Terrific broth [TB]) (25) at 30 or 37°C in Erlenmeyer flasks with baffles on a rotary water bath shaker (G76; New Brunswick Scientific Co, Edison, N.J.) at 150 rpm. *P. simplicissimum* CBS170.90 was grown in a minimal medium as described previously (8). Ampicillin was used at a final

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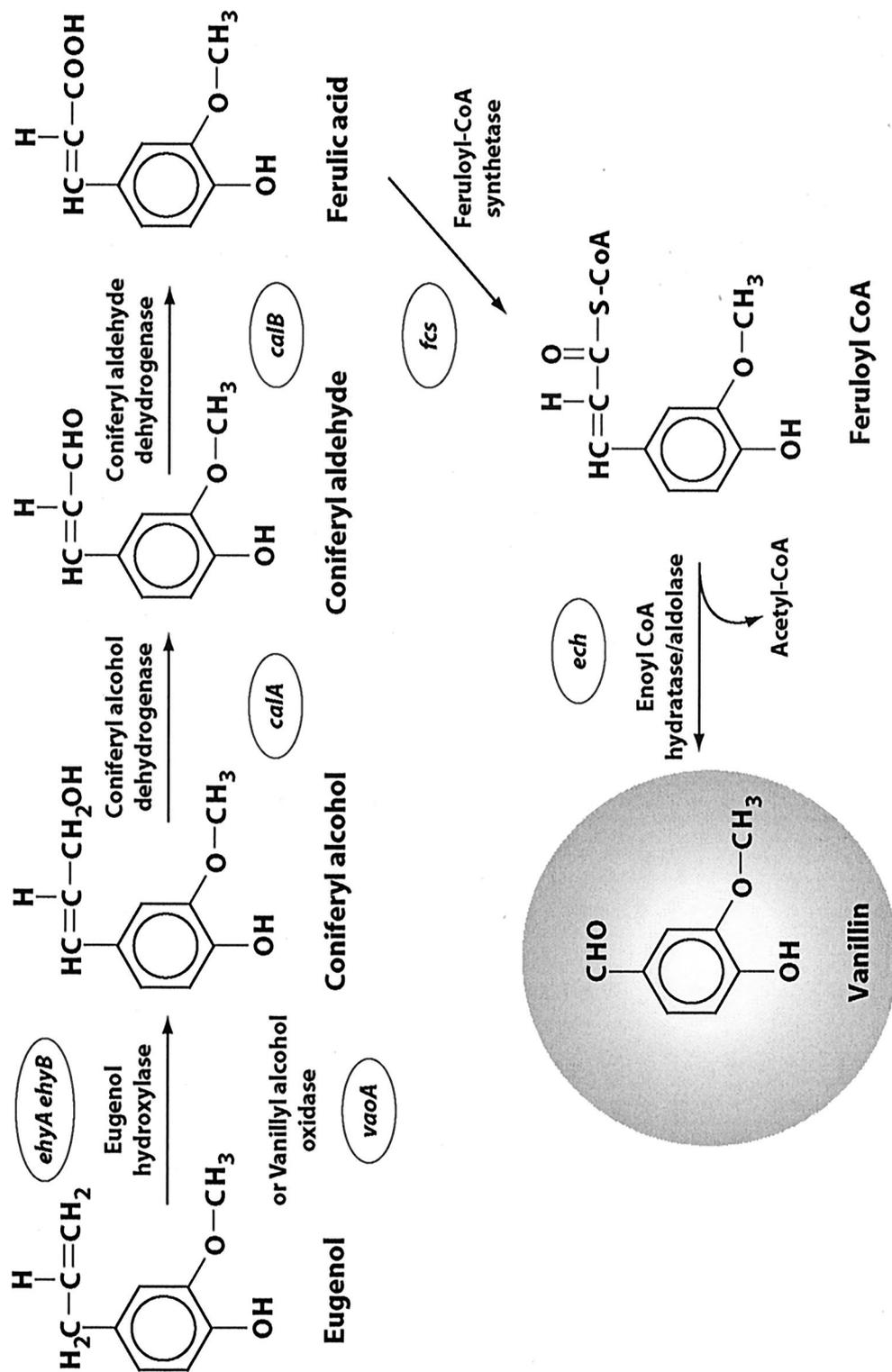


FIG. 1. Biotransformation of eugenol to vanillin.

TABLE 1. Strains and plasmids used in this study

Strain or plasmid	Relevant characteristics	Source or reference
Strains		
<i>E. coli</i> XL1-Blue	<i>recA1 endA1 gyrA96 thi-1 hsdR17</i> ($r_K^- m_K^+$) <i>supE44 relA1</i> $\lambda^- lac$ [F' <i>proAB lacI^q Z</i> ΔM15 Tn10(Tc ^r)]	6
<i>P. simplicissimum</i> CBS 170.90		ATCC 90172
Plasmids		
pBluescript SK ⁻ pSKcalA	Ap ^r <i>lacPOZ</i> , T ₇ and T ₃ promoter pBluescript SK(-) harboring the PCR product comprising <i>calA</i> as a <i>NotI-SpeI</i> fragment	Stratagene, San Diego, Calif. 19
pSKcalAm	pBluescript SK(-) harboring the PCR product comprising <i>calA</i> as a <i>NotI-SpeI</i> fragment with the <i>EcoRI</i> restriction site eliminated	This study
pSKcalB	pBluescript SK(-) harboring the PCR product comprising <i>calB</i> as a <i>SpeI-BamHI</i> fragment	19
pSKcalAmcalB	pBluescript SK(-) harboring the PCR products comprising <i>calAm</i> and <i>calB</i> as a <i>NotI-BamHI</i> fragment	This study
pSKvao	pBluescript SK(-) harboring the PCR product comprising <i>vao</i> as a <i>SacI-NotI</i> fragment	This study
pSKvaom	pBluescript SK(-) harboring the PCR product comprising <i>vao</i> as a <i>SacI-NotI</i> fragment with the <i>EcoRI</i> and <i>BamHI</i> restriction sites eliminated	This study
pSKvaomPcalAmcalB	pBluescript SK(-) harboring the PCR products comprising <i>vaom</i> , <i>PlacZ</i> , <i>calA</i> , and <i>calB</i>	This study
pSKechE/Hfcs	pBluescript SK(-) harboring the PCR product comprising <i>ech</i> as an <i>EcoRI-HindIII</i> fragment and <i>fcs</i> as a <i>PstI</i> fragment	18

concentration of 100 µg/ml for recombinant strains of *E. coli*. Eugenol was directly added to the medium at final concentrations of 0.01 to 0.1% (vol/vol). Growth was monitored by measuring the turbidity of the cultures at 600 nm.

Analytical methods. Culture supernatants were analyzed for excreted intermediates of eugenol catabolism by liquid chromatography without prior extraction, using a Knauer (Berlin, Germany) high-pressure liquid chromatography (HPLC) apparatus. Intermediates were separated by reverse-phase chromatography on a Nucleosil-100 C₁₈ column (particle size, 5 µm; column, 250 by 4.0 mm) with a gradient of 0.1% (vol/vol) formic acid (eluant A) and acetonitrile (eluant B) in a range of 27 to 100% (vol/vol) eluant B and at a flow rate of 1 ml/min. For quantification, all intermediates were calibrated with external standards. The compounds were identified by their retention times, as well as the corresponding spectra, which were recorded with a diode array detector (Well-Chrom Diodenarray-Detektor K-2150; Knauer).

Isolation, manipulation, analysis, and transfer of DNA. All genetic techniques were performed as described by Sambrook et al. (25). DNA sequences of constructed hybrid plasmids were confirmed by DNA sequencing according to the chain termination method, using an automatic sequencer (Li-Cor model 4000L; MWG-Biotech, Ebersberg, Germany).

Isolation of total RNA and cloning of vanillyl alcohol oxidase gene *vaoA*. Total RNA was isolated from *P. simplicissimum* CBS170.90 cells after 48 h of growth on minimal medium containing veratryl alcohol as inducer of vanillyl alcohol oxidase expression using a Qiagen RNeasy minikit. The *vaoA* comprising cDNA that encoded vanillyl alcohol oxidase was amplified in a reverse transcription-PCR using the primers *vaoU* (5'-AAAAGAGCTCTAAGGAGGTGACAACA TGTCGAAGACACAGGAATTTAGGCC-3') and *vaoD* (5'-AAAAGCGGCC CCTTACAGTTTCCAAGTAACATGACTG-3'), which were designed from the published *vaoA* sequence (3). These primers were used together with the isolated total RNA applying a Qiagen OneStep reverse transcription-PCR kit. For an enhanced expression of the *vaoA* gene in *E. coli*, a consensus Shine-Dalgarno sequence (TAAGGAGGTGA) was introduced four nucleotides from the *vaoA* start codon in the upstream primer *vaoU*. The PCR product was digested with *SacI* (site underlined in the upstream primer) and *NotI* (site underlined in the downstream primer) and cloned in pBluescript SK(-). In the resulting hybrid plasmid pSKvao, the *vaoA* gene was colinear to and downstream of the *lacZ* promoter of the vector.

PCR-directed mutagenesis for elimination of interfering restriction sites. The *vaoA* gene contained an *EcoRI* restriction site at position 70 (referring to the published coding region) (3) and a *BamHI* site at position 1608, while *calA* contained an *EcoRI* site at position 462. For further subcloning of *vaoA* and *calA*, it was necessary to eliminate these interfering restriction sites. Because of the

terminal positions of the *EcoRI* and *BamHI* restriction sites in *vaoA*, these two sites were eliminated using the primers *vaoU-m* (5'-AAAAGAGCTCTAAGG AGGTGAAACATGTCCAAGACACAGGAATTTAGGCCCTTTGACACTGC CACCCAAGCTGTGCTTAAGTGACTTCAATGAGTTCATC-3') and *vaoD-m* (5'-AAAAGCGGCCGCTTACAGTTTCCAAGTAACATGACTGTATTGAC TCGGCCAAACACCAGACTTCCCGGGGCAATGATGCCATTAGGGTCC ACCG-3') in a PCR with pSKvao as the template DNA. The recognition sequences of the restriction enzymes were mutated by silent nucleotide exchanges in the primers used (underlined). The PCR product was digested with *SacI* and *NotI* and cloned in pBluescript SK(-), resulting in pSKvaom.

The *EcoRI* restriction site in *calA* was eliminated by using a unique site elimination mutagenesis kit from Amersham Pharmacia (Freiburg, Germany) with the primers *calAm* (5'-CCCGAGTTCTGCTACCAGTATTT-3') and *Pmut* (5'-GAACTCGATATCAAGCTTATCGGTAC-3'), resulting in hybrid plasmid pSKcalAm.

Construction of hybrid plasmid pSKvaomPcalAmcalB. The coniferyl aldehyde dehydrogenase gene *calB* was isolated from *SpeI-BamHI*-digested pSKcalB, which has been described recently (19), and was subsequently cloned in *SpeI-BamHI*-digested pSKcalAm, resulting in hybrid plasmid pSKcalAmcalB. In order to increase the activities of the coniferyl alcohol and coniferyl aldehyde dehydrogenases in corresponding recombinant *E. coli* strains, an additional promoter was integrated upstream of *calAm*. This was achieved by amplification of *calAm-calB* together with the *lacZ* promoter in a PCR using the primers *lacZU* (5'-AAAGGATCCACCGAGCGCAGTGAGTCACTG-3') and *calBD2* (5'-AAAA AGAATTCCTCACTACCAACGGTTCTAACACTCCG-3'). The PCR product was digested with *BamHI* (site underlined in the upstream primer) and *EcoRI* (site underlined in the downstream primer) and cloned in *BamHI-EcoRI*-digested pSKvaom, resulting in hybrid plasmid pSKvaomPcalAmcalB.

Enzyme assays. Cells of recombinant strains of *E. coli* were grown for 12 h at 37°C in LB or TB in the presence of 1 mM IPTG (isopropyl-β-D-thiogalactopyranoside). Cells were harvested by centrifugation and resuspended in a suitable buffer with respect to the subsequent enzyme assay. Disruption of the cells was performed either by a twofold French press passage at 96 MPa or by sonication (1 min/ml of cell suspension with an amplitude of 40 µm) with a Bandelin Sonoplus GM200 ultrasonic disintegrator. The soluble fractions of crude extracts were obtained by centrifugation at 100,000 × g at 4°C for 1 h. Vanillyl alcohol oxidase, coniferyl alcohol dehydrogenase, and coniferyl aldehyde dehydrogenase were measured in optical enzymatic tests as described previously (1, 3, 19). The amount of soluble protein was determined as described by Bradford (4).

Cultivation on a 30-liter scale. A Biostat DL30 stainless steel reactor (B. Braun Biotech International, Melsungen, Germany) with a total volume of 42

liters (28-cm inner diameter and 71-cm height) and a relation of stirrer diameter to vessel diameter of 0.375 was used for cultivations on the 30-liter scale. This bioreactor was equipped with three stirrers, each containing six paddles and a Funda-Foam mechanical foam destroyer (B. Braun Biotech International). In addition, sterilizable probes were used to measure dissolved oxygen (pO₂) (model 25; Mettler-Toledo GmbH, Steinbach, Switzerland), pH (model Pa/25; Mettler-Toledo GmbH), foam (model L300/Rd. 28; B. Braun Biotech International), temperature (pt 100 electrode; M. K. Juchheim GmbH, Fulda, Germany), and optical density at 850 nm (model CT6; Sentex/Monitek Technology Inc.). The fermentation process was controlled and recorded by a digital control unit in combination with the MFCS/win software package (B. Braun Biotech International).

Carbon dioxide and oxygen concentrations in the exhaust gas were measured with a URAS 10 P NDIR spectrophotometer (Mannesmann, Hartmann and Braun, Frankfurt, Germany) or a Magnos 6 G oxygen analyzer (Mannesmann, Hartmann and Braun), respectively. Cultivations were performed at 30°C and at a pO₂ range of 0 to 100% air saturation of the medium, which was controlled by agitation rates between 200 and 500 rpm and aeration rates of 0.4 and 1.0 volume per volume per minute. The pH in the medium was controlled between 7.1 and 7.3 by addition of 4 N HCl or NaOH. If the mechanical foam destroyer insufficiently removed foam, the antifoam agent Silikon Antischaum Emulsion SLE (Wacker, Darwin Vertriebs GmbH, Ottobrunn, Germany) was added. Small cell-free samples of the culture fluid for analytical purposes were prepared by 10 min of centrifugation at 3,500 × g.

Materials. Restriction endonucleases, T4 DNA ligase, lambda DNA, and substrates used in the enzyme assays were obtained either from Roche Molecular Biochemicals (Mannheim, Germany) or from Invitrogen (Karlsruhe, Germany). Agarose type NA was purchased from Amersham Pharmacia Biotech (Freiburg, Germany). Synthetic oligonucleotides were purchased from MWG-Biotech. All other chemicals were from Merck Eurolab (Darmstadt, Germany), Serva Feinbiochemica (Heidelberg, Germany), or Sigma-Aldrich Fine Chemicals (Deisenhofen, Germany).

Nucleotide sequence accession numbers. The nucleotide sequence accession numbers of the *calA* (named CADH in the patent), *calB*, and *vao* genes are A92130, AJ006231, and Y15627, respectively.

RESULTS

Tolerance of *E. coli* XL1-Blue towards different eugenol concentrations. *E. coli* is able neither to use the aromatic substrates eugenol or ferulic acid as the carbon source for growth nor to convert or degrade ferulic acid. Thus, it is an eligible host for establishing a biochemical pathway enabling biotransformation of eugenol to ferulic acid.

Eugenol is a highly toxic substance for most bacteria (11). To investigate the eugenol tolerance of the selected host *E. coli* XL1-Blue, several 50-ml cultures in 250-ml Erlenmeyer flasks without baffles containing LB medium and different concentrations of eugenol ranging from 0.01% (vol/vol) up to 0.1% (vol/vol) were inoculated with an overnight preculture. The cultures were incubated for 48 h at 37°C, and the optical densities of the cultures at 600 nm were determined daily. As shown in Table 2, growth of *E. coli* XL1-Blue was only slightly affected by eugenol concentrations of up to 0.025% (vol/vol). There was a notable inhibition of *E. coli* XL1-Blue at a eugenol concentration of 0.05% (vol/vol), and the strain was no longer able to grow at concentrations higher than 0.075% (vol/vol).

Cloning and functional expression of the vanillyl alcohol oxidase gene (*vaoA*) from *P. simplicissimum* CBS 170.90 in *E. coli* XL1-Blue. In initial experiments, *ehyAB*, *calA*, and *calB*, encoding eugenol hydroxylase, coniferyl alcohol dehydrogenase, and coniferyl aldehyde dehydrogenase in *Pseudomonas* sp. strain HR199, respectively, were expressed in *E. coli* to establish conversion of eugenol to ferulic acid. However, no biotransformation was achieved with this strain, although the corresponding enzyme activities were detectable in protein

TABLE 2. Tolerance by *E. coli* XL1-Blue of different eugenol concentrations^a

Eugenol concn (% [vol/vol])	OD ₆₀₀ after an incubation period of ^b :		
	0 h	24 h	48 h
0	0.07	3.5	3.4
0.010	0.08	3.1	2.9
0.025	0.08	2.6	2.4
0.050	0.08	0.07	0.7
0.075	0.07	0.06	0.05
0.100	0.07	0.05	0.05

^a Cells of *E. coli* XL1-Blue were precultured overnight in LB medium. The cells were used for inoculation of a series of 50-ml cultures in 250-ml unbaffled Erlenmeyer flasks containing LB medium and different concentrations of eugenol.

^b Data are means for three independent determinations. OD₆₀₀, optical density at 600 nm.

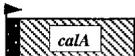
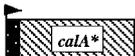
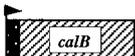
extracts of the recombinant cells in vitro (data not shown). Even the coexpression of *azu* from *Pseudomonas* sp. strain OPS1, which encodes the potential physiological electron acceptor of the eugenol hydroxylase (5), was in vain (data not shown). Thus, the initial reaction leading from eugenol to coniferyl alcohol could not be established by expression of the eugenol hydroxylase genes in *E. coli*.

Since the hydroxylation of eugenol to coniferyl alcohol is also catalyzed by vanillyl alcohol oxidase (*VaoA*) from *P. simplicissimum* CBS 170.90 (8, 10), the corresponding gene *vaoA* was chosen to establish the initial eugenol biotransformation step in *E. coli*. *vaoA* was amplified as cDNA and cloned in pBluescript SK(-), and site-specific silent mutations for elimination of an *EcoRI* and a *BamHI* restriction site in the gene were introduced as described in Materials and Methods. The resulting hybrid plasmid pSK*vaoM*, harboring *vaoA* colinear to and downstream of the *lacZ* promoter, conferred vanillyl alcohol oxidase activity to recombinant strains of *E. coli* XL1-Blue (Table 3). Furthermore, with IPTG-induced resting cells of *E. coli* XL1-Blue(pSK*vaoM*), a conversion of 3.25 mM eugenol (0.05% [vol/vol]) to coniferyl alcohol was obtained within 1 h. In addition, coniferyl aldehyde was detected in the medium, which was most probably due to an unspecific alcohol dehydrogenase activity of the host cells.

Since the expression of *vaoA* enabled *E. coli* XL1-Blue(pSK*vaoM*) to convert eugenol to coniferyl alcohol, coexpression of *vaoA*, *calA*, and *calB* was expected to enable *E. coli* to convert eugenol to ferulic acid efficiently. For this reason, the hybrid plasmid pSK*vaoM*P*calA*m*calB* was constructed. Recombinant cells of *E. coli* XL1-Blue harboring pSK*vaoM*P*calA*m*calB* were grown at 30°C, and expression of the introduced genes was induced by IPTG addition as described above. Protein extracts derived from these cells exhibited vanillyl alcohol oxidase, coniferyl alcohol dehydrogenase, and coniferyl aldehyde dehydrogenase activities, as determined by applying the corresponding enzyme assays (Table 3).

Biotransformation of eugenol to ferulic acid in 50-ml cultures. To investigate the biotransformation of eugenol to ferulic acid, cells of *E. coli* XL1-Blue(pSK*vaoM*P*calA*m*calB*) were grown overnight at 30°C in 50 ml of TB medium containing tetracycline, ampicillin, and IPTG. When the culture entered the stationary growth phase, 25-μl portions of eugenol (final concentration, 0.05% [vol/vol]) were added hourly during a

TABLE 3. Organization of genes in hybrid plasmids and in vitro enzyme activities^a

Hybrid plasmid	Organization of genes ^b	In vitro activity (U/mg of protein) of:			Reference
		Vanillyl alcohol oxidase	Coniferyl alcohol dehydrogenase	Coniferyl aldehyde dehydrogenase	
pSKvao		0.02 ± 0.01	ND ^c	ND	This study
pSKvaoM		0.02 ± 0.01	ND	ND	This study
pSKcalA		ND	1.16 ± 0.05	ND	19
pSKcalAm		ND	1.16 ± 0.05	ND	This study
pSKcalB		ND	ND	0.06 ± 0.01	19
pSKvaoMcalAmcalB		0.02 ± 0.01	3.7 ± 0.15	0.28 ± 0.02	This study

^a Cells were grown and enzyme activities were determined as described in Materials and Methods. Experiments and enzyme assays were done in triplicate, and the values are means ± deviations calculated by using standard statistics ($P < 0.05$).

^b Dark areas with arrows represent *lac* promoters.

^c ND, not determined.

15-h biotransformation phase, and the appearance of coniferyl alcohol, coniferyl aldehyde, and ferulic acid in the culture supernatant was analyzed by HPLC. The time course of the production of ferulic acid from eugenol is summarized in Fig. 2. Within 15 h a ferulic acid concentration of 8.6 g liter⁻¹ was obtained, corresponding to a molar yield of 90.8% with respect

to the added amount of eugenol. Cultures of *E. coli* XL1-Blue harboring only the vector pBluescript SK(-), which were treated in the same way as a negative control, showed no conversion of eugenol.

Technical-scale production of ferulic acid from eugenol. To test the capability of *E. coli* XL1-Blue(pSKvaoMcalAmcalB)

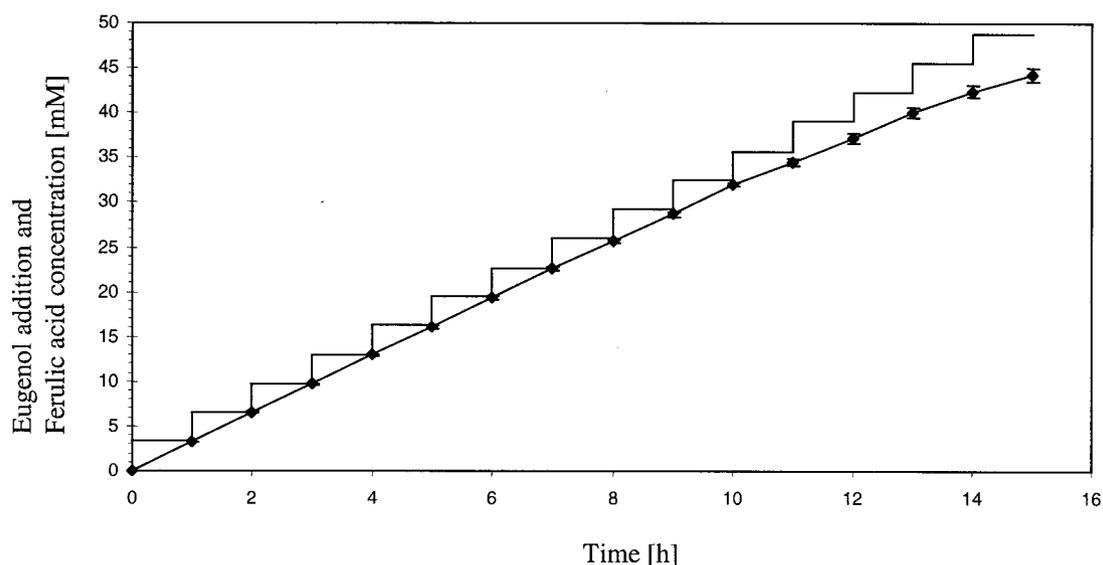


FIG. 2. Production of ferulic acid from eugenol by *E. coli* XL1-Blue(pSKvaoMcalAmcalB). Cells were grown in 50 ml of liquid TB medium in the presence of tetracycline, ampicillin, and IPTG overnight at 30°C. Portions (25 μ l) of eugenol (final concentration, 0.05% [vol/vol]) were added to the culture hourly. Samples were taken, and ferulic acid concentrations were determined by HPLC. Data are means for three independent experiments. The variance is indicated by error bars. —, total amount of added eugenol; \blacklozenge , ferulic acid concentration.

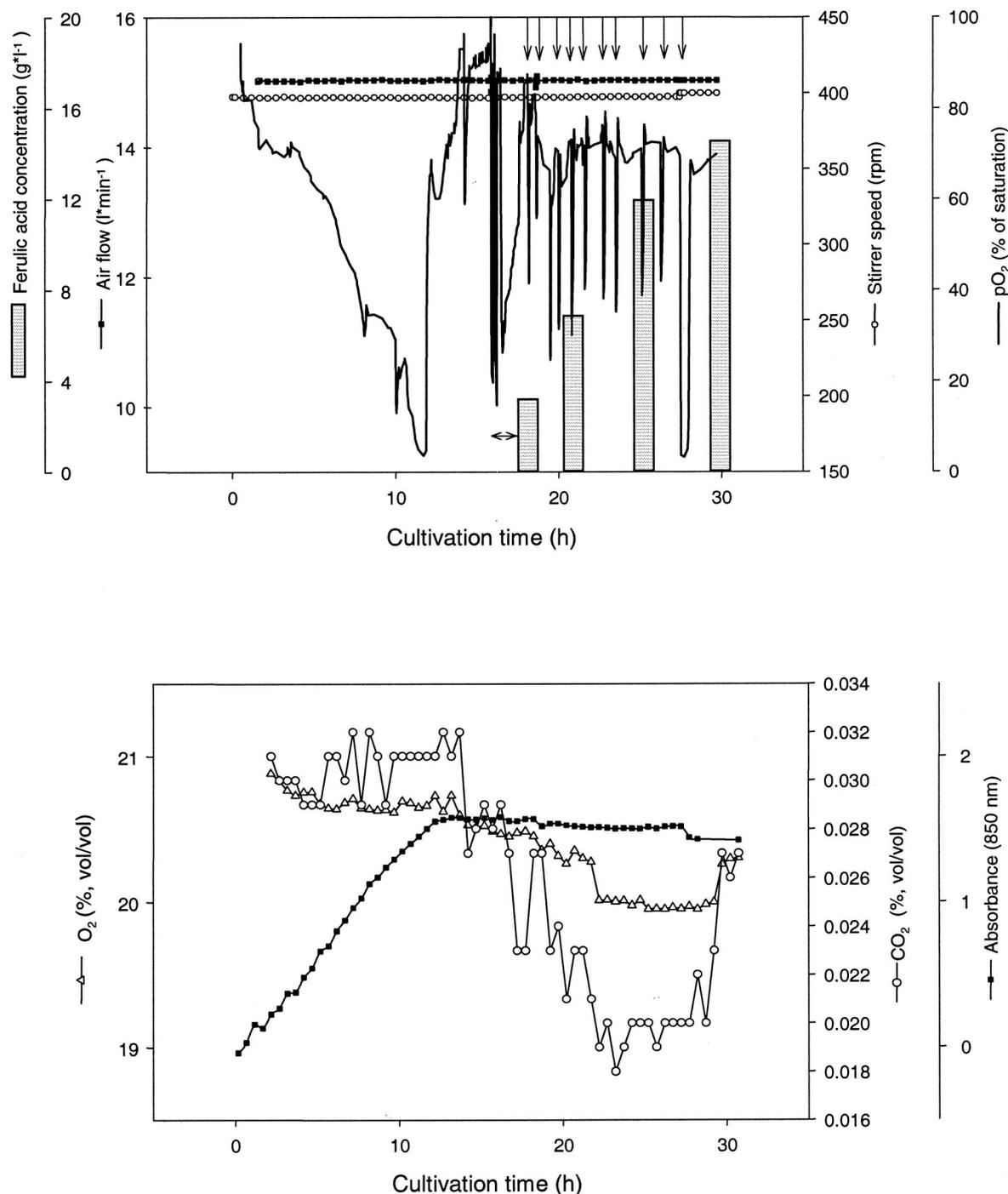


FIG. 3. Fed-batch fermentation of *E. coli* XL1-Blue(pSKyaomPcalAmcalB) on the 30-liter scale in TB medium. The cultivation was done in a 42-liter stirred-tank reactor, and the cultivation parameters were pH 7.2, aeration at 0.8 to 1.0 volume per volume per minute, and agitation at 200 to 400 rpm. Aeration and agitation were adjusted according to the oxygen demand of the culture. The temperature was decreased from 30 to 28°C at the beginning of the biotransformation process. Eugenol (100 ml) was added to the culture continuously over 2 h (\leftrightarrow), maintaining a final concentration of approximately 0.1% (vol/vol) in the medium. Subsequently, eugenol was added in pulses of 30 ml to the medium (\downarrow).

to transform eugenol to ferulic acid in a technical-scale production process, 28 liters of TB medium containing tetracycline, ampicillin, and IPTG was inoculated with a 2-liter pre-culture of this strain grown overnight in the same medium. The time course of this fermentation is shown in Fig. 3. After 16 h,

when the cells had been in the stationary growth phase for approximately 4 h, 100 ml of eugenol was fed to the culture continuously over 2 h, maintaining a final eugenol concentration of approximately 0.1% (vol/vol) in the medium. Subsequently, eugenol was fed in 10 pulses of 30 ml to the culture as

indicated in Fig. 3. To ensure optimal conditions for the coniferyl aldehyde dehydrogenase, the temperature was decreased from 30 to 28°C at the beginning of the biotransformation. In this experiment a maximum production rate of 14.4 mmol of ferulic acid per h per liter of culture was observed in the biotransformation phase in the time frame from 0 to 90 min, when eugenol was added continuously to the medium. The maximum concentration of ferulic acid obtained was 14.7 g liter⁻¹ after a total fermentation time of 30 h, which corresponded to a total amount of 441 g and a molar yield of 93.3% with respect to the amount of eugenol added.

Two-step biotransformation of eugenol to vanillin with recombinant strains of *E. coli*. As described above, the quantitative conversion of eugenol to ferulic acid was achieved with *E. coli* XL1-Blue(pSKvaomPcalAmcalB). In a recent study, it was shown that the coexpression of *fcs* and *ech*, encoding feruloyl-CoA synthetase and enoyl-CoA hydratase/aldolase, respectively, enabled recombinant *E. coli* XL1-Blue strains to convert ferulic acid to vanillin (18). Thus, we established a two-step biotransformation of eugenol to vanillin with recombinant *E. coli* XL1-Blue strains harboring the hybrid plasmids pSKvaomPcalAmcalB and pSKechE/Hfcs (18), respectively.

Cells of *E. coli* XL1-Blue(pSKvaomPcalAmcalB) were incubated on the 50-ml scale as described above until a ferulic acid concentration of 5 g liter⁻¹ was reached. Subsequently, washed cells of a 50-ml culture of *E. coli* XL1-Blue(pSKechE/Hfcs), which had been grown to the late exponential growth phase and induced by the addition of IPTG, were added to the culture. In order to inactivate the *calB* gene product, which besides coniferyl aldehyde dehydrogenase also possesses vanillin dehydrogenase activity (17), the temperature was raised to 37°C. Further incubation of this mixed culture and analysis of culture supernatants via HPLC revealed a production of 0.3 g of vanillin liter⁻¹ after 2 h. At this time point, 4.6 g of ferulic acid liter⁻¹ and 0.1 g of vanillyl alcohol liter⁻¹ were detected in the culture supernatant. During further incubation, however, the vanillin produced was completely reduced to vanillyl alcohol and no more ferulic acid was converted to vanillin, indicating the loss of biotransformation capability of this mixed culture.

DISCUSSION

With an increasing interest in natural products, biotechnological production of vanillin has gained more interest in recent years, since it offers an alternative way to produce this aromatic substance as a “natural” flavor compound. Until today, only the biotransformation of ferulic acid to vanillin had been developed to an economically feasible process. Using *Amycolatopsis* sp. strain HR167, 11.5 g of vanillin liter⁻¹ was produced from 19.9 g of ferulic acid liter⁻¹ within 32 h on a 10-liter scale, corresponding to a molar yield of 77.8% (Rabenhorst and Hopp, patent application). A biotransformation with similar efficiency based on the same substrate using *S. setonii* as a biocatalyst was developed (14; Müller et al., patent application). However, these biotransformations depend on the use of the expensive substrate ferulic acid.

A different source for a natural ferulic acid feedstock is eugenol, which is a commercially available natural raw material extracted from clove tree leaves. The current market price is

about \$5 kg⁻¹ (22). Eugenol can be enzymatically converted to ferulic acid via coniferyl alcohol and coniferyl aldehyde. The genes *ehyAB*, *calA*, and *calB*, which are involved in this biotransformation, were investigated in detail in *Pseudomonas* sp. strain HR199 and *Pseudomonas* sp. strain OPS1 (1, 5, 20). *Pseudomonas* sp. strain HR199 has been used to produce natural ferulic acid (23); however, the yield of 6.7 g of ferulic acid liter⁻¹ from 13.9 g of eugenol liter⁻¹ within 78 h (R. Hopp and J. Rabenhorst, European Patent Office patent application EP0583687, 1994) was too low for a commercial process. To achieve higher yields, the said genes of *Pseudomonas* sp. strain HR199 were expressed in *R. eutropha* H16, which is not able to degrade ferulic acid (19). With this recombinant strain, 3.5 g of ferulic acid liter⁻¹ from 3.2 g liter⁻¹ eugenol was obtained within only 20 h, which corresponded to a molar yield of 93.8% (19). The major difficulty of this biotransformation process was the sensitivity of *R. eutropha* H16 to eugenol, demanding a stringent eugenol addition mode to keep the final concentration of eugenol in the medium below 3.25 mM (corresponding to 0.05% [vol/vol]).

In the present study, we used *E. coli* XL1-Blue as an alternative host strain for gene expression, since it exhibited a significantly higher eugenol tolerance than *R. eutropha* H16 (Table 2) (19). However, the expression of the *Pseudomonas* sp. strain HR199 genes *ehyAB*, *calA*, and *calB* in this strain did not lead to a suitable biocatalyst, even after coexpression of *azu*, encoding the putative physiological electron acceptor of eugenol hydroxylase (7). A suitable alternative for *ehyAB* was found in the gene *vaoA* from *P. simplicissimum* CBS 170.90, encoding vanillyl alcohol oxidase (8). This well-characterized flavoprotein has a wide substrate specificity and is able to catalyze the hydroxylation of eugenol to coniferyl alcohol in addition to the oxidation of vanillyl alcohol to vanillin and other reactions (8, 10). This enzyme has even been used for the in vitro enzymatic synthesis of vanillin (26). By expressing the *vaoA* gene together with *calA* and *calB* in *E. coli* XL1-Blue, we were able to obtain 8.6 g of ferulic acid liter⁻¹ within 15 h at a molar yield of 90.8% with respect to the substrate eugenol. This biotransformation process was successfully transferred to the 30-liter scale, where we obtained a ferulic acid concentration of 14.7 g liter⁻¹ after a total fermentation time of 30 h. The molar yield of this biotransformation was 93.3% and even higher than at the 50-ml scale. Thus, *E. coli* XL1-Blue(pSKvaomPcalAmcalB) is a highly efficient biocatalyst for producing natural ferulic acid from eugenol.

Based on this successful conversion, we established a two-step biotransformation process leading from eugenol to vanillin. In a recent study it was shown that the coexpression of the *fcs* and *ech* genes of *Pseudomonas* sp. strain HR199, encoding feruloyl-CoA synthetase and enoyl-CoA hydratase/aldolase, respectively, enabled recombinant *E. coli* strains to convert ferulic acid to vanillin (18). The sequential application of the two recombinant *E. coli* strains harboring the hybrid plasmids pSKvaomPcalAmcalB and pSKechE/Hfcs initially led to the production of 0.3 g of vanillin liter⁻¹. However, during further incubation vanillin was reduced to vanillyl alcohol and no more ferulic acid was converted to vanillin. The reduction of vanillin represents a detoxification mechanism, which was also observed with *E. coli* XL1-Blue cells during incubation with vanillin (17).

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REFERENCES

- Achterholt, S., H. Priefert, and A. Steinbüchel. 1998. Purification and characterization of the coniferyl aldehyde dehydrogenase from *Pseudomonas* sp. strain HR199 and molecular characterization of the gene. *J. Bacteriol.* **180**:4387–4391.
- Achterholt, S., H. Priefert, and A. Steinbüchel. 2000. Identification of *Amycolatopsis* sp. strain HR167 genes, involved in the bioconversion of ferulic acid to vanillin. *Appl. Microbiol. Biotechnol.* **54**:799–807.
- Benen, J. A. E., P. Sanchez-Torres, M. J. M. Wagemaker, M. W. Fraaije, W. J. van Berkel, and J. Visser. 1998. Molecular cloning, sequencing, and heterologous expression of the *vaoA* gene from *Penicillium simplicissimum* CBS 170.90 encoding vanillyl-alcohol oxidase. *J. Biol. Chem.* **273**:7865–7872.
- Bradford, M. M. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* **72**:248–254.
- Brandt, K., S. Thewes, J. Overhage, H. Priefert, and A. Steinbüchel. 2001. Characterization of the eugenol hydroxylase genes (*ehyA/ehyB*) from the new eugenol degrading *Pseudomonas* sp. strain OPS1. *Appl. Microbiol. Biotechnol.* **56**:724–730.
- Bullock, W. O., J. M. Fernandez, and J. M. Stuart. 1987. XL1-Blue: a high efficiency plasmid transforming *recA Escherichia coli* strain with beta-galactosidase selection. *BioTechniques* **5**:376–379.
- Causser, M. J., D. J. Hopper, W. S. McIntire, and T. P. Singer. 1984. Azurin from *Pseudomonas putida*: an electron acceptor for *p*-cresol methylhydroxylase. *Biochem. Soc. Trans.* **12**:1131–1132.
- De Jong, E., W. J. van Berkel, R. P. van der Zwan, and J. A. de Bont. 1992. Purification and characterization of vanillyl-alcohol oxidase from *Penicillium simplicissimum*. A novel aromatic alcohol oxidase containing covalently bound FAD. *Eur. J. Biochem.* **208**:651–657.
- Fraaije, M. W., and W. J. van Berkel. 1997. Catalytic mechanism of oxidative demethylation of 4-(methoxymethyl)phenol by vanillyl-alcohol oxidase. Evidence for formation of a *p*-quinone methide intermediate. *J. Biol. Chem.* **272**:18111–18116.
- Fraaije, M. W., C. Veeger, and W. J. van Berkel. 1995. Substrate specificity of flavin-dependent vanillyl alcohol oxidase from *Penicillium simplicissimum*. Evidence for the production of 4-hydroxycinnamyl alcohols from 4-allylphenols. *Eur. J. Biochem.* **234**:271–277.
- Friedman, M., P. R. Henika, and R. E. Mandrell. 2002. Bactericidal activities of plant essential oils and some of their isolated constituents against *Campylobacter jejuni*, *Escherichia coli*, *Listeria monocytogenes*, and *Salmonella enterica*. *J. Food Prot.* **65**:1545–1560.
- Furukawa, H., M. Wieser, H. Morita, T. Sugo, and T. Nagasawa. 1998. Purification and characterization of eugenol dehydrogenase from *Pseudomonas fluorescens* E118. *Arch. Microbiol.* **171**:37–43.
- Gasson, M. J., Y. Kitamura, W. R. McLaughlan, A. Narbad, A. J. Parr, E. L. Parsons, J. Payne, M. J. Rhodes, and N. J. Walton. 1998. Metabolism of ferulic acid to vanillin. A bacterial gene of the enoyl-CoA hydratase/isomerase superfamily encodes an enzyme for the hydration and cleavage of a hydroxycinnamic acid SCoA thioester. *J. Biol. Chem.* **273**:4163–4170.
- Muheim, A., and K. Lerch. 1999. Towards a high-yield bioconversion of ferulic acid to vanillin. *Appl. Microbiol. Biotechnol.* **51**:456–461.
- Narbad, A., and M. J. Gasson. 1998. Metabolism of ferulic acid via vanillin using a novel CoA dependent pathway in a newly-isolated strain of *Pseudomonas fluorescens*. *Microbiology* **144**:1397–1405.
- Overhage, J., A. U. Kresse, H. Priefert, H. Sommer, G. Krammer, J. Rabenhorst, and A. Steinbüchel. 1999. Molecular characterization of the genes *pcaG* and *pcaH*, encoding protocatechuate 3,4-dioxygenase, which are essential for vanillin catabolism in *Pseudomonas* sp. strain HR199. *Appl. Environ. Microbiol.* **65**:951–960.
- Overhage, J., H. Priefert, J. Rabenhorst, and A. Steinbüchel. 1999. 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.* **52**:820–828.
- Overhage, J., H. Priefert, and A. Steinbüchel. 1999. Biochemical and genetic analyses of ferulic acid catabolism in *Pseudomonas* sp. strain HR199. *Appl. Environ. Microbiol.* **65**:4837–4847.
- Overhage J., A. Steinbüchel, and H. Priefert. 2002. Biotransformation of eugenol to ferulic acid by a recombinant strain of *Ralstonia eutropha* H16. *Appl. Environ. Microbiol.* **68**:4315–4321.
- Priefert, H., J. Overhage, and A. Steinbüchel. 1999. Identification and molecular characterization of the eugenol hydroxylase genes (*ehyA/ehyB*) of *Pseudomonas* sp. strain HR199. *Arch. Microbiol.* **172**:354–363.
- Priefert, H., J. Rabenhorst, and A. Steinbüchel. 1997. Molecular characterization of genes of *Pseudomonas* sp. strain HR199 involved in bioconversion of vanillin to protocatechuate. *J. Bacteriol.* **179**:2595–2607.
- Priefert, H., J. Rabenhorst, and A. Steinbüchel. 2001. Biotechnological production of vanillin. *Appl. Microbiol. Biotechnol.* **56**:296–314.
- Rabenhorst, J. 1996. Production of methoxyphenol type natural aroma chemicals by biotransformation of eugenol with a new *Pseudomonas* sp. *Appl. Microbiol. Biotechnol.* **46**:470–474.
- Rosazza, J. P. N., Z. Huang, L. Dostal, T. Volm, and B. Rousseau. 1995. Review. Biocatalytic transformations of ferulic acid: an abundant aromatic natural product. *J. Ind. Microbiol.* **15**:457–471.
- Sambrook J., E. F. Fritsch, and T. Maniatis. 1989. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
- van de Heuvel, R. H., M. W. Fraaije, C. Laane, and W. J. van Berkel. 2001. Enzymatic synthesis of vanillin. *J. Agric. Food Chem.* **49**:2954–2958.