

Biotransformation of Eugenol via Protocatechuic Acid by Thermophilic *Geobacillus* sp. AY 946034 Strain

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The metabolic pathway of eugenol degradation by thermophilic *Geobacillus* sp. AY 946034 strain was analyzed based on the lack of data about eugenol degradation by thermophiles. TLC, GC-MS, and biotransformation with resting cells showed that eugenol was oxidized through coniferyl alcohol, and ferulic and vanillic acids to protocatechuic acid before the aromatic ring was cleaved. The cell-free extract of *Geobacillus* sp. AY 946034 strain grown on eugenol showed a high activity of eugenol hydroxylase, feruloyl-CoA synthetase, vanillate-O-demethylase, and protocatechuate 3,4-dioxygenase. The key enzyme, protocatechuate 3,4-dioxygenase, which plays a crucial role in the degradation of various aromatic compounds, was purified 135-fold to homogeneity with a 34% overall recovery from *Geobacillus* sp. AY 946034. The relative molecular mass of the native enzyme was about 450 ± 10 kDa and was composed of the non-identical subunits. The pH and temperature optima for enzyme activity were 8 and 60°C, respectively. The half-life of protocatechuate 3,4-dioxygenase at the optimum temperature was 50 min.

Keywords: Thermophilic bacteria, eugenol, protocatechuate 3,4-dioxygenase, purification, metabolism

Introduction

Aromatic compounds play an important role in food, flavor, fragrance, and chemical industries. They are traditionally produced from petroleum by chemical synthesis, which nowadays faces resource and energy shortages and environmental pollution. Biodegradation and biotransformation of plant-related phenylpropanoid compounds such as eugenol are very important because they are natural renewable resources that can be used for the production of useful chemicals. Thus, biotransformation of eugenol has always been a hot topic because it is a natural renewable resource and the transformation processes are environmentally friendly. Eugenol is used in the production of biodegradable polymers and it has a great potential as a starting material for the synthesis of aromatic flavorings and aromas. Vanillin (4-hydroxy-3-methoxybenzaldehyde), one of the most important aromatic flavor compounds used in the food and perfume industries, is an intermediate in microbial degradation of several substrates such as ferulic acid,

phenolic stilbenes, lignin, eugenol, and isoeugenol [7, 27, 36, 39]. The biotransformation of ferulic acid has been widely investigated [8, 11, 16, 23, 25]. However, natural ferulic acid is expensive and is not available in sufficient quantities. A new method for producing ferulic acid and vanillin is the enzymatic oxidative transformation of eugenol. A method of eugenol biotransformation to vanillin was developed by Rabenhorst [28] based on a new *Pseudomonas* sp. strain. Some microorganisms, including *Pseudomonas fluorescens*, *P. putida*, *Corynebacterium* sp., *Fusarium solani*, *Byssoschlamys fulva*, and *Penicillium simplicissimum*, can degrade eugenol as well [9, 15, 27, 34]. Eugenol is catabolized by *Pseudomonas* and *Corynebacterium* via ferulic, vanillic, and protocatechuic acids, where the latter is the substrate for further ring cleavage and the β -keto adipate pathway [15, 34]. A few eugenol-converting enzymes have been described, including vanillyl alcohol oxidase from *Penicillium simplicissimum* [13], vanillin dehydrogenase from *Pseudomonas* sp. strain HR [24], and eugenol oxidase from *Rhodococcus* sp. strain RHA1 [14].

Industrial interest in *Geobacillus* species has grown because of their potential applications in biotechnological processes; for example, as a source of various thermostable enzymes, such as proteases, amylases, lipases, and others [19]. *Geobacillus* species also have potential in generating products for industrial uses such as exopolysaccharides [22]. Many of the species of *Geobacillus* have been isolated from oil fields and more specifically from deep oil wells. Not surprisingly, these organisms show well-developed abilities to degrade a range of hydrocarbons. The ability of *Geobacillus* spp. to degrade such compounds has not been fully investigated. There are a few reports on the range of hydrocarbons utilized by *G. caldoxylosilyticus*, *G. toebii*, and *G. thermoleovorans* [6, 38].

In the present study, the principal metabolic stages of eugenol biotransformation by thermophilic *Geobacillus* sp. AY 946034 were analyzed, and the protococatechuate 3,4-dioxygenase (3,4-PCDase), which plays a crucial role in the degradation of aromatic compounds, was purified and partially characterized.

Materials and Methods

Bacterial Growth

The thermophilic strain designated as *Geobacillus* sp. AY 946034 was isolated from an oil field of Lithuania [5]. For enzyme assays and metabolites determination, the thermophilic strain was grown in medium as follows (g/l): KH_2PO_4 – 7.5, $\text{Na}_2\text{HPO}_4 \cdot 12\text{H}_2\text{O}$ – 3.5, NaCl – 0.5, $(\text{NH}_4)_2\text{SO}_4$ – 0.4, $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ – 0.5, $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ – 0.5, $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ – 0.5, $\text{CuSO}_4 \cdot 2\text{H}_2\text{O}$ – 0.5, ZnCl_2 – 0.5, yeast extract – 0.5, and eugenol (0.01%) as an inducer of the metabolic pathway and the carbon source. The pH of the medium was adjusted to 7.0. The culture flasks were inoculated with 10% (v/v) of preculture grown overnight to an OD_{590} of 0.7 in mineral salt medium at 60°C. Batch culture was incubated in darkness without shaking at 60°C, reaching a maximum in the late exponential growth phase (OD_{590} of 1.01 ± 0.08).

Isolation and Identification of Metabolites

After eight days of growth (the late exponential phase), the metabolites were isolated from the culture supernatants. Supernatants were extracted with chloroform (3×10 ml) and then acidified to pH 2 with 2 M HCl and extracted again in the same manner. The combined extract was dried over anhydrous Na_2SO_4 , evaporated, and the residue was redissolved in ethyl alcohol. The extract was analyzed on TLC plates (Silica Gel F₂₅₄) using a chloroform – ethyl acetate – acetic acid solvent system (85:25:1). The metabolites were visualized under UV light at 254 nm and by exposure to iodine vapor. The compounds were identified by their retardation factor (R_f) and absorbance spectra in comparison with those of authentic compounds.

For GC-MS analysis, the supernatant was extracted four times (1:1 (v/v)) with ethyl acetate, twice at neutral pH and twice after acidification to pH 2. The acidic and neutral extracts were dried over Na_2SO_4 and analyzed separately. The volatile compounds of the extract were analyzed as obtained and after methylation with dimethylsulfate [5]. For GC analysis, 5 μl aliquots of extract were taken. Analyses were carried out on a HP 5890 (Hewlett Packard) gas chromatograph equipped with a flame ionization detector. The separation was performed on a silica capillary column, CP-Sil 8CB (50 m \times 0.32 mm i.d.). The injection temperature was 280°C and the detection temperature was 300°C. The GC oven temperature was programmed as follows: from 100°C to 180°C at the rate of 5°C/min and to 300°C at the rate of 10°C/min and a final temperature was maintained for 10 min. The same capillary column and temperature program as in the GC analysis was used in GC-MS. Mass spectra in electron mode were generated at 70 eV. Qualitative analysis was based on retention indexes, mass spectra comparison with data in the literature and mass spectral libraries, and comparison on the mass spectra with those of commercially available compounds.

Biotransformation of Postulated Metabolites

Resting cells were harvested in the late exponential growth phase by centrifugation (7,000 $\times g$, 10 min, 4°C) and washed twice in phosphate buffer (50 mM, pH 7.0). Cells were resuspended in the same buffer to concentration of about 3 g (dry cell weight)/l. Then resting cells were incubated in the presence of 5 g of ferulic, vanillic, or protococatechuic acid per 1 L. The biotransformation of each acid was conducted in 30 ml of buffer at 60°C. The time-course samples were taken and analyzed by a UV spectrophotometric method. A control experiment was carried out in phosphate buffer under the same testing conditions without adding resting cells. Experiments were performed three times under the same testing conditions.

Enzyme Assay

The cells were harvested in the late exponential growth phase by centrifugation at 7,000 $\times g$ for 10 min at 4°C, washed twice in 10 ml of 50 mM sodium phosphate buffer (pH 7.0) containing 10% glycerol, and then resuspended in the same volume of the buffer. The cells were disrupted by ultrasonic treatment for 5 min at 22 kHz by using a sonicator. The cell debris were removed by centrifugation at 4°C for 25 min at 14,000 $\times g$. The supernatant was used as the crude cell-free extract.

Enzyme activities were assayed by monitoring the disappearance of substrate or appearance of products with a UV-visible spectrophotometer (Perkin Elmer) with a thermostated cuvette at 60°C. The following enzymes were assayed according to the reported methods given in the references: eugenol hydroxylase [10], feruloyl-CoA synthetase [25], vanillate-*O*-demethylase [21], protococatechuate 3,4-dioxygenase [12], catechol 1,2-dioxygenase [1], and catechol 2,3-dioxygenase [20]. One unit of enzyme activity (U) was defined as that catalyzing either the degradation of 1 μmol of

substrate or the formation of 1 μmol of degradation product per minute.

Protein concentrations were determined spectrophotometrically from the absorbance at 280 nm during the purification procedure and by the standard Bradford method [2] for a pure enzyme.

Enzyme Purification

The purification procedure was carried out in 50 mM sodium phosphate buffer (pH 7.0) containing 10% (v/v) glycerol at room temperature. To remove DNA, the crude cell-free extract was stirred while 20% (w/v) streptomycin sulfate solution was added to a final concentration of 1%. The solution was stirred for 30 min and subjected to centrifugation at 15,000 $\times g$ for 20 min. The supernatant was used as a cell-free extract.

Ammonium sulfate fractionation. The cell-free extract was fractionated by ammonium sulfate precipitation. The ammonium sulfate was added to the cell-free extract and the fraction that precipitated between 30% and 70% saturation was collected by 60 min centrifugation at 15,000 $\times g$ and dialyzed for 20 h against the same buffer.

DEAE cellulose fractionation. A dialyzed protein that precipitated between 30% and 70% ammonium sulfate was applied to a DEAE cellulose column (1.5 \times 8.5 cm; Sigma) equilibrated with 50 mM sodium phosphate buffer, and the enzyme was eluted with a linear gradient from 0.1 to 0.6 M NaCl in 200 ml of the same buffer. The flow rate was maintained at 1 ml/min, and 3 ml fractions were collected.

Sephadex G-200 fractionation. The DEAE cellulose eluent was concentrated by tangential flow filtration (Vivaflow 50) to 2 ml and applied to a Sephadex G-200 column (1 \times 30 cm) equilibrated in 50 mM sodium phosphate buffer and eluted at 1 ml/min with 50 mM phosphate buffer containing 0.15 M NaCl. One milliliter fractions were collected.

Enzyme Characterization

Electrophoresis and molecular mass determination. Purified enzyme was monitored for purity by nonreducing and reducing conditions in vertical gel systems containing 7.5% acrylamide [18]. Proteins were stained with silver.

The native enzyme molecular mass was measured by gel filtration on Sephadex G-200 with thyroglobulin (670 kDa), ferritin (450 kDa), catalase (240 kDa), and aldolase (160 kDa) as standards. The column was calibrated by determining the elution volumes of standard proteins and then calculating the elution volume of each protein with respect to the elution volume of Blue Dextran.

Determination of pH and temperature optima. The effect of pH on enzyme activity was measured at various pH values within the range of 4.0 to 11.0 by using sodium acetate, sodium phosphate, and glycine buffer systems. The pH values were equilibrated at 60°C. The remaining activity was assayed under the standard conditions described above.

The temperature dependence of the protococatechuate oxidation reaction at pH 7.0 was investigated in the range 20–90°C by means

of a thermostated reaction cuvette. The enzyme and substrate solutions were preincubated for 10 min, mixed, and the enzymatic reaction was then carried out at the same temperature.

The thermal stability of the enzyme was determined by incubating their solutions at 60°C for 2 h and measuring activity under standard conditions.

Inhibitors of enzyme activity. The reaction mixture in 50 mM sodium phosphate buffer (pH 7.0), having enzyme of 100 μg , was incubated for 10 min at room temperature in the presence of the inhibitor (1.0 mM) and the reaction started by adding protococatechuate (3 μM). The activity was then measured at 60°C as described above and expressed as a percentage of the activity obtained in the absence of the added compounds.

Results and Discussion

Eugenol and isoeugenol are produced by plants as defence compounds against various microorganisms. The growth of six subsurface thermophilic bacterial strains isolated from oil fields of Lithuania in media with eugenol as the carbon and energy source was examined. The only strain of *Geobacillus* sp. able to grow with eugenol as the carbon resource was strain AY 946034. The minimal eugenol concentration (0.005%) was inadequate carbon resource for strain growth. The eugenol concentration of 0.01% (v/v) enhanced bacterial growth about eight times. The increasing of substrate concentration to 0.02% (v/v) inhibited the bacterium growth. Based on these data (Table 1), stringent eugenol addition to keep the final concentration of eugenol in the growth medium below 0.02% (v/v) has to be controlled. The preliminary qualitative analysis of culture, extracts of *Geobacillus* sp. AY 946034 strain grown on eugenol, by TLC revealed the main metabolites. The R_f values of metabolites corresponded with those of authentic compounds protocatechuic acid (0.14), vanillic acid (0.51), coniferyl alcohol (0.67), ferulic acid (0.71), and eugenol (0.80). The other metabolites were of low concentrations, being hardly visible by TLC and difficult to separate. Vanillin was not detected in the culture extract. Since vanillin is very reactive, it exhibits a toxic effect on most microorganisms. Thus, a rapid conversion of vanillin was

Table 1. Tolerance of *Geobacillus* sp. AY 946034 strain to different eugenol concentrations.

Eugenol concentration, (%, v/v)	Biomass amount (OD ₅₉₀)		
	0 h	96 h	192 h
0.005	0.1	0.15	0.15
0.01	0.1	0.38	0.81
0.02	0.1	0.10	0.15

Table 2. Detection of eugenol metabolites by GC-MS.

Aromatic compound	Retention time (min)	Mass to charge ratio (m/z) of major ion peaks (%) ^a
Eugenol (substrate)	20.89	164(M^+) (100), 149(20), 131(50), 103(25), 93(20), 77(15), 55(9)
Coniferyl alcohol	21.99	181(M^+) (65), 166(20), 153(65), 138(49), 76(56), 204(100)
Vanillic acid	25.57	166(M^+) (100), 151(69), 133(30), 126(40), 73(26), 50(12)
Ferulic acid	25.57	194(M^+) (100), 191(15), 146(30), 73(25)
Protocatechuic acid	24.05	154(M^+) (100), 138(11), 137(10), 109(34), 81(12), 63(7), 55(6), 51(9)

^aThe ion abundance percentages are shown in parentheses.

commonly observed and if vanillin is detected at all, only trace amounts occur during degradation of ferulic acid [25]. GC-MS data also confirmed these results. Four metabolites were detected in GC-MS chromatograms directly (Table 2) and as methylated derivatives. Identification of all these compounds was carried out by comparing their MS data

and retention time with those of standards. It is quite possible that thermophilic bacterium is able to degrade eugenol, like strains of *Pseudomonas*, *Corynebacterium*, *Penicillium simplicissimum*, and *Byssoschlamys fulva*, which can also convert eugenol or isoeugenol *via* coniferyl alcohol, and ferulic, vanillic, and protocatechuic acids [27], the latter of which is the substrate of further ring cleavage and the β -keto adipate pathway.

To clarify the metabolic pathway of eugenol biotransformation, supposed metabolites such as ferulic acid, vanillic acid, and protocatechuic acid were investigated using resting cells (Fig. 1). The aromatic acids were quickly oxidized by resting cells; at the late stage of biotransformation, the aromatic ring was broken and no UV absorption was observed (Figs. 1B and 1C). Changes in the concentration of the above-mentioned aromatic acids were not observed in the control experiment. Thus, in the biotransformation of eugenol, the main metabolites were the corresponding benzoic acids – ferulic, vanillic, and protocatechuic.

A further experiment was performed in order to characterize the enzymatic system responsible for eugenol biotransformation, because a few eugenol-degrading enzymes have been described. The crude cell-free extracts of *Geobacillus* sp. AY 946034 grown on eugenol should have high activities of eugenol hydroxylase, feruloyl-CoA synthetase, vanillate-*O*-demethylase, and protocatechuate 3,4-dioxygenase (Table 3). The crude cell-free extracts of glucose-grown

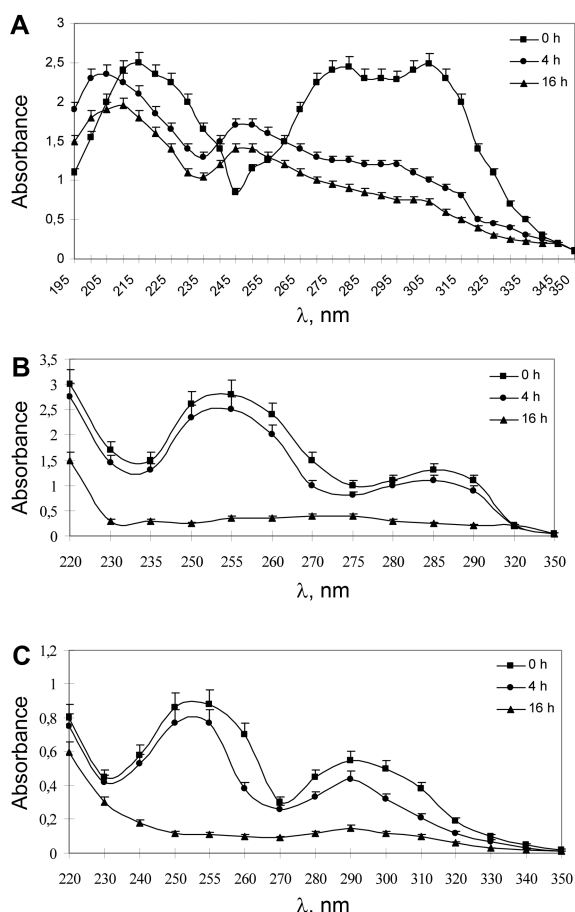


Fig. 1. The shift in UV absorption spectra during the biodegradation of ferulic acid, λ_{\max} - 225 (A), vanillic acid, λ_{\max} - 255 (B), and protocatechuic acid, λ_{\max} - 290 (C) by resting cells of *Geobacillus* sp. AY 946034 strain.

Table 3. Specific enzyme activities in the crude cell-free extract of *Geobacillus* sp. AY 906034 strain grown on eugenol.

Enzyme	Activity, $\mu\text{mol min}^{-1}$ (mg of protein) ⁻¹
Eugenol hydroxylase	0.59 ± 0.06
Feruloyl-CoA synthetase	0.91 ± 0.09
Vanillate- <i>O</i> -demethylase	0.90 ± 0.07
Protocatechuate 3,4-dioxygenase	0.45 ± 0.04
Catechol 1,2-dioxygenase	0
Catechol 2,3-dioxygenase	0

Table 4. Purification of the protocatechuate 3,4-dioxygenase of *Geobacillus* sp. AY 946034 strain.

Fraction	Volume (ml)	Total protein (mg)	Activity (U)	Specific activity (U/mg)	Yield (%)	Purification (fold)
Cell-free extract	15	127.8	65.2	0.51	100	1
Ammonium sulfate precipitation	6	31.35	57	1.82	87	3.6
DEAE cellulose	12	5.2	29	5.58	45	11
Sephadex G-200	4	0.32	22	68.8	34	135

cultures did not contain any of these enzyme activities. These results have indicated that enzymes were induced by growth of the bacteria on eugenol. All purified and characterized enzymes of the eugenol-degrading microorganisms [10, 13, 14, 26] have oxidation of the side chain of aromatic compounds. Degradation of aromatic compounds are mediated by a panoply of enzymatic machinery, which consists of hydroxylating, dehydrogenating, and hydrolyzing systems along with the further complete ring-cleavage systems. Protocatechuate 3,4-dioxygenase is one ring-cleavage enzyme that plays a crucial role in the degradation of aromatic compounds, and is an important component of the bacterial metabolism of these compounds.

The purification of protocatechuate 3,4-dioxygenase from *Geobacillus* sp. AY 946034 grown on eugenol is summarized in Table 4. The enzyme with the specific activity of 68.8 U/mg was purified 135-fold from a cell-free extract with a yield of

34%. The homogeneity of the purified enzyme preparation was examined by electrophoresis on polyacrylamide gels. The native enzyme appeared as one major protein band on the gel (Fig. 2A). SDS-PAGE revealed the enzyme as a multimer composed of two subunit types, α and β (Fig. 2B). The relative molecular masses of these components were estimated using commercial molecular mass standards as 30 kDa for α and 55 kDa for β . The molecular mass of the enzyme, measured by gel filtration, was about 450 ± 10 kDa (Fig. 3). Therefore, it is likely the native enzyme has a composition of $(\alpha\beta\text{Fe}^{3+})_5$ [30].

The 3,4-PCDase was tested for activation or inactivation after an incubation (10 min) with various compounds: oxidants/reductants and specific (dithiothreitol) and non-specific (EDTA) iron chelators. Fe^{2+} inhibited the activity slightly (20%) and Fe^{3+} increased it (15%), which suggests that the dioxygenase contains Fe^{3+} and is consistent with what has been found with other 3,4-PCDases. The fact that Fe^{3+} did not greatly increase the enzyme activity suggests that the purified enzyme has a close to optimal iron content. Enzyme activity was slightly reduced (8%) by ascorbate and phenanthroline, but no activity reduction was observed with dithiothreitol, H_2O_2 , or EDTA. The slight decrease of activity due to ascorbate, a reductant, is a property consistent with a prosthetic Fe^{3+} responsible for

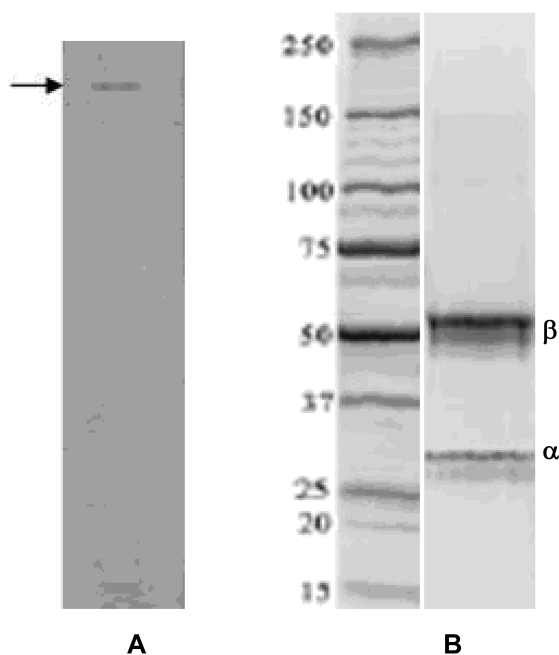


Fig. 2. Polyacrylamide gel electrophoresis of 3,4-PCDase. (A) Native state, arrow: native enzyme; (B) denatured with SDS, α : alpha subunit, β : beta subunit.

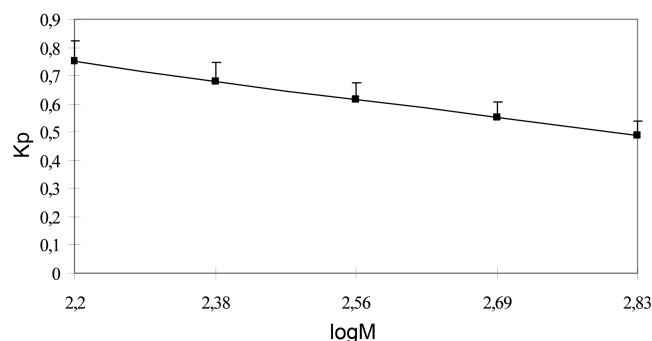


Fig. 3. A semilogarithmic plot of molecular mass as a function of the distribution coefficient, K_p (from left to right: aldolase, catalase, ferritin, 3,4-PCDase, and thyroglobulin).

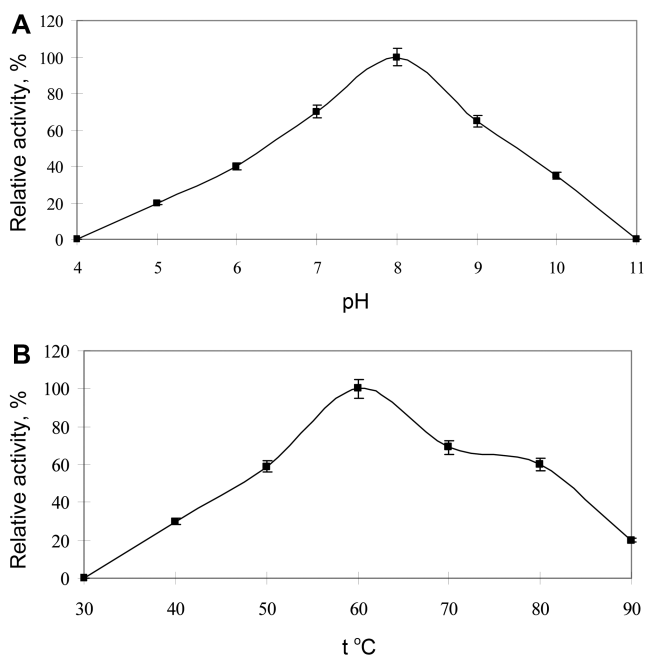


Fig. 4. Effects of pH (A) and temperature (B) on the activity of 3,4-PCDase.

catalysis. Fe^{2+} -containing enzymes are sensitive to oxidizing agents whereas Fe^{3+} -containing enzymes are often activated by them [29].

The effects of pH and temperature on enzyme activity were examined. The optimal pH for enzyme activity was found to be about 8 (Fig. 4A). The pH optimum may be higher, but protocatechuic acid above pH 9.0 undergoes nonenzymatic oxidation. The enzyme completely lost its activity at a pH below 4.0 and above 11.0 after heating at

60°C for 10 min. The optimum temperature for enzyme activity was 60°C (Fig. 4B). No catalytic activities were detected at 30°C and 90°C. The thermal stability of enzyme at 60°C was analyzed and the half-life of 3,4-PCDase at this temperature was 50 min. The 3,4-PCDase retained 100% activity after being stored for two months at 4°C and was stable at room temperature for several weeks. Properties such as temperature, pH optima, and stability are important determinants to consider when making decisions about the use of enzymes.

Previously, we purified 3,4-PCDase from thermophilic bacterial *Geobacillus* sp. AY 946034 strain grown on naphthalene [4]. The current purified enzyme has a specific activity and yield 2-fold higher than our previously reported data about 3,4-PCDase from *Geobacillus* sp. strain grown on naphthalene. Two alternative forms of 3,4-PCDase were induced in *Moraxella* sp. strain GU2 by different aromatic substrates [33]. The occurrence of two forms of enzyme was explained by the presence of two different but closely related sets of cistrons coding for α and β chains, one pair being expressed while the other was not. Another possibility was the occurrence of some posttranslational modifications of a common set of α and β chains, leading to changes in their net charges. Thus, in order to explain the difference of activity and yield of 3,4-PCDases from *Geobacillus* sp. AY 946034 grown on eugenol and naphthalene, further investigations are needed into the amino acid composition and kinetic data between the two dioxygenases.

In summary, the investigation data described above suggest that the degradation pathway of eugenol in the gram-positive thermophilic *Geobacillus* sp. AY 946034 strain

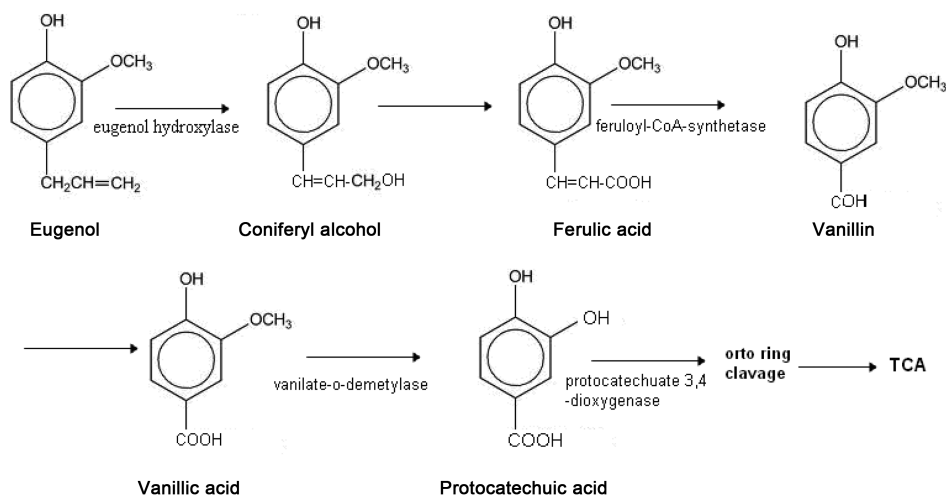


Fig. 5. Postulated degradation pathway of eugenol by thermophilic *Geobacillus* sp. AY 946034.

may be like the pathway found in gram-negative mesophilic *Pseudomonas* sp. [3, 35]. Therefore, we propose the pathway from eugenol to protocatechuic acid in thermophilic bacterium (Fig. 5). As far as we know, the metabolic pathway of eugenol in a thermophilic bacterium was never described previously. Thus, the *Geobacillus* sp. AY 946034 has potential as a biocatalyst for the preparative synthesis of ferulic and vanillic acids – products of great industrial interest. The increasing knowledge of metabolic pathways for vanillin production as well as the identification and characterization of the corresponding genes offers new opportunities for metabolic engineering of industrially important starter cultures. The production of natural vanillin through biotechnological processes using cheaper industrial wastes and major substrates such as lignin, eugenol, isoeugenol, and ferulic acid helps to increase its importance in the flavor industry [17, 37].

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