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Biodegradation of Eugenol by *Bacillus Cereus* Strain PN24

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Abstract: *Bacillus cereus* strain PN24 was isolated from soil by a conventional enrichment culture method using eugenol as a sole source of carbon and energy. The organism also utilized eugenol, 4-vinyl guaiacol, vanillin, vanillic acid and protocatechuic acid as growth substrates. The organism degraded eugenol to protocatechuic acid, which was further metabolized by a β-ketoadipate pathway. On the other hand, the intermediate of the eugenol-degrading pathway, such as ferulic acid was not detected in the culture medium as an intermediate, as evidenced by isolation and identification of metabolites and enzyme activities in the cell-free extract. Such a bacterial strain could be used for phenolic environmental clean-up given optimal nutrient conditions.

Keywords: Biodegradation, Eugenol, *Bacillus cereus* strain PN24.

Introduction

Biodegradation and biotransformation of lignin related phenylpropanoid compound such as phenol has attracted attention as natural renewable resources for the production of useful chemicals[1]. Eugenol is the main component of essential oil of clove tree (*Syzygium aromatium*) degraded[2] by *Pseudomonas* sp. strain HR 199, it is used in the production of biodegradable polymers. And it has great potential as a starting material for the synthesis of aromatic flavorings and aroma such as vanillin[3-5]. Most of the L-Dopa sold commercially is produced from vanillin and hydantoin[6,7]. L-Dopa has been preferred drug for treatment of Parkinson's disease. It is used for controlling the myocardium following neurogenic injury. The world market for L-Dopa is about 250 tons per year. Ferulic acid and lignin were found to be potential substrate for biotransformation processes[8] and there is a growing interest in producing natural vanillin by biotransformation[9,10]. Numerous bacteria and fungi capable of degrading eugenol have been isolated and studied[8,9,11-16]. *Schizyophyllum commune* organism degrade

eugenol *via* ferulic acid and then 4-vinyl guaiacol, in the initial step, the double bond transforming hydroxylation catalysed by eugenol dehydrogenase, which was able to produce methoxyphenol type of aromatic compounds vanillin, vanillate and protocatechuate, which is further metabolized by *ortho*-cleavage of the aromatic ring. Eugenol degradation pathway study is important due to the importance of certain degradation metabolites as fine chemicals or as precursors for industrially important products. In the present study, we report the isolation and characterization of a *Bacillus* sp strain PN 24 that degrade eugenol, since it is cheap and abundant in soil.

Experimental

Eugenol, ferulic acid**,** vanillin, vanillic acid, 4-vinyl guaiacol, protocatechuate, catechol and *α, α′*-bipyridyl was obtained from Sigma Chemical Co., (St. Louis, MO). Other chemicals used were of highest purity obtainable commercially.

Organism and growth conditions

The organism was isolated from soil samples by an enrichment culture technique. It was grown on Seubert's mineral salts medium[17] containing eugenol (0.1% w/v) as sole source of carbon, in a 500 mL Erlenmeyer's flask on a rotary shaker (150 rpm) at room temperature. Growth was monitored turbidometrically at 660 nm. The culture was maintained on agar slants.

The identification of eugenol degrading organism was done on the basis of its morphological, cultural and physiological characteristics. The biochemical tests were carried out according to Pelczar; Holding and Collee[18,19]. DNA isolation and determination of G+C contents from melting temperature was done as described by Marmur; Mandel and Marmur[20,21]. The compound was incorporated in mineral salts medium 0.1% (w/v).

Nucleotide sequence accession number

The nucleotide gene sequences were analyzed in NCCS Pune (India) and nucleotide sequence data bases are deposited in the Gene Bank under Accession No.DQ423485.1.

Isolation and identification of metabolites

The metabolites were isolated from culture filtrate of the organism grown on eugenol by extraction with ethylacetate. The residues were analyzed for metabolites by Thin Layer Chromatography (TLC) on Silica gel G plates using the following solvent systems: (A) Benzene-methanol-acetic acid (40:20:1 v/v). (B) *n*-Butanol-acetic acid-water (4: 1: 2.2, vol/vol); (C) Benzene-dioxane-acetic acid (90: 25: 4 v/v). The metabolites were visualized under UV light at 254 nm or by exposure to iodine vapors and also by spraying with 1% $FeCl_3$-$K_3Fe(CN)_6$ solution in water. Vanillin gave blue colour when treated with phosphomolybdo-phosphotungstic acid. Phenolic compounds gave a blue colour on spraying with 1% Folin-Ciocalteu's phenol reagent and a bluish-green colour with 100 mM $FeCl_3$, which turned red on exposure to ammonia. Aldehydes were detected by spraying with a solution of 2, 4-dinitrophenylhydrazine (0.1%) in 2M HCl. UV visible absorbance spectra were recorded with a Hitachi 150-20 spectrophotometer. Metabolites were analyzed by reversed phase high performance liquid chromatography (HPLC) with a 5-μ-sperisorb-ODS (C18) column and acetonitrile-phosphate buffer (50 mM, pH 7.0) as the mobile phase. The flow rate was 1 mL/min. The peaks were detected at 280 nm. The mass spectra were recorded using GCMS-QP 15700 Shimadzu Japan made 2001.

Enzyme assay

Cell free extracts were prepared from the washed cells suspended on three volumes of 50 mM phosphate buffer, pH 7.0 by sonication (ultrasonic processor model XL 2010) for 5 min. and centrifugation at 10,000 g for 1 h at 4 ^{o}C. The clear supernatant was used as crude extract for enzyme assays. The following enzymes were assayed on spectrophotometrically according to the reported method of Rabenhorst and Overahge[1,8]: eugenol dehydrogenase, 4-vinyl guaiacol dehydrogenase, vanillin dehydrogenase and vanillate-*o*-demethylase. The protocatechuate 2,3-dioxygenase and protocatachuate, 3,4-dioxygenase enzyme activity was measured according to the method of Hayashi *et al*[22]. Protein was determined by the method of Lowry[23]. One unit of enzyme activity is defined as the amount required catalyzing the formation or consumption of 1 µmol of product or substrate per minute.

Results and Discussion

Characterization of organism

The eugenol degrading *Bacillus cereus* strain PN24 was an aerobic, gram positive, motile and rod shaped. It showed catalase, oxidase, DNase and citrate activities. The organism was able to reduce nitrite and nitrate, produced acid from glucose and sucrose but not lactose, MR-VP test positive, hydrolyzed starch and casein but not gelatin. The strain was able to grow in medium containing 5% NaCl but not in 7.5% NaCl. The G+C content of DNA from the bacterial strain was found to be 30-40 moles %. Thus according to 16S rRNA partial gene sequence data analysis the strain was identified as PN24.

Identification of metabolites

The analysis of culture extracts of *Bacillus cereus* strain PN24 grown on eugenol by TLC revealed compounds. The R_f values of compounds corresponded with those of authentic compounds 4-vinyl guaiacol, vanillin, vanillate and protocatechuate respectively (Table 1). These compounds were purified by preparative TLC and analyzed by HPLC, UV and IR data. The GC/MS spectrum of the isolated compound corresponded well to that of authentic protocatechuic acid (Figure 1). The IR spectrum showed the presence of hydroxyl group (3560-3520 cm^{-1}) and aromatic (1615-1600 cm^{-1}) group and a vinyl double bond at (990-905 cm^{-1}) (Figure 2). And the GC/MS spectrum showed the occurrence of 4-vinyl guaiacol, or 4-hydroxy-3-methoxy styrene (Figure 3).

Table 1. Chromatographic and spectral properties of metabolites of eugenol

Properties	Isolated metabolites				Authentic compounds			
R_f values in diff. solvent system	4-4-vinyl guaiacol	vanillin	vanillic acid	Protocate-chuic acid	4-vinyl guaiacol	vanillin	vanillic acid	Protocate-chuic acid
A	0.87	0.97	0.67	0.70	0.87	0.97	0.67	0.70
B	0.82	0.75	0.26	0.40	0.82	0.75	0.26	0.40
C	0.84	0.86	0.72	0.43	0.84	0.86	0.72	0.43
MP /BP, ^{0}C	205	84	213	202	205	84	213	202
HPLC: Rt, min.	2.10	1.90	2.00	2.17	2.10	1.90	2.00	2.17
λ_{max}, in methanol	277	310	286	260	277	310	286	260

(A) Benzene-dioxane-acetic acid (90: 25: 4, vol/vol); (B) n-Butanol-acetic acid-water (4:1: 2.2, v/v); (C) Benzene-methanol-acetic acid (40: 10: 1, vol/vol)

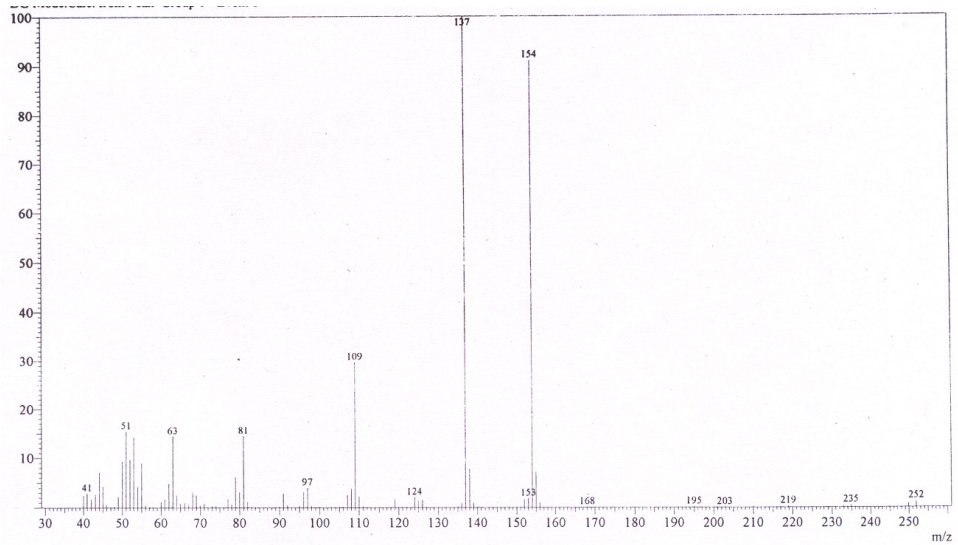

Figure 1. Mass spectrum of isolated metabolite protocatechuic acid

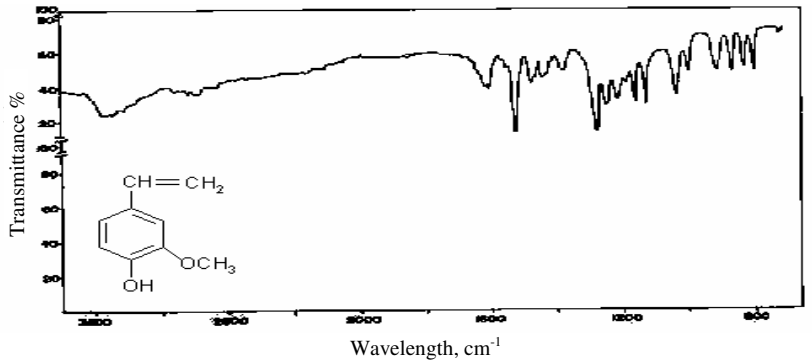

Figure 2. IR spectra of isolated metabolite 4-vinyl guaiacol

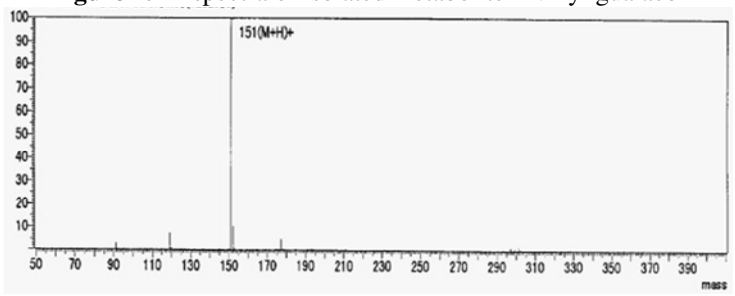

Figure 3. GC/MS spectra of the isolated metabolite 4-vinyl guaiacol

Enzyme activities in cell-free extracts

The cell free extract of the *Bacillus cereus* strain PN 24 grown on eugenol contained the activities of eugenol hydroxylase, 4-vinyl guaiacol dehydrogenase, vanillin dehydrogenase, vanillate-*o*-demethylase and protocatechuate 3,4-dioxygenase. But activities of protocatechuate 4, 5-dioxygenase and catechol 2, 3-dioxygenase were not detected in cell-

free extracts (Table 2). The cell free extract of glucose-grown cells did not contain any of these enzyme activities. These results have indicated that these enzymes were induced by the growth of organism on eugenol.

Table 2. Specific activities of enzyme in the cell-free extract of *Bacillus cereus* strain PN24 grown on eugenol

Enzymes	Specific activity (units/mg of protein)
Eugenol dehydrogenase	0.59
4-Vinyl-guaiacol dehydrogenase	0.71
Vanillin dehydrogenase	0.90
Vanillin-O-demethylase	0.90
Protocatechuate 3,4-dioxygenase	0.65
Protocatechuate 4,5-dioxygenase	ND
Catechol 2,3-dioxygenase	ND

ND= Not Determined

The present studies have demonstrated that the *Bacillus cereus* strain PN 24 is able to utilize eugenol as the sole carbon source for growth. On the basis of 16S rRNA sequence data analysis, strain PN 24 did not show any similarity of the sequence to reported eugenol degrader. These observations suggest that strain PN 24 is a newly isolated eugenol degrader placed in the genus *Bacillus*. Strain PN 24 is now deposited in the Gene Bank database under accession number DQ423485.1. The bacterium was inoculated in the mineral salts medium containing 1g/L eugenol compound. The bacterial growth was measured turbidometrically at 660 nm. The isolated strain PN 24 was capable of degrading the eugenol completely within 48 h (Figure 4).

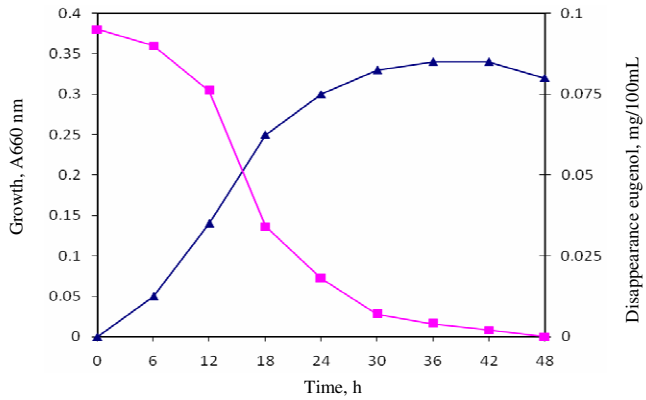

Figure 4. Utilization of eugenol (■-■) during growth (▲-▲) of *Bacillus cereus* strain NP 24

The initial steps of eugenol transformation in *Bacillus cereus* strain PN 24, appears to proceed in an unusual fashion, namely the initial non-oxidative shortening of the side chain of eugenol by a one carbon fragment to yield 4-vinyl guaiacol and oxidative two carbon fragmentation of the side chain of the vinyl bond was cleaved to vanillin directly[2,5,24-,26]. Vanillin as transformation products followed vanillic acid[10] and protocatechuic acid which are the intermediates in eugenol degradation. The catabolism of eugenol to ferulic acid, vanillin and vanillic acid has been reported by Tadasa[27]. In *F. solani,* formation of 4-vinyl guaiacol from eugenol proceeds *via* ferulic acid, although the latter could not be detected in the medium[2,13]. During our observations, we were not identified or detected ferulic acid as

intermediate or metabolite in the culture medium. This suggests that eugenol is directly converted to 4-vinyl guaiacol by double bond transforming hydroxylation catalyzed by eugenol dehydrogenase, as shown in Figure 5.This degradation pathway of eugenol probably similar to that of *F. solani*[13]. Vanillin dehydrogenase and vanillate-*o*-demethylase respectively, which are responsible for the conversion of vanillin to protocatechuate, have been identified[20]. It appeared that the *p*-hydroxyl group was essential for the initial decarboxylation of side chain in this organism.

When α α^1-bipyridyl was added to the medium to block dioxygenase activity, cultures grown on eugenol, protocatechuate accumulated in the medium. The accumulated Protocatachuate further metabolizes by the formation of ring cleavage product and then enters β-ketoadipate pathway[4,12,22,28] indicating that the *ortho*-cleavage metabolic pathway is inducible[13]. However, this pathway requires the identification of the intermediate such as ferulic acid for further conformation. Since the glucose grown cells did not contain any activities of these enzymes.

Enzymatic studies have showed that eugenol was degraded through 4-vinyl guaiacol, it has been observed that *ortho*-clevage pathway[4,29] Figure 5. *Bacillus cereus* strain PN 24 has been isolated and it is able to completely degrade eugenol, 4-vinyl guaiacol, vanillin, protocatechuic acid as the sole source of carbon and energy. Eugenol degradation pathway study is important due to the importance of certain degraded metabolites used as fine chemicals or as precursors for industrially important products.

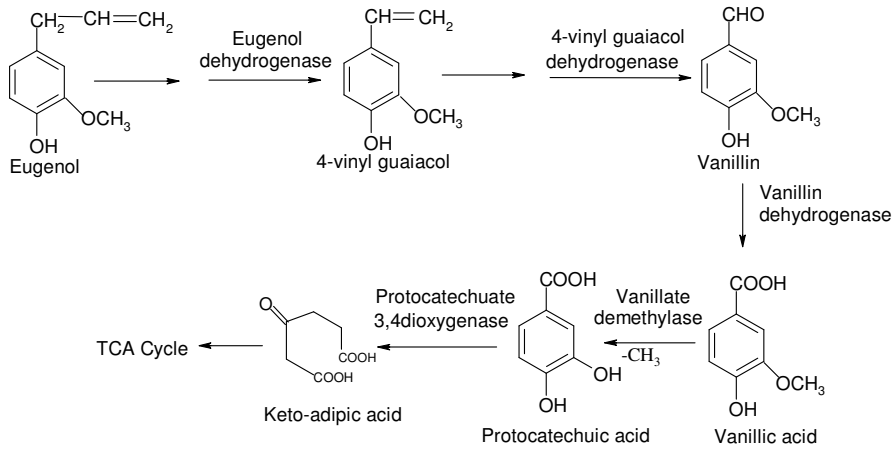

Figure 5. Proposed pathway for the degradation of eugenol by *Bacillus cereus* strain NP24

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