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Rapid conversion of ferulic acid to 4-vinyl guaiacol and vanillin metabolites by *Debaryomyces hansenii*

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Abstract

Debaryomyces hansenii, an isolated yeast strain metabolized ferulic acid to an intermediate compound, namely 4-vinyl guaiacol, by the non oxidative decarboxylation of its side chain. This bioconversion is a highly value added process as 4-vinyl guaiacol is nearly 40 times costlier than ferulic acid. The major degradation products were isolated and identified by thin layer chromatography, high performance liquid chromatography and ^{1}H and ^{13}C nuclear magnetic resonance (NMR) spectroscopy. *Debaryomyces* produced 1470 mg l^{-1} of vinyl guaiacol at the tenth hour itself, corresponding to a molar yield of 95% while the production of vanillin reached a maximum of 169 mg l^{-1} at the fifth hour.

Keywords: Biotransformation; Ferulic acid; Vinyl guaiacol; *Debaryomyces*; 3-Methoxy 4-hydroxystyrene

1. Introduction

Ferulic acid is one of the major phenolic lignin monomers found in woods, grasses, corn hulls, cereal brans and sugar beet pulp [1,2]. These sources provide several billion pounds of ferulic acid as a renewable aromatic feed stock for the conversion into other useful and value added chemicals like guaiacol, vanillin, vanillic acid and protocatechuic acid [3,4].

Ferulic acid is a precursor of 4-vinyl guaiacol (3-methoxy 4-hydroxystyrene), the commercial cost of which is nearly 40 times more than that of ferulic acid. 4-Vinyl guaiacol possesses a spicy clove-like aroma and is an appreciable flavor constituent in Belgian wheat and German Rauch beers [5], wine and soy sauce. It is used in the fragrance and perfume industry [6] and can be converted through biocatalytic routes into products like acetovanillone and ethyl guaiacol that are used in perfumery. Biodegradable, oxygenated polystyrenes with mechanical strength superior to other styrenes [7,8] have been obtained chemically from vinyl guaiacol.

The decarboxylation of ferulic acid to 4-vinyl guaiacol [3,5,9] and its further oxidation to vanillin and vanillic acid [10] has been reported earlier. Immobilized *Haematococcus pluvialis* cultures produced 10.6 mg l^{-1} of vanillin and 5.4 mg l^{-1} of vanillic acid when supplemented with 1 mM ferulic acid [11]. *Streptomyces halstedii* converted ferulic acid into vanillic acid (80% molar conversion) with transient formation of 150 mg l^{-1} of vanillin from 1 g l^{-1} of ferulic acid [12]. The US Legislation incorporates under the term 'natural products' those obtained from biological sources like living cells or their components and there has been an increasing demand for natural vanillin in the past few years.

The present study has dealt with the microbial transformation of ferulic acid, a renewable material, as a means of generating higher value added products.

2. Materials and methods

2.1. Chemicals

Ferulic acid, ABTS (2,2′-azino-bis(3-ethylbenz-thiazoline-6 sulfonic acid)) (Sigma Chemical Co., St. Louis, MO, USA), Vanillin (Fluka, Sigma–Aldrich, USA), Vanillic acid (SD Fine Chemicals, Mumbai, India), Vanillyl alcohol and 4-vinyl guaiacol (Lancaster Synthesis Ltd., Lancashire, UK) were of analytical grade and of the highest purity. Peptone and yeast extract were obtained from Himedia Laboratories, Mumbai, India. Solvents used were of the HPLC grade.

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2.2. Fungal strain

A yeast culture isolated in the laboratory and identified as *Debaryomyces hansenii* and assigned number 539 (MTCC, IMTECH culture collection, Chandigarh, India) was used for the study. The culture was maintained on Chloramphenicol rose bengal agar and stored at 4 °C.

2.3. Medium and culture conditions

A two-stage fermentation protocol [13] was followed for the biotransformation studies. Stage I cultures were grown in 30 ml of sterile Yeast extract peptone glucose medium (YEPG) [14] held in a 150 ml fernbach flask and shaken at 120 rpm and 30 °C. A 5% inoculum derived from 48 h old stage I culture was used to start stage II cultures made of 100 ml of sterile YEPG medium in 500 ml flasks that were incubated for 24 h. Ferulic acid dissolved in *N*,*N*-dimethyl formamide, was filter sterilized and added to the medium to a final concentration of 2 g l^{-1} and incubated on a rotary shaker at 120 rpm for 72 h. The final concentration of dimethyl formamide in the medium was 2% (v/v).

2.4. Growth measurements

Growth was measured in terms of optical density of the culture samples at 600 nm in a UV–Vis Spectrophotometer (Shimadzu UV 2100).

2.5. Substrate utilization and transformation studies

Culture samples were withdrawn at definite time intervals up to 72 h after the addition of the substrate for the determination of pH, turbidity and concentration of the transformed products. The disappearance of ferulate was monitored in the 200–400 nm range as ferulate absorbs maximally at 286 and 310 nm.

2.6. Preparation of yeast resting cells and cell free extract

The yeast cells were harvested after 10 hours of incubation with the phenolic substrate, by centrifugation at 10,000 × *g* at 4 °C and the pellet was washed (×3) with 50 mM Bis–Tris propane (BTP) buffer of pH 6.0. The resultant pellet resuspended in 5 ml BTP buffer constituted the yeast resting cells. A similar pellet of yeast cells was freeze-dried (6 h), mixed with an equal volume of sand and ground using a mortar and pestle. The ground cells were resuspended in 50 mM Bis–Tris propane buffer of pH 6.0 (5.0 ml) and centrifuged at 12,000 × *g* for 20 min to remove the cell debris, and the supernatant was referred to as the cell free extract. The total protein concentration was determined by the Lowry's method [15] with bovine serum albumin as the standard.

2.7. Enzyme assays

The culture supernatant collected after centrifugation was checked for the presence of laccase and peroxidase enzymes as they are found to catalyze the polymerization of aromatic precursors [16] and thereby reduce the efficiency of breakdown of ferulic acid. The measurement of laccase activity was done using ABTS as substrate (5 mM final concentration) [17] in 100 mM sodium acetate buffer of pH 5.0. The total reaction volume of 3 ml comprised of 2 ml buffer, 0.750 ml of 20 mM ABTS and 0.250 ml of culture supernatant and the optical density of the oxidation product was read at 420 nm (ε_{max} = 36,000 M^{-1} cm^{-1}).

The peroxidase activity was also measured using ABTS as the substrate [18]. The assay mixture consisted of the culture supernatant, 0.6 mM ABTS and 1.2 mM H_2O_2 in 67 mM phosphate buffer of pH 6.0. Activity was calculated from the change in absorbance at 420 nm min^{-1}.

The ferulic acid decarboxylase activities of the yeast cell free extract and yeast resting cells were analysed. The assay reaction mixture contained 50 mM BTP buffer, pH 6.0 containing ferulic acid at a concentration of 2 mg $ml^{-1}$and 0.2 ml of cell free extract/cell suspension. Reaction was started by the addition of cell suspension/cell free extract (20%, v/v) and incubating the mixture at 30 °C for 30 min, and the change in absorbance at 260 nm was recorded. One unit of activity is defined as the activity required for the formation of 4-vinyl guaiacol at 1 μmol min^{-1}.

2.8. Analytical methods and instrumentation

Four millilitre of the culture medium was removed at different time intervals and extracted with equal volumes of ethyl acetate: propanol mixture (9:1, v/v) (×3) and the combined extracts were dried over sodium sulfate and concentrated in vacuo in a rotavapor at 50 ± 1 °C (Buchi Model R-205, Germany). For preparative scale experiments, stage II incubations were carried out in 100 ml of media held in 500 ml flasks. The samples were withdrawn 10 h after the addition of the substrate and centrifuged at 10,000 × *g* for 10 min. The culture supernatants were extracted with the solvent and concentrated in vacuo at 50 ± 1 °C.

The solvent systems employed in the TLC analyses were toluene–acetic acid (11.5:0.4, v/v) and benzene: dioxane: acetic acid (90:25:4, v/v/v). The components in the organic extract were separated by silica gel column chromatography (60–120 mesh) using hexane: ethyl acetate as the eluting system in a glass column of inner diameter 2.2 cm and length 30 cm.

2.8.1. NMR analyses

The major compound that eluted out from the silica gel column in 1% ethyl acetate was analysed by nuclear magnetic resonance (NMR) spectroscopy. NMR spectra were recorded on a high field spectrometer (Bruker Advance DPX 300) operating at 300 MHz for ^{1}H and at 75 MHz for ^{13}C NMR. A homogenous suspension of the isolated compound was prepared in deuterated chloroform and a 10 parts per million (ppm) scan width was used for ^{1}H and 200 ppm scan width for ^{13}C. Tetramethyl silane was used as the internal standard. Chemical shift values are reported in ppm and the coupling constants (joule values) are given in hertz. Abbreviations for NMR are as follows: s, singlet; d, doublet; m, multiplet. The ^{1}H NMR spectrum indicated one methoxyl group (δ 3.82, s, 3H), one hydroxyl group (δ 5.60, s, 1H), three aromatic protons (δ 6.8, m, 3H) and the

olefinic protons ((δ 5.06, d, 1H, J_{B-X} = 10.8 Hz), (δ 5.53, d, 1H, J_{A-X} = 17.5 Hz) and (δ 6.65, d, 1H, J_{A-X} = 17.5 Hz)). These values are nearly identical to those reported earlier [19]. The ^{13}C NMR spectrum comprised of peaks at 77.5 ($CDCl_3$), 55.9 (O–CH_3), 108.9 (C-2′), 110.0 (C-2), 115.0 (C-5′), 120.0 (C-6′), 130.2 (C-1′), 136.8 (C-1), 145.0 (C-4′) and 147.5 (C-3′) ppm identifying the compound as 4-vinyl guaiacol.

2.8.2. HPLC analyses of the phenolic metabolites

Ferulic acid and the transformed products were analysed by reverse phase HPLC on a Lichrocart 250-4 mm ODS C_{18} column (Merck, Darmstadt, Germany) using an HPLC model LC 10AD, Shimadzu at room temperature, equipped with dual pumps and an SPD-10A UV–Vis detector. The mobile phase comprised of water, methanol and acetic acid (70:30:1, v/v/v), and the flow rate employed was 1 ml min^{-1}. The UV detector was maintained at 280 nm. The samples were filtered through 0.22 μm PTFE membrane before analysis. The standard deviations of the analyses were less than 5%. The chromatograph was connected to the CSL Data Processing software and the quantification was performed using external standards. The compounds eluted out in the following order. The R_t values were vanillyl alcohol (4.2), vanillic acid (6.1), vanillin (8.7), *trans*-ferulic acid (12.4), *cis*-ferulic acid (14), 4-vinyl guaiacol (36) and two unidentified compounds.

3. Results and discussion

3.1. Preliminary analyses

Yeast cultures incubated with ferulic acid showed distinct changes in the UV absorption pattern (Fig. 1). The decarboxylation of ferulic acid to 4-vinyl guaiacol leads to a hypsochromic shift in the peak maxima. The transformation of ferulic acid (λ_{max} 290, 310 nm) to a product with absorption maxima (λ_{max} 210, 260 nm) could be ascribed to the formation of 4-vinyl guaiacol as the λ_{max} of vinyl guaiacol in methanol was observed at 265 and 226 nm.

3.2. Ferulic acid metabolism

A prominent reduction in the ferulic acid concentration (>50% reduction) was observed in the first few hours after supplementation. The production of 4-vinyl guaiacol increased steadily over the first few hours after ferulic acid supplementation, reaching a maximum of 1470.8 mg l^{-1} with a corresponding molar yield of 95.07% at the tenth hour itself (Fig. 2). The rate of degradation of ferulic acid in most of the earlier reported cases was very slow with low yield of metabolites [10,20]. *Candida lambica* isolated from unpasteurized apple juice produced a maximum of only 310 μg ml^{-1} of 4-vinyl guaiacol upon supplementation with 5 mg ml^{-1} of ferulic acid after 40 h [21]. Only very few cases have reported high levels of conversion of ferulic acid to vinyl guaiacol (more than 90% molar yield) in a short period such as in *Bacillus subtilis* [19].

Decarboxylation function represents a step of secondary metabolism that could have evolved in response to the toxicity of the compounds [22]. The mechanism of conversion of ferulic acid to 4-vinyl guaiacol, might involve quinoid and vinylogous β-keto acid intermediates which are prone to decarboxylation [20].

The measurement of phenolic compounds by HPLC showed the presence of vanillin, vanillic acid and vanillyl alcohol in addition to vinyl guaiacol, but in lower quantities. Vinyl guaiacol might undergo an oxidative two-carbon fragmentation of the side chain to yield vanillin [23]. Vanillin was detected in the culture supernatant starting from 30 min and reached maximum at the fifth hour (169.09 mg l^{-1}) while vanillic acid started to accumulate from the second hour and reached its maximum also at the fifth hour (93.2 mg l^{-1}). The latter observation can

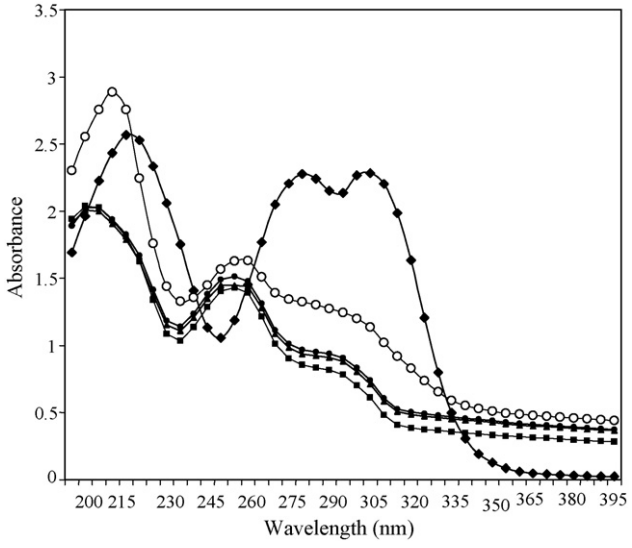

Fig. 1. UV spectra of the YEPG medium inoculated with *D. hansenii* and supplemented with ferulic acid at different hours of growth: (♦) 0 h; (◯) 0.5 h; (●) 6 h; (▲) 10 h; (■) 24 h.

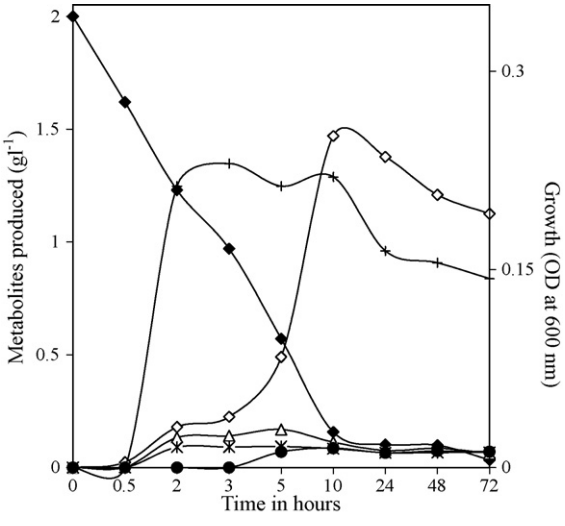

Fig. 2. Growth (+) and ferulic acid breakdown by *D. hansenii* in YEPG medium supplemented with 2 mg ml^{-1} ferulic acid (concentrations of 4-vinyl guaiacol (□), vanillin (△), vanillic acid (*), vanillyl alcohol (●) and ferulic acid (♦) as measured by HPLC).

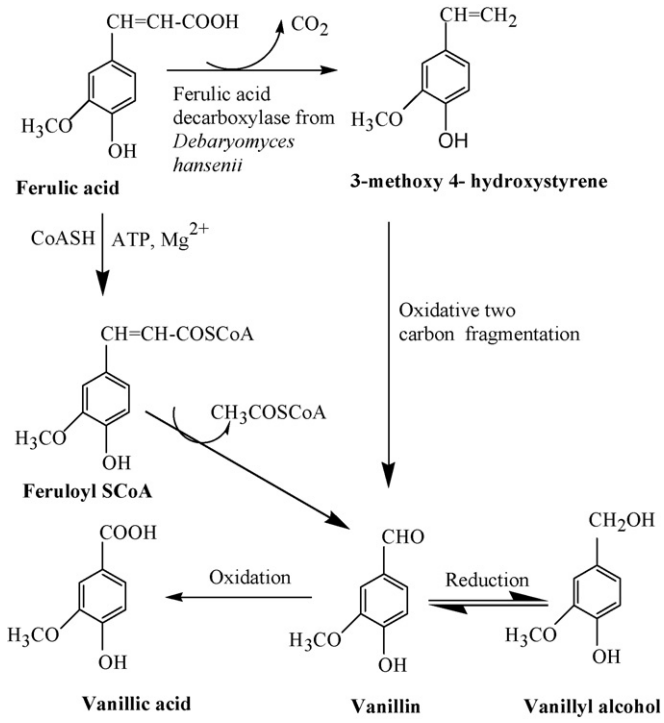

Fig. 3. Proposed pathway for the catabolism of ferulic acid by *D. hansenii* (539).

be explained by the oxidation of the reactive aldehyde to the corresponding acid. This is in line with the previous findings that vanillin is an intermediate of ferulic acid degradation to vanillic acid [24]. The presence of vanillyl alcohol was detected only from the fourth hour and reached a maximum of 14.74 mg l^{-1} at the tenth hour. The culture reduced the vanillin further to vanillyl alcohol via a reductive pathway. The production of vanillyl alcohol and vanillic acid from vanillin has been reported in *Saccharomyces cerevisiae* [25]. The reduction of aromatic acids to alcohols might involve aryl alcohol dehydrogenases [26]. The reduction steps in the fungal metabolism of aromatic compounds would be a part of the detoxification system to maintain the level of inhibitory compounds under a threshold concentration [27]. Vanillin dehydrogenase activity has been found to be responsible for the conversion of vanillin to vanillic acid in *Pseudomonas* sp. strain HR199 [28]. To analyse whether vanillin was formed by the oxidation of vinyl guaiacol, the yeast strain was grown on YEPG medium supplemented with 1 g l^{-1} of 4-vinyl guaiacol. The vinyl guaiacol concentration reduced to 92% within few hours followed by an increase in the concentration of vanillin, vanillic acid and vanillyl alcohol. The intermediates found in the culture medium are the result of the cleavage of the vinyl bond of 4-vinyl guaiacol produced in the culture media as a result of the C-1 cleavage of the ferulic acid side chain. However, the possibility of formation of vanillin from ferulic acid, involving the hydration and retro-aldol cleavage of feruloyl CoA [23] cannot be ruled out due to the presence of a free OH group at the C-4 position of ferulic acid which makes the mechanism quite feasible. A tentative scheme for the catabolism of ferulic acid by *D. hansenii* (539) has been given in Fig. 3, the major one being the decarboxylation step.

3.3. Enzyme activity

Laccase and peroxidase activity was absent in the culture supernatant. Ferulic acid decarboxylase activity was not detected in the culture supernatant; however, the cell free extract showed ferulate decarboxylase activity with the ability to transform ferulic acid into 4-vinyl guaiacol. The specific activity of ferulate decarboxylase enzyme was found to be 1.02 U mg protein^{-1}. The production of this enzyme seems to be induced by the presence of ferulic acid in the culture broth [29]. Crude cell extracts of *B. subtilis* showed a specific activity of 0.20 μmol min^{-1} mg of protein^{-1} for ferulic acid decarboxylase [30]. A specific activity of 1.2 U mg protein^{-1} has been reported from the crude extracts of *Bacillus pumilis* while purified enzyme had a specific activity of 72 U mg of protein^{-1}[29]. The specific activities for ferulic acid decarboxylase of cell free extracts from *C. lambica*, *Rhodotorula rubra* and *Rhodotorula minuta* were found to be 52.6, 24.5 and 12 mU mg protein^{-1}, respectively [21].

Huang et al. in 1993 found that the ferulic acid decarboxylase activity of *S. cerevisiae* was associated with the resting cells of yeast, which support the absence of decarboxylase activity in the culture supernatant [3]. It would appear that the enzyme is membrane-associated or might be intracellular and therefore released by the disruption of yeast cells.

4. Conclusion

The main goal of this study was the identification of a microbial species capable of converting ferulic acid into commercially valuable products, as it forms a readily available natural raw material. *D. hansenii* appears as a good candidate as far as the time duration for the conversion of ferulic acid into vinyl guaiacol and the concentration of vinyl guaiacol formed is concerned, as time duration and concentration are two important factors in industrial processes. However, the production of vanillin from 4-vinyl guaiacol through this biotechnological route is not very economical as the vanillin levels were 169 mg l^{-1} at the fifth hour. Extensive screening of microbial cultures needs to be performed in order to produce strains capable of releasing appreciable amounts of vanillin.

Products obtained from the microbial and enzymatic bioconversion processes are rendered the GRAS status; thus, if ferulic acid, obtained from cereal brans/sugar beet pulp, is used as substrate in the bioconversion process, catalysed by *D. hansenii*, the products formed, namely vanillin and vinyl guaiacol would also be natural. This biotransformation could be of importance in improving the ruminal digestive function and also as a means of generating value added chemicals such as guaiacol derivatives and flavor compounds from ferulic acid. The key advantage of biocatalysis is that enzymes and microorganisms catalyse reactions specifically under mild conditions thereby saving energy [31]. The role and utility of this reaction needs further investigation. A more detailed study on the yeast metabolic pathway and the enzymes involved in the various stages of degradation is needed.

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