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Biotransformation of Ferulic acid to Vanillin by the fungus, *Schizophyllum commune* Fr.

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ABSTRACT

The abundant and readily available precursor Ferulic acid (4-hydroxy-3-methoxycinnamic acid) was utilized to evaluate the biotransformation potential of S. commune in catabolizing the precursor Ferulic acid, Ferulic acid metabolism is expected to occur via. propanoic chain degradation to form **Vanillic acid** which was further metabolized to Protocatechuic aldehyde (3,4-dihydroxybenzaldehyde) via. Protocatechuic acid (3, 4-dihydroxybenzoic acid) or by reductive pathway forming **Vanillin** (4-hydroxy-3-methoxybenzaldehyde) The percentage of biotransformation was found to be 93 in production of Vanillic acid (4-hydroxy-3-methoxybenzoic acid) from Ferulic acid for bio-mass of 0.025gms (fresh weight) at a concentration of 02.5mM Ferulic acid in 50ml. Minimal media, at 370 C, pH (6.0) and an incubation period of 10 days. The presence of Vanillin is detected. along with Protocatechuic acid. Vanillin accumulation was found to be in remarkable amounts when Vanillic acid was fed as sole carbon source. Vanillic acid is an intermediate metabolite of Ferulic acid and immediate precursor of Vanillin. The potential of the fungus to convert Vanillic acid (10.0mM) concentration was explored and an yield of 4.5gl-1 was found on 12th day of incubation. Formation of Protocatechuic acid alone from Ferulic acid was noticed at pH (8.0) under static experimental conditions. This indicates that *S. commune* follows an entirely novel path of catabolism in alkaline conditions in comparison to acidic environment.

Key words: Biotransformation, Schizophyllum commune, Ferulic acid, Vanillic acid, Vanillin,

1. INTRODUCTION

Strong market demand for natural and eco-friendly products has spawned efforts to produce aromatic flavors or pharmaceuticals of interest by microbial transformations. The abundance, ready availability, in-expensiveness, solubility in aqueous media of the precursors and less reports on reduction of carboxylic acids to aldehydes due to their difficulty to achieve by chemical means necessitates the use of Ferulic acid and Vanillic acid as best precursors in biotransformation. Invitro studies on the strain AN103 of Pseudomonas fluorescens reveal the side chain cleavage of ferulic acid to produce Vanillin [1], Later Phenyl propanoid chain cleavage is studied expressing the enzyme 4-Hydroxycinnamoyl CoA hydratase/lyase in Hairy Root cultures of Datura stramonium [2].

S. commune, an edible mushroom of family Schizophyllacae is the fungus of interest. It has a remarkable biotransformation and biocatalytic potential with wide distribution of cellobiose oxidizing enzymes indicating its physiological importance.

In our present investigation the substrates (Ferulic acid and Vanillic acid) as well as the products (Vanillic acid, Vanillin, Protocatechuic acid, Protocatechuic aldehyde) were found to be Host defence potentiators. They are said to exhibit apoptotic, anti-inflammatory, anti-carcinogenic, anti-oxidative, anti-mutagenic properties besides having various applications in Nutraceutical and Pharmaceutical industries. The glycoside, Vanillin is a preferred food flavoring agent with high commercial value.

2. MATERIALS AND METHODS

2.1.1. Micro-organism:

The basidiocarps of S. commune were collected from wooden logs in campus of IIT-Kharagpur.

Figure. 1.1: *Schizophllum commune* grown on wooden logs

2.1.2. Precursors:

Ferulic acid, Vanillic acid

2.2. Initiation and enrichment of cultures:

The fruiting bodies were surface sterilized in $HgCl_2$ (0 .1% v/v) solution. Minute tissue internal to the fruiting body was aseptically transferred to the Petri plates of Potato Dextrose Agar and incubated for 3 days at 37^0C and pH (6.0) For luxuriant growth the mycelium was transferred to Potato Dextrose Broth and placed on orbital shaker. Stock cultures were maintained at 40^0 C on Potato Dextrose Agar. Sub-cultivation was done in Minimal media [1] containing the macronutrients KH_2PO_4, K_2HPO_4, $(NH_4)_2SO_4$, NaCl and micronutrients $FeSO_4.7H_20$, $MgCl_2$ at pH (6.0) and incubated for 3days at 37^0 C.

Figure. 1.2: Mycelial culture of *S. commune*

2.3. Evaluation of the biotransformation potential of S. commune:

Experiments were performed in triplicate in conical flasks containing 50ml. of Minimal media, pH (6.0), 0.025gms. of biomass (fresh weight) was transferred to all except the controls. Further analysis was performed in two steps.

2.3.1. Feeding Ferulic acid as sole carbon source:

Ferulic acid (from Hi-Media Company) was dissolved in aqueous methanol (2% v/v) and filter-sterilized. Various concentrations (02.5mM, 05.0mM, 07.5mM, 10.0mM) were added to each flask. Incubated for 07, 14, 21 and 28 days at 37^0C and analyzed at subsequent intervals.

2.3.2. Feeding Vanillic acid as sole carbon source:

Vanillic acid (from Hi-Media Company) was dissolved in aqueous Methanol(2%v/v) and filter sterilized. 10mM Vanillic acid was added to each flask and incubated at uniform time intervals of 04, 08, 12 days.at 37^0 C. More-over, one set was maintained as aerobic on orbital shaker and the other set as anaerobic cultures.

2.4. Characterization of Phenolics by TLC and Spectrophotometry:

Mycelial mass was removed. Medium was filtered and centrifuged at 5000rpm for 10 min. Supernatant was collected and acidified to pH (2.0). Gently, added Ethyl acetate (1:2), centrifuged at 5000rpm for 10min. and left to settle for 30min. It fractionated into two layers. The upper hydrophobic layer expected to contain the phenolics of interest is separated and subjected to dryness in Rotary Vacuum Evaporator. The concentrate was transferred to Eppendorf tubes and dissolved in 1ml. of aqueous methanol (50% v/v) and subjected to Thin Layer Chromatography. The samples along with standards were loaded on Silica plates, left to dry and developed in saturation chamber of aqueous Formic acid (2% v/v). When Relative Front (Rf) was met the plates were removed, dried and fluorescence under dual wavelength (254/365nm) UV lamp (UVitec, Cambridge, U.K). The band separation of different phenolics was marked against authentic standards. Each band was separately scrapped and dissolved in aqueous Methanol (50% v/v), centrifuged at 5000rpm for 10min. The supernatant was collected and scanned. The scan of samples was compared against the standard scans in UV-Vis. Spectrophotometer 117(Systronics) within a scan range of 200-400nm.

3. RESULTS AND DISCUSSION

While optimizing the biotransformation process it was found that using S. commune has several

advantages over the other organisms reported in the bio-catalysis so far, and the results are very much encouraging.

3.1. Effect of concentration on biotransformation of Ferulic acid:

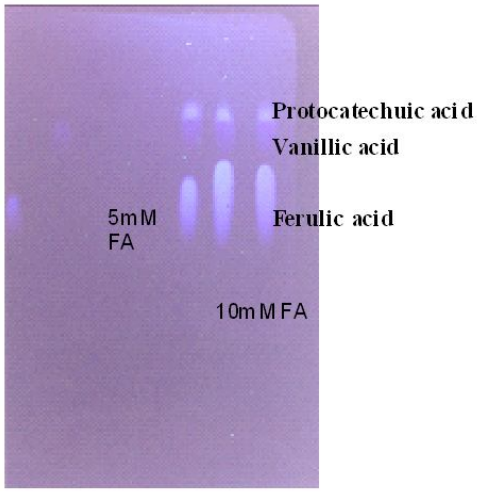

Figure. 2: TLC chromatogram of Ferulic acid, Vanillic acid, Protocatechuic acid detected on 10 days fed Ferulic acid

The biotransformation of Ferulic acid to Vanillic acid was found to be 93% when compared to 38% biotransformation in 10mM. concentration of Ferulic acid. It was also observed that the concentration is too low the conversion to phenolics of interest is low and there is a problem of detection and if too high the concentration it became a toxic precursor and decreased the cell yields.

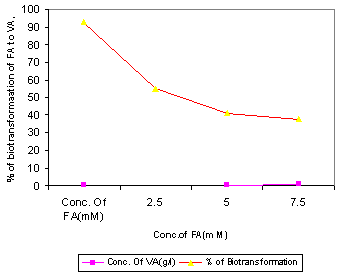

Figure 3: Effect of Concentration on Biotransformation of Ferulic acid to Vanillic acid in 10 days.

It was found that adequate supply of oxygen to the cultures helped in the increase of the biomass since Schizophyllum is an obligate aerobe and in turn increased the enzyme activity of the organism for the formation of Vanillic acid. Aerobic conditions favored formation of acids and anaerobic conditions favored the formation of aldehydes by reduction.

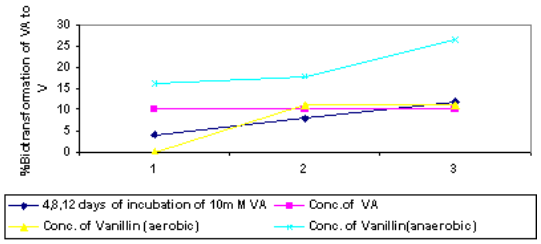

Figure 4: Effect of oxygen on Biotransformation of Vanillic acid (VA) to Vanillin(V)

3.2. Effect of incubation period on biotransformation of Ferulic acid

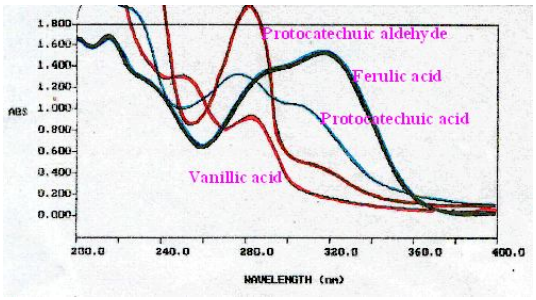

Figure 5: U.V. scan of the metabolites formed from Ferulic acid after 7 days of incubation**.**

The growth of Schizophyllum was active over a wide range of pH but for Vanillic acid formation from Ferulic acid pH range of 6.0-7.0 was found to be more favorable. Vanillic acid is the highly and immediately detected metabolite when Ferulic acid catabolizes in acidic range of near neutral pH.

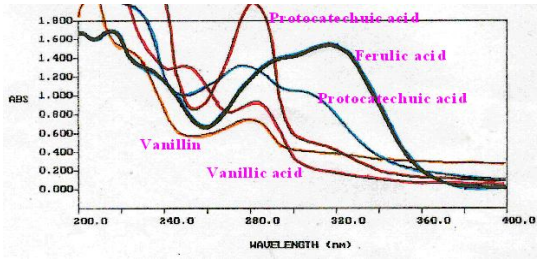

Figure 6: U.V. scan of the metabolites detected after 15, 21, 28 days by biotransformation of Ferulic acid

Vanillic acid formation from Ferulic acid in the first fifteen days was found to be increasing with an yield of 45% but in later stages there was a decrease. This may be due to the further degradation of secondary metabolite formed. So, for Vanillic acid a harvest with-in a fort-night is preferable.

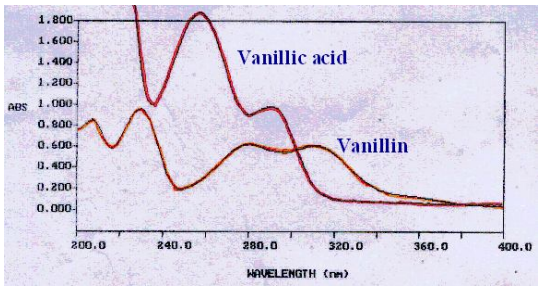

Figure 7: U.V. scan of the Vanillin found after 4 days of incubation from Vanillic acid(10.0mM) as sole carbon source.

Vanillin is the only detected metabolite. Precursor Vanillic acid is highly favorable for production of flavonoid, Vanillin since there isn't any interference of co-products.

3.3. Catabolism of Ferulic acid by S. commune at an acidic pH (6.0):

The biological reactions in the biotransformation of macromolecular substrate, Ferulic acid to Vanillin and Protocatechuic acid is proceeding through an intermediate Vanillic acid. Protocatechuic acid is further reduced to Protocatechuic aldehyde.

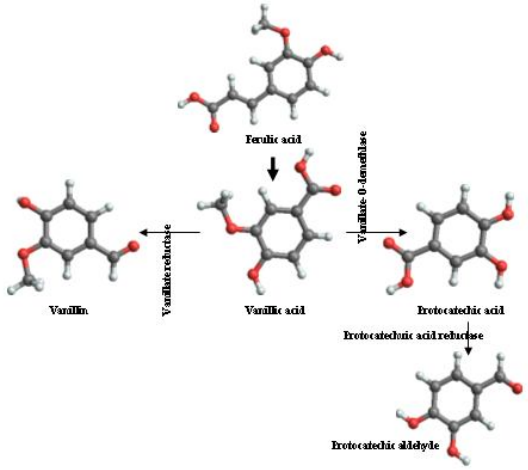

Figure 8: Proposed pathway of Ferulic acid catabolism by S. commune

3.4. Catabolism of Ferulic acid by S. commune at an alkaline pH-(8.0):

Protocatechuic acid is the only product observed. This can be inferred as alkaline medium elicits a novel pathway of biotransformation since the alkalinity may induce different enzymes from that of acidic pH in S. commune and alter contact-catalysis.

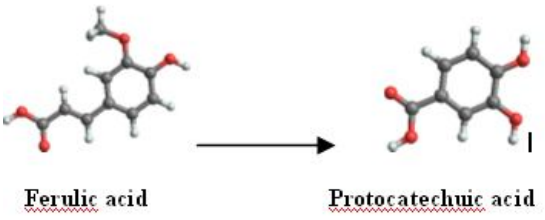

Figure 9: Proposed pathway of Ferulic acid by S. commune at pH-8.0

4. CONCLUSION

From the experimental results it can be concluded that the best medium for transformation is minimal medium devoid of readily available carbon source reduced exo-polysaccharide accumulation of hyphae and hence, increased the surface area of contact catalysis and thus catabolized the fed substrates into the metabolites of interest.

The ratio of concentration of the substrate fed must always be proportionate to the amount of biomass taken. The days of incubation must be proportional to the amount of the biomass, the nature of the substrate taken and also the concentration of the substrate fed. aerobic or anaerobic conditions prevailing.

Though some parameters are similar for the biotransformation of Ferulic acid to Vanillic acid and Vanillic acid to Vanillin there is a marked difference. In the initial step of biotransformation oxidase group of enzymes takes the lead but in later stages enzyme reductase expected to play a role.

Thus, it can be stated that proper monitoring of all these parameters influencing the biotransformation, S. commune can serve as an excellent source for biotransformation.

ACKNOWLEDGMENTS

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