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Original Article

Biodegradation of Ferulic Acid Using *Aspergillus fumigatus*

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Abstract

The soil samples collected from the dumped sites of coffee industry residues contaminated with ferulic acid at Keezhkadu, Kodaikanal were subjected to serial dilution and the development of fungal colonies in PDA plates. One of the colonies was selected and identified as *Aspergillus fumigatus* using Lacto phenol cotton blue staining method. The efficiency of the fungal strain on the degradation of different concentrations of the ferulic acid was studied using 5, 10, 15 and 20 ppm of ferulic acid. The changes in pH, COD, and biomass observed were proved statistically significant using ANOVA. It was also confirmed by the appearance of new peaks in HPLC analysis after ten days of treatment. Hence this strain can be used efficient in degrading the phenolic compound.

1. Introduction

The increase in human population and industrialization led to the deterioration of the natural resources and release of many new waste products into the environment resulting in pollution. A wide range of anthropogenic chemicals designed for use in industry, agriculture, pest control, consumer goods, and emissions from the combustion of fossil fuels are the main sources for soil and water pollution. There are a number of pollutants that are released from industries including toxic chemicals, heavy metals, dyes, phenolic compounds, polymers and some organic compounds. Phenolic compounds are the priority pollutants due to their toxic and harmful effects even at low concentrations. Environmental pollution due to the release of natural phenolic compounds from agro-industrial operations has become widespread in the world. They are found in many industrial effluents and residues like those produced in wine distillery, olive oil extraction, cork preparation, wood debarking and coffee production [1, 2].

The standard limits established for the release of phenolic compounds are normally lower than 0.5mg/L but the content of phenolic compounds in industrial waste waters amounts to the range of 200 to 2000 mg/L. In case of single or small releases, the half-life of phenolic compounds in water is between 2 to 20 days which is long in comparison to its half-life in air and soil. If a large amount is released at one time or if a steady amount is released over a long time, the half-life will increase. Therefore the surface waters and surrounding air would be more contaminated [3]. Ferulic acid is a major phenolic compound of lignin in plants [4, 5]. A number of industrial and food related applications have been reported for ferulic acid, especially based on its microbial degradation to vanillic acid. Though ferulic acid is known to play many beneficial roles, it also had its toxic effects on various organisms. It acts as allelochemical for plants. Aromatic acids like ferulic acid are more toxic than aliphatic compounds [6, 7]. Ferulic acid and other phenolic acids decrease plant growth in part by decreasing the absorption of mineral nutrients and water [8]. Under laboratory conditions a treatment with ferulic acid significantly reduced the radicle length of spring barley [9]. Physical and chemical methods that are used currently to remove phenolic compounds are costly and less effective. Alternatively biological methods including biodegradation and bioremediation are more advantageous since they are cost effective and do not produce any toxic byproducts. Ferulic acid is an important intermediate of lignin degradation [10]. Hence this acid can be used potentially as a carbon source by many bacteria and wood rotting fungi. Filamentous fungi can be an important source of ferulic acid as they grow frequently in wood where phenolic structures are present. In the present study an attempt to degrade the phenolic compound ferulic acid has been made using a fungus isolated from the soil contaminated with ferulic acid.

2. Materials and Methods

The soil samples were collected from the dumped sites of coffee industry residues contaminated with ferulic acid at Keezhkadu, Kodaikanal in sterile screw capped bottles and transported to the laboratory immediately for analysis. The samples were serially diluted and 0.1 ml from 10^{-4}, 10^{-5} and 10^{-6} dilutions were taken and spread plated on minimal medium containing 10 ppm of ferulic acid as a sole carbon source and incubated at 37^0 C for 96 hours and the colonies were isolated. The pure cultures of the colonies were made with potato dextrose agar and they were stored in slants and petridishes for further studies.

The isolated organisms were inoculated into the minimal broth containing 15 ppm of ferulic acid and incubated at room temperature. The fungus with maximum growth was chosen for the study of degradation. A drop of Lacto phenol cotton blue was placed on a glass slide and a small portion of the colony was placed and the culture was spread evenly. A cover slip was placed on the sample and after thirty minutes the slide was observed under the microscope. The species of the fungal isolate was confirmed in Bose Clinical Laboratory, Madurai.

The isolated fungus was inoculated on minimal broth containing different concentrations of ferulic acid like 5, 10, 15 and 20 ppm. The flasks were incubated at room temperature for a period of ten days and the changes in pH, and COD were observed periodically at an interval of forty eight hours. At specific intervals samples were taken centrifuged at 4000 rpm for ten minutes and the supernatant was taken and analyzed for pH.

To ten ml of the sample, 1ml of sulphuric acid and 5ml of 0.25N potassium dichromate were added and refluxed in a water bath for few minutes and it was titrated against ferrous ammonium sulphate. Ferroin is used as the indicator and appearance of red brown colour is the end point. The following formula was used for the determination of COD.

$$\text{COD (mg/L)} = \frac{(a-b) \times \text{Normality of ferrous ammonium sulphate} \times 8000}{\text{Sample volume (ml)}}$$

Where a =volume of ferrous ammonium sulphate used for blank,
b = volume of ferrous ammonium sulphate used for sample

For biomass estimation, samples were taken and centrifuged. The pelleted biomass was taken and the wet biomass was calculated. After drying them in hot air oven, the dry biomass was noted. The samples containing the minimal broth with 20ppm of ferulic acid taken on 0^{th} day and 10^{th} day were subjected to HPLC analysis. Two way ANOVA was performed for the parameters pH, COD and biomass, using Microsoft excel. Variability was considered significant only when the statistic value was greater than the tabulated value at P is less than or equal to 0.05.

3. Results

A fungus was isolated from the phenolic acid contaminated soil and it was tested for its resistance and ability to degrade the phenolic compound, ferulic acid. The fungal strain was exposed to 10ppm concentration of the ferulic acid and this isolate was found to be effective in the degradation of ferulic acid after four days and hence it was selected for further studies of biodegradation (Plate 1 and 2). The colonies were green in color and Lacto phenol cotton blue staining showed that the conidial head was columnar and compact and conidiophore terminated into club or flask shaped vesicle. Hence it was identified as *Aspergillus fumigatus* (Plate 3).

Plate 1: Growth of *Aspergillus fumigatus* in Potato Dextrose Agar

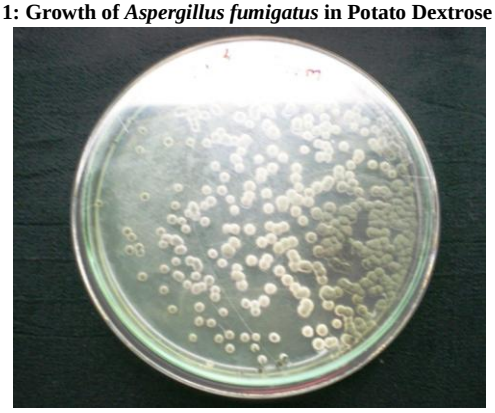


Plate 2: Growth of *Aspergillus fumigatus* in minimal medium containing 10ppm of ferulic acid

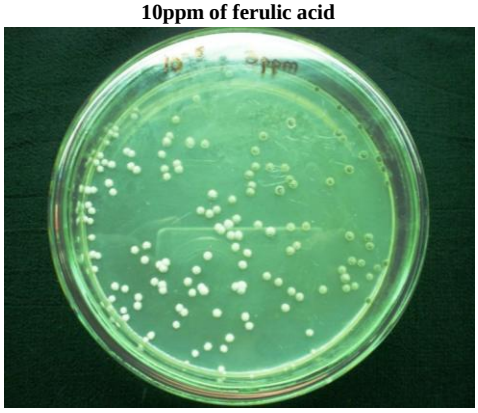


Plate 3: Microscopic view of *Aspergillus fumigatus* showing the conidium

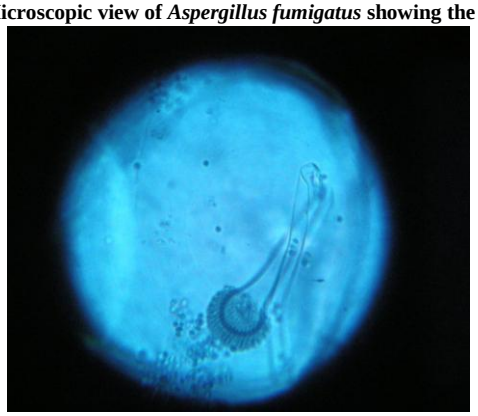
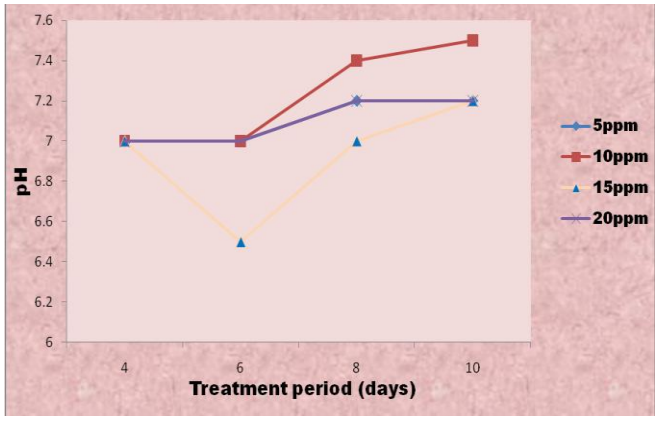


Figure 1 exhibits the changes in the pH of the medium during the degradation of ferulic acid by *Aspergillus fumigatus*. The pH is found to be neutral during the first six days and seemed to be increasing thereafter. The pH increased drastically indicating the degradation of ferulic acid.

Fig. 1: Changes in pH during degradation of ferulic acid by *Aspergillus fumigatus*



Biomass changes during the degradation of ferulic acid by *A. fumigatus* is illustrated in Fig.2. There was a drastic increase in biomass for 10ppm concentration and linear increase in biomass was observed for 20ppm concentration of ferulic acid. Figure 3 shows the changes in COD level during ferulic acid degradation by *A. fumigatus*. Decline in COD was observed with the increase in treatment period.

Fig. 2: Changes in biomass (g dry weight /L) during degradation of ferulic acid by *Aspergillus fumigates*

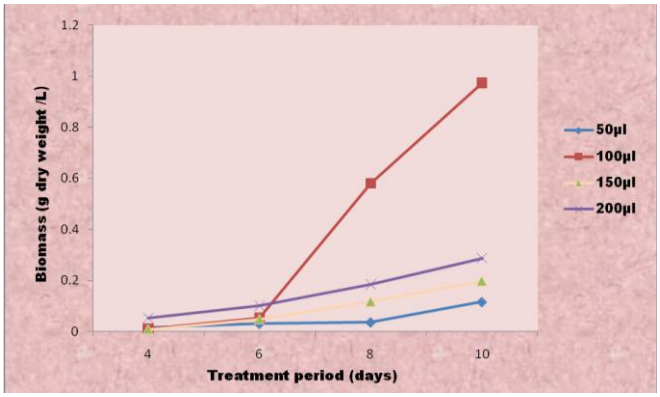


Fig.3: Changes in COD during the degradation of Ferulic acid by *Aspergillus fumigatus*

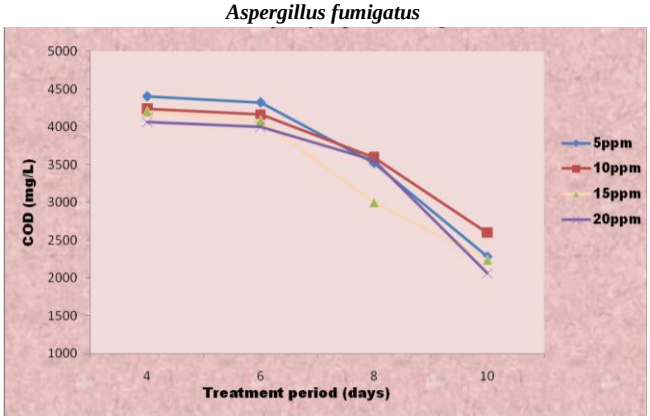


Figure 4 shows the HPLC analysis for 20ppm of ferulic acid on the 0th day of treatment. The degradation of 20ppm of ferulic acid after four days of treatment by *Aspergillus fumigatus* is shown in Fig.5. The degradation of 20ppm of ferulic acid after ten days of treatment by *Aspergillus fumigatus* is depicted in Fig. 6.

The peaks after ten days of treatment were different in their retention times compared to the peaks after four days of treatment indicating the formation of intermediates as well as the disappearance of parent compound. Table 2 exhibits the two way analysis of variance for the factors pH, biomass and COD with the variables, treatment period and ferulic acid concentration for *Aspergillus fumigatus*.

Fig. 4: HPLC analysis report for 20 ppm Ferulic acid (control)

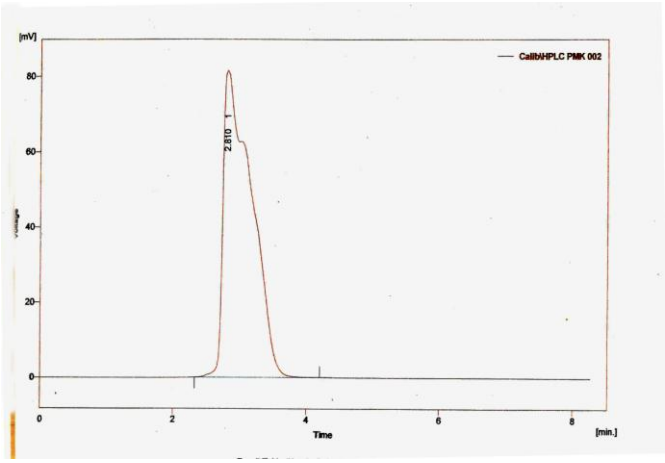


Fig. 5: HPLC analysis report for 20 ppm Ferulic acid treated with *Aspergillus fumigatus* for 4 days

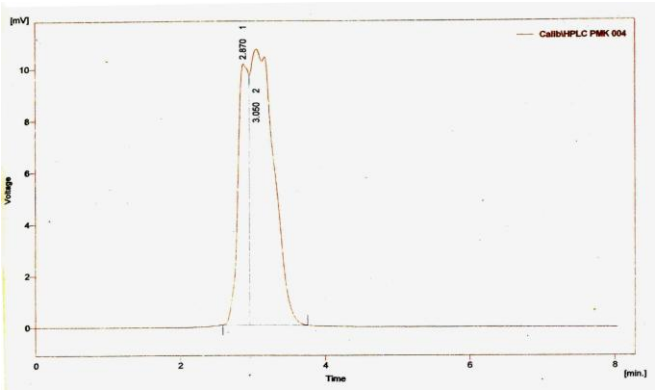


Fig. 6. HPLC analysis report for 20 ppm Ferulic acid treated with *Aspergillus fumigatus* for 10 days

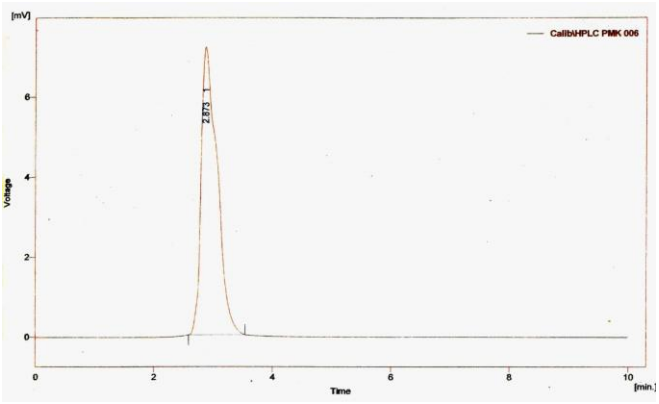


Table-2: Two way analysis of variance for the factors with the variables, treatment period and ferulic acid concentration for *A. fumigates*

Factor	Source of variation	df	MS	Calculated F value	Table F value	Level of Significance
pH	Treatment period	3	0.0608	3.590	3.862548	Not Significant
	Ferulic acid concentration	3	0.134	7.918	3.862548	Significant
biomass	Treatment period	3	0.1003	2.652	3.259167	Not significant
	Ferulic acid concentration	3	0.1173	3.101	3.862548	Not Significant
Carbon dioxide	Treatment period	3	78133	2.602	3.862548	Not Significant
	Ferulic acid concentration	3	3189	106.2	3.862548	Significant

4. Discussion

The processing of plant foods results in the production of by-products that are rich sources of organic compounds including phenolic compounds. Ferulic acid is one of the major phenolic compounds released from a wide range of industries which were proved to be toxic to aquatic organisms [3]. As an allelochemical, ferulic acid has growth inhibitory effects on plants [11]. So the removal of high concentrations of ferulic acid from the environment is necessary.

In the present work, an *Aspergillus fumigatus* isolated from the phenolic acid contaminated soil was tested for its resistance and ability to degrade ferulic acid. The fungal isolate was found to be effective in the degradation of ferulic acid after four days and was used for further studies of biodegradation. Fungi like *Pycnoporus cinnabarinus* and *Schizophyllum commune* degrade ferulic acid via phenyl propenoic side chain pathway [12]. To degrade plant phenolic compounds, fungi produce a wide range of hemicelluloses and cinnamic acid esterases Several cinnamic acid esterases have been isolated from *Aspergillus niger* [13].

The optimum pH for the activity of cinnamic acid esterases was found to be 6.8 [14]. In the present study, there was a mild increase in pH of the medium during the degradation of ferulic acid. The increase in pH of the culture medium may be due to the production of metabolites and formation of intermediate degradation products which might have led to the alkaline condition of the medium. It was found that the efficiency of degradation increases as the treatment period increases. This may be due to the increase in biomass during the treatment period. There was a drastic increase in biomass for 10ppm concentration and linear increase in biomass was observed for 20ppm concentration of ferulic acid. Increase in biomass indicated increased fungal growth due to the utilization of ferulic acid as carbon source.

The chemical oxygen demand is a measure of the oxidizability of a substance, expressed as the equivalent amount in oxygen of an oxidizing reagent consumed by the substance under fixed laboratory conditions. The reduction in organic matter can be measured by chemical oxygen demand [15]. Biodegradation of phenolic compounds usually depends upon the oxidative activities of microorganisms and the amount of COD reduced is directly correlated with degradation efficiency of the organism showing the utilization of phenolic compounds by the organism [16]. There is a gradual reduction in COD during the degradation of higher concentrations of ferulic acid by *A. fumigatus* and COD reduction was greater for lower concentrations of ferulic acid.

In HPLC analysis, the peaks after ten days of treatment were different in their retention times compared to the peaks after four days of treatment indicating the formation of intermediates as well as the disappearance of parent compound. The change in the retention time after four and ten days of treatment exhibited difference which may be attributed to the transformation of ferulic acid into different intermediates. The variations due to the treatment period were statistically not significant for pH, biomass and COD but they were significant due to ferulic acid concentration for pH and COD.

5. Conclusion

The changes in pH, COD and biomass during the degradation of ferulic acid by *A. fumigatus* indicated its greater efficiency in degrading ferulic acid. Thus in bioremediation of ferulic acid, *A. fumigatus* can be employed to clean up ferulic acid contaminated sites as it has better degrading ability. Understanding the genetic aspects of *A. fumigatus* will be more helpful for further research on strain improvement.

Acknowledgments

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