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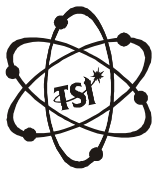

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Coir pith degradation by freshwater cyanobacteria: Targeting value added products

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ABSTRACT

The main objective of the study was to characterize the phenolic compounds released during the degradative action of fresh water cyanobacterium on coir pith. The major lignolytic enzymes, laccase and polyphenol oxidase and accessory enzyme esterase, expressed induction on exposure to the lignocellulosic material. Spectral analysis, thin layer chromatography and high performance liquid chromatography results coincided with the standard chemicals- guaiacol and 4-hydroxy benzaldehyde. The results showed that this fresh water cyanobacterium, *Phormidium* sp. BDU-5 can be used as a driving force to release industrially important phenolic compounds from lignocellulosic waste like coir pith, avoiding chemical delignification, pollution and above all oxygenating the environment during the degradation process. Coir pith, a lignocellulosic waste, which is dumped in ton every year, can be utilized as a natural and cheap source for industrially important compounds like guaiacol and 4-hydroxybenzaldehyde. © 2009 Trade Science Inc. - INDIA

KEYWORDS

Lignin;
Biodegradation;
Cyanobacteria;
Laccase;
Polyphenol oxidase;
Esterase;
Guaiacol;
4-hydroxybenzaldehyde.

INTRODUCTION

As the cyanobacteria have simple growth requirements, grow to high densities and use light, CO_2 and other inorganic nutrients efficiently, they could be attractive hosts for production of valuable organic products[1]. Cyanobacteria have been shown to degrade naturally occurring hydrocarbons[2], coir pith[3], phenol[4], xenobiotics[5] and organophosphorus pesticides[6].

Coconut (*Cocos nucifera*) is one of the most useful and extensively cultivated palm in tropical countries. The fibrous mesocarp of the fruit known as coir is a useful by-product used to make ropes. The wastes of coir yarn industry get accumulated to the tune of 1 lakh ton per year making their disposal difficult, although a limited quantity is used as soil conditioner[7]. A symbiotic cyanobacterium *Anabaena azollae* ML2 was reported to degrade lignin during application of coir pith as carrier material[3]. Apart from this report there is so far no report on application of cyanobacteria for solid waste management.

The degradation of phenolic pollutants in aquatic and terrestrial environments is not limited to any particular group of microorganisms; it occurs widely within bacteria[8,9], fungi[10,11], algae[12] and actinomycetes[13]. Even though cyanobacteria are ubiquitous in their distribution and play a major role in fixation and turnover of carbon and other nutrient elements, recognition of their heterotrophic abilities by the environmental microbiologists remain poor.

In nature, cellulose, hemicellulose and lignin are the major sources of plant biomass; therefore, their recycling is indispensable for the carbon cycle. The removal of pollutants by physico-chemical treatments are energy and cost intensive and generate secondary pollution[14]. Each polymer is degraded by a variety of microorganisms which produce a battery of enzymes that work synergistically. In the near future, processes that use lignocellulolytic enzymes or microorganisms could lead to new, environmental friendly technologies[15].

The recycling of carbon cycle is indispensable. Removal of pollutants by physico-chemical treatments are energy and cost intensive and generate secondary pollution[14]. As the cyanobacteria have simple growth requirements, grow to high densities and use light, CO_2 and other inorganic nutrients efficiently, they could be attractive hosts for production of valuable organic products[1]. Cyanobacteria have been shown to degrade naturally occurring hydrocarbons[2], organophosphorus pesticides[6], phenol[4], xenobiotics[5] and distillery effluent[16].

Each polymer is degraded by a variety of microorganisms which produce a battery of enzymes that work synergistically. In the near future, processes that use lignocellulolytic enzymes or microorganisms could lead to new, environmental friendly technologies[15].

Our study proved that cyanobacteria are a cheap source to degrade the industrial waste coir pith in an environmental friendly manner. To the best of our knowledge this is the first attempt to identify the phenolic compounds released during the degradative action of cyanobacteria on coir pith. Global attention is now focused on production of industrially important phenolic compounds from natural source cheaply. The cyanobacterial degradation of coir pith may be the ray of hope in the near future.

MATERIALS AND METHODS

Coir pith and other chemicals

Coir pith was collected from a coir factory located in Tiruchirapalli, Tamilnadu, India. The chemical standards used for identifying the compounds were purchased from Sigma Aldrich chemicals, Germany and were of HPLC grade.

Physical pretreatment of coir pith

After thorough washing under running tap water, detergent and then with double distilled water the coir pith was oven dried at 60°C overnight. Then it was autoclaved at 121°C 15psi for 20 min in SANYO MLS-3788 autoclave. The purpose of autoclaving the coir pith was to ensure the removal of other coir pith degrading organisms. Chemical pretreatment of coir pith was totally avoided to explore the degradative action of cyanobacteria to the maximum.

Organism and maintenance

Phormidium sp. BDU-5 was obtained from the germplasm collection of National Facility for Marine Cyanobacteria, Bharathidasan University, Tiruchirapalli, Tamilnadu, India. This strain was selected since it was fast growing and they were able to grow luxuriantly in presence of lignocellulosic material. Maintenance of the culture and the experiment was performed in BG-11 medium[17]. The culture was tested for its axenic nature by inoculating a loop full of culture in 1% peptone-glucose broth and observed for bacterial growth for up to 5 days by incubating at room temperature. Bacterial presence was also monitored microscopically before and during the experiments[18].

Biodegradation experiment with *Phormidium* sp. BDU-5

The degradation experiments were carried out in 250ml sterile Erlenmeyer flasks. Each flask received 70ml of autoclaved BG-11 medium, 10ml of culture suspension (of approximately 50mg dry weight) and 500mg of coir pith (dry weight). Control cyanobacteria and coir pith alone were also maintained separately. All flasks were maintained at 25±2^{0}C and a light regime of 14 h light and 10 h dark. The light intensity was 28.5μmol (photons) $m^{-2}s^{-1}$ set using a Kyoritzu, illuminator, Japan. The experiment was carried out for 30 days and

analytical experiments were performed periodically in triplicates.

Analytical methods

Periodically cultures were centrifuged and pellets were subjected to chlorophyll estimation[19]. Spectral analysis in the wavelength range from 200-350 nm (Jasco V- 550 spectrophotometer, Japan) and reducing sugar estimation[20] were performed in the supernatant.

Lignin determination

The method of Crawford and Crawford[21] was employed for Klason lignin determination. Content of Klason lignin was estimated in both control coir pith and coir pith treated with cyanobacteria.

Enzyme assays

The pellets were washed repeatedly in medium and sonicated in a Labsonic 2000 UB, Braun sonicator, Germany. Supernatant was subjected to protein quantification by Lowry *et al.*,[22] after centrifuging the contents at 10,000 rpm for 10min. Equal amount of protein was used to detect the activity of laccase[23], polyphenol oxidase[24] and esterase[25] in nondenaturing gel electrophoresis.

Compound study

The supernatant was dried and extracted twice using 70% ethanol. Extracted supernatants were pooled and air dried in order to concentrate the phenolic compounds.

Spectral analysis

The concentrated sample was redissolved in methanol and its spectra were compared with that of the eight phenolic standards in the range 200-350 nm using (Jasco V- 550 spectrophotometer, Japan). The standard chemicals which were used for the compound study were guaiacol, vanillic acid, ferulic acid, veratraldehyde, 4-hydroxybenzaldehyde, vanillin, 3,4-diethoxybenzoic acid and coumaric acid. The spectra of the standard chemicals were taken by dissolving them in methanol keeping methanol as blank.

Thin layer chromatography (TLC) analysis.

TLC was carried out on glass plates (20x20cm^2) coated with 0.4mm layer of silica gel slurry (0.5g/ml) prepared using a spreader. The plates were activated at 100ºC overnight. The ethanol extracted sample was dissolved in methanol and spotted in the plate along with the chemical standards. The mobile phase used was benzene: ethyl acetate: acetic acid (85: 14: 1). Plates were exposed to iodine vapor to check the presence of organic compounds[26].

High performance liquid chromatography (HPLC)

HPLC analysis was performed with a Spherisorb C18 (surface area of 220m^2/g) column (ISCO product) in an ISCO model 3250, instrument. Separation was achieved by isocratic elution in methanol: water (90:10), with a flow rate of 0.4ml/min. UV absorbance detector was set at 285nm[27].

RESULTS

Phormidium sp. BDU5 showed luxuriant growth in presence of the coir pith and also degraded it substantially. The filamentous cyanobacterium showed higher chlorophyll *a* content (Figure1) in presence of the lignocellulosic material when compared to control cyanobacteria. Introduction of cyanobacteria with coir pith resulted in greater extent of reducing sugar release into the medium (Figure 2) than the controls (cyanobacteria and coir pith alone in media).

Verification of composition of control and test wood sample revealed 22% lignin degradation whereas in control sample the degradation was negligible. However, the holocellulose estimation revealed that only 6.2% was degraded (Figure 3).

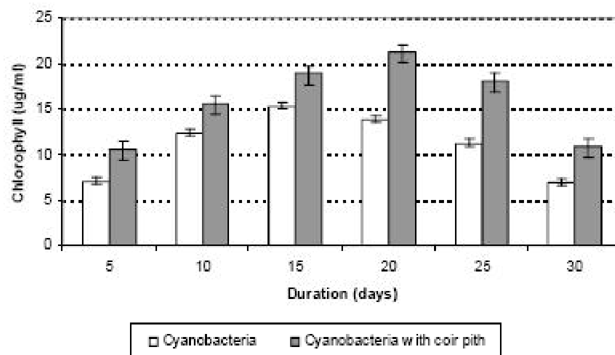

Figure 1 : Periodical analysis of chlorophyll *a* content in control (cyanobacteria) and test (cyanobacteria with coir pith). Results are average of three replicates.

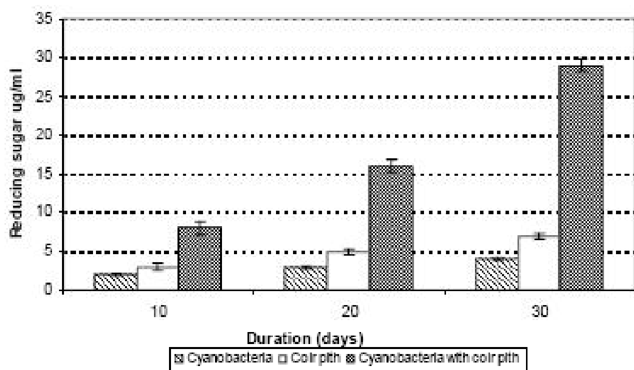

Figure 2 : Periodical estimation of reducing sugar content in supernatant of control cyanobacteria, coir pith and test sample. Results are average of three replicates.

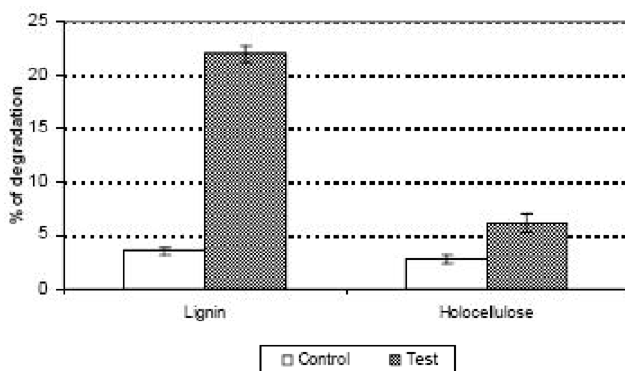

Figure 3 : Degradation of lignin and holocellulose in the presence and absence of cyanobacteria. Percentage of degradation calculated as the difference in lignin and holocellulose content between initial and the final. Results are average of three replicates.

The major lignolytic enzymes, laccase and polyphenol oxidase showed induction on exposure to coir pith.

Figure 4 showed that laccase is inducible whereas polyphenol oxidase is constitutive in this fresh water cyanobacterium. Even the accessory enzyme, esterase responded with pronounced induction.

Spectral analysis of the ethanol extracted sample (Figure 5) from test supernatant and standard chemicals (guaiacol and 4-hydroxybenzaldehyde) showed absorbance in the range 270–290nm. TLC plate (Figure 6) revealed that the Rf values of the spots in test sample coincided with that of guaiacol and 4-hydroxybenzaldehyde. Comparison of HPLC chromatogram with standard chemicals reconfirmed the release of guaiacol and 4-hydroxybenzaldehyde in test sample.

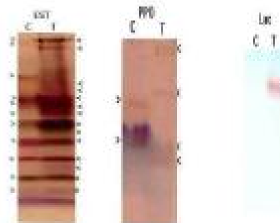

Figure 4 : Non denaturing gels showing the induction in 3a- EST- esterase, 3b- PPO-polyphenol oxidase and 3 c- Lac- Laccase. C- cyanobacteria, T – test (cyanobacteria exposed to coir pith)

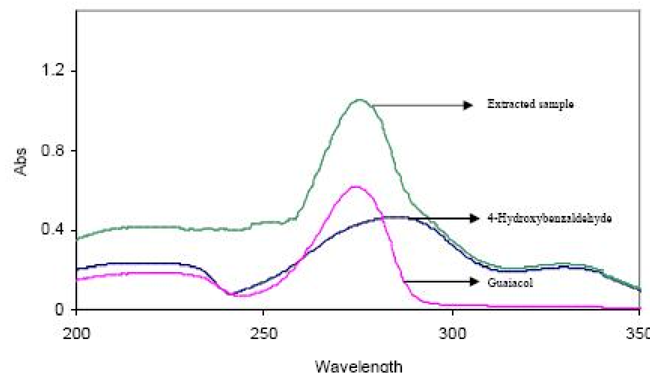

Figure 5 : Spectral analysis showing comparison of extracted sample and chemical standards guaiacol and 4-hydroxybenzaldehyde

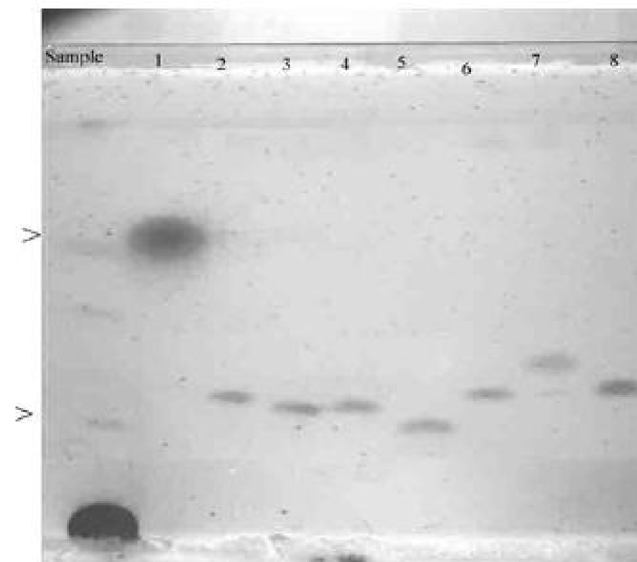

Figure 6 : TLC plate showing the spots that appeared for the sample and the corresponding standards. 1) guaiacol, 2) vanillic acid, 3) ferulic acid, 4) veratraldehyde, 5) 4-hydroxybenzaldehyde, 6) vanillin, 7) 3,4- diethoxybenzoic acid and 8) coumaric acid

DISCUSSION

The pronounced increase in chlorophyll *a* indicated that (1) the presence of lignocellulosic material did not inhibit the growth of the cyanobacterium (2) the cyanobacterium utilized the coir pith as nutrient source hence, the depletion of nutrients in media, after 15 days, did not affect the growth of cyanobacteria in test samples. Subramanian *et al.*,[6] demonstrated that addition of organophosphorus pesticide has enhanced cyanobacterial growth by many folds even though the lag phase is extended up to 10 days, which is evident by increase in chlorophyll content in test samples.

The increase of reducing sugar in media was attributed to the degradative action of cyanobacteria on holocellulosic part of coir pith. Our results were in accordance with El-Gammel *et al.,*[28] who reported variation in percentage of sugar released by the lignolytic activity of a *Streptomyces* sp. and three white rot fungi on various lignocellulosic substances. The ester linkage between lignin and the holocellulose prevents the normal attack of microorganisms on cellulose and hemicellulose, which is more prone to attack among the three components in the lignocellulose.

The higher degradation rate of lignin when compared to the holocellulose by cyanobacteria may be due to the photoautotrophic nature of the organism. Scrutiny of literature indicated the ability of a marine filamentous, non heterocystous form *Oscillatoria boryana* BDU 92181 to use the recalcitrant biopolymer melanoidin as nitrogen and carbon source leading to decolourization[16]. Mineralization of organic compound was possible by marine cyanobacterium *Phormidium valderianum* BDU 30501 in nitrogen supplied medium and also against the usual nitrogen starved condition[4].

Induction of two isoforms on exposure to lignocellulosic material indicated that the polyphenol oxidase in *Phormidium* sp. BDU5 is an inducible enzyme. The brown band that appeared confirmed polyphenol oxidase activity[24]. Shasirekha *et al.,*[4] reported, during the phenol degradation study, the role of laccase and polyphenol oxidase, in a marine cyanobacterium *Phormidium valderianum* BDU 30501 which were intracellular and inducible. The intracellular activity of laccase was explored by Froehner and Eriksson[23], Arcand and Archibald[29], Rigling and Alfen[30], Scholosser *et al.*,[31] and Burke and Cairney[32].

Hemicellulose biodegradation needs accessory enzymes such as esterases acting synergistically to efficiently hydrolyze wood xylans and mannans[33]. Degrassi *et al.*,[34] and Rumbold *et al.*,[35] reported that the presence of corncob powder and birch wood xylan was able to induce esterase. The results obtained in the present work are in agreement with literature data mentioned for the induction of esterase in the test sample. The phenolic compounds released during the incubation of the cyanobacterium with coir pith would have induced the isoforms of esterase.

Thin layer chromatography plate resolved into brown spots on a yellow background on exposure to iodine vapor which confirmed the presence of organic compounds[26,36]. The phenolic compounds recorded under conditions of TLC analysis were 4-hydroxybenzaldehyde and guaiacol confirmed by co-chromatography. Rf values of the spots in the test sample, on exposure to iodine vapor, matched exactly with that of standard chemicals (Figure 6). Dey *et al.*,[37] identified 4-hydroxybenzoic acid and ferulic acid, from coconut husk using preparative TLC. They reported the occurrence of 4-hydroxybenzoic acid in coconut husk raised the possibility of its bioconversion to 4-hydroxybenzaldehyde. This report was an obvious proof for our finding. HPLC reconfirmed the presence of the compounds. Two peaks detected in test sample matched with the retention time of the standard chemicals guaiacol and 4-hydroxybenzaldehyde (RT 6.55 and 7.15 min).

CONCLUSIONS

Phenolic compounds are important for plant metabolism and have also become important for humans due to their health characteristics, particularly related to their antioxidant properties[38]. According to Sun and Cheng[39] and Galbe and Zacchi[40] chemical pretreatment of lignocellulosic materials is considered as the easiest mode of releasing phenolic compounds which enhances enzymatic degradation also[41]. However, the present study successfully demonstrated the release of value added phenolic compounds from the lignocellulosic material without any chemical pretreatment to elimi-

nate environmental pollution caused by the release of chemicals used for the pretreatment.

Our study proved that 4-hydroxy benzaldehyde and guaiacol could be produced, from a cheap source like coir pith, which is of high economic value. It would be a boon for industries because of the fact that 4-hyroxy benzaldehyde, the second abundant compound of *Vanilla* bean extracts, contributes to the natural flavor of vanilla. It is also a very popular flavoring agent used in large range of foods and as fragrance ingredients[42]. Guaiacol is a synthetic intermediate compound that is important in the production of flavorings, fragrances and pharmaceuticals[43].

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