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Dye industry effluent treatment using free and immobilized fungus

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Abstract

Paper and pulp mills, textile and dye factories, distilleries, and the leather industry are just few of the businesses that produce brightly colored effluents. The lignin-degrading enzymes laccase, manganese-peroxidase, and lignin peroxidases produced by terrestrial white-rot basidiomycetous fungus may be used to neutralize dyes and other xenobiotics in industrial effluents.Thirteen unique fungal species were identified from the wastewater for this investigation. Aspergillus was found to be the most common fungal genus in the waste water.In comparison to the control group, all variables except dissolved oxygen exhibited a reduction.Both the BOD and the COD levels dropped dramatically. Aspergillus that has been immobilized was more effective in cleaning up pollution than free-living Aspergillus cells.

Key-Words: Aspergillus, BOD, COD

Introduction

It has been predicted that by 2010 the United States would produce 253 million tons of municipal solid garbage annually (USEPA, 2007). With a growing rate of roughly 3% per year (PCD, 2005)[1], solid waste production in Thailand's metropolitan regions exceeds 14.3 million tons per year, or 39,200 tons per day. About 65% of garbage is disposed of in open dumps while the remaining 35% is handled via sanitary landfill. Basidiomycete fungi are well-known for producing ligninolytic enzymes. White rot fungus's fermented culture broth is rich in lignin modifying enzymes (LMEs) [2,3], whereas bacteria, yeast, and other fungi seldom make LMEs. The efficacy of treating Olive Oil mill wastewaters (OOMW) with four different white rot fungi (WRF) strains was evaluated in relation to their cultivation mode, specifically as free mycelium, mycelium immobilized in alginate beads, and solid state cultivation on Petri dishes[4].

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Material and methods

Isolation of fungi

Ten ml of the effluent sample was taken in a 250ml conical flask containing 90ml sterile distilled water. The flask was shaken on an elastic shaker to get a homogenous suspension and transferring serially 10ml of the effluent suspension to 90ml of sterile distilled water made different dilutions viz., 10-1, 10-2 and 10-

3. 0.1 ml of 10^{-3} dilution was plated in petridishes containing Potato Dextrose Agar medium (PDA). **Immobilization of fungi in Alginate beads** (Fig.2) Commercial grade sodium alginate was used as theimmobilizing agent for bead preparation. Exponentially growing Aspergillus flavus (Fig.1) were harvested bycentrifugation (5000 x g for 10 min) and resuspendedin 50ml of sterile water. To this 50ml of 4% alginate solution was mixed thoroughly to get a final alginate concentration of 2%. The alginate-bacterial mixturewas then added drop wise into $CaCl_2$ (0.1 M) solution.The beads were kept in the same solution for 30 mins at 4°C for hardening.

Experimental condition (Fig.3)

For the present study the following treatments wereemployed

- Effluent sample without inoculation-control I
- Effluent sample inoculated with *Aspergillusflavus*-treatment I
- Effluent sample inoculated with immobilized *A. flavus*- treatment II

The experiment was conducted in patch cultures in duplicates for a total period of 15 days in 250ml Erlenmeyer flasks. Effluent samples (control and treated) were periodically (every 5th days) analyzed for various physico-chemical parameters and recorded.

Physico-chemical analysis of effluent (APHA, 1995)

Determination of pH

pH was recorded at the collection site with pH indicator paper. At laboratory the pH was checked again with pH meter (Elico-India).

Estimation of alkalinity (Carbonate and Bicarbonate)

To 10ml of filtered sample, 2 drops of phenolphthalein indicator was added and titrated against 0.1N HCl. Thissample was used for both phenolphthalein and total alkalinity.

Estimation of Free Carbon dioxide

To 10ml of filtered sample a few drops phenolphthalein indicator was added and titrated against 0.05 N NaOH. The end point was the appearance of pink color.

Estimation of Dissolved oxygen

Standard flasks (15 ml) were filled with filtered samples without air bubbles. Then the samples were Winklerised by adding 0.2 ml of manganous sulphate followed by 0.2 ml of alkaline iodide. The precipitate was dissolved by the addition of 0.2 ml of sulphuric acid. The clear yellow color solution was titrated against sodium thiosulphate and starch was used as an indicator. The end point was the disappearance of blue color.

Estimation of Nitrate

A series of 50ml standard solutions of the nitrate from the standard nitrate solution were prepared to obtain a range of conc. (10 to 100µg). 2ml aliquots of the standard solutions were pipetted into different dry 100ml beakers provided with glass rods. To each beaker 1ml brucine sulphanilic acid reagent and 10ml sulphuric acid solution was added. Stirred gently for about 5 minutes. The beakers were covered with the watch glasses and kept in dark for 10 minutes during which, a yellow colour develops. 10ml distilled water was to added each of the beakers and incubated in the dark for 30 min. The absorbance of each solution was then measured at 410nm. Plotting the absorbance values against the nitrate concentrations of the standard solutions draws a graph. The amount of nitrate content of the effluent was determined using the standard graph**Estimation of Nitrite**

To 10ml of filtered sample, 0.2ml of sulphanilamide solution was added. After 10 minutes 0.2ml of NED was added. The pink colour appeared was measured at 543nm in spectronic 20 against reagent blank. Theamount of Nitrite (mgl-1) was calculated from the standard graph prepared by using sodium nitrite.

Estimation of Ammonia

To 10ml of filtered sample 0.4ml phenol reagent was added and stirred, then 0.4ml of nitroprusside reagent was added and mixed. Finally, 1ml of the oxidizing reagent was added and mixed thoroughly and then incubated at room temperature for an hour. Absorbency was measured at 630nm in spectronic 20. The amount of ammonia was calculated by using a standard graph prepared by using ammonium chloride.

Estimation of total phosphorus

To 10ml of filtered sample and 2 ml of 5% potassium persulphate followed by 2ml of mixed reagent was added and diluted to 15ml with distilled water. The reading was taken after 10 minutes in spectronic 20 against the reagent blank at 882 nm. The amount of inorganic phosphate was calculated using a standard graph prepared by using K_2HPO_4.

Estimation of inorganic phosphate

To 10ml of filtered sample, 2ml of mixed reagent was added and diluted to 15ml with distilled water. The reading was taken after 10 minutes in spectronic 20 against the reagent blank at 882 nm. The amount of inorganic phosphate was calculated using a standard graph prepared by using K_2HPO_4.

Estimation of Organic phosphate

Organic phosphate was estimated by subtracting inorganic phosphate from the total phosphorus.

Estimation of Hardness

To 10ml of filtered sample 0.2ml of buffer solution and50mg of Erichrome black T were added. The solution turned wine red. This was treated against EDTA (0.01 M) solution. At the end point, colour change from winered to blue was noted.

Estimation of Calcium

To 10 ml of filtered sample, 0.4 ml of 0.1 N NaOH and
50 mg of murexide indicator were added. This was titrated against EDTA (0.01 M) solution until the pink colour solution changed to purple.

Estimation of Magnesium

Amount of magnesium was estimated by the following methods

$$Mg^{++}\ (mgl^{-1}) = \frac{Y - X \times 400.8}{\text{Volume of sample}}$$

Where, Y=EDTA used in hardness determination, X =
EDTA used in calcium determination for thesame volume of the sample.

Estimation of chloride

To 10ml of filtered sample in an Erlenmeyer flask, fewdrops $K_2Cr_2O_4$ were added and titrated against silver

nitrate (0.0141 N) solution until a persistent red tinge colour appeared. The amount of chloride was calculated as follows.

Determination of Biological Oxygen Demand

pH of the water sample was adjusted to neutrality using1 N acid or 1 N alkali. BOD bottles were filled with water sample without air bubbles. One ml of allylthiourea was added to each bottle. Dissolved oxygen content for 3 of the bottles were estimated by Winkler's method (DO estimation) and the mean of thethree bottles were taken (D1). Rest of the bottles (3) was incubated at 27°C in a BOD incubator for 3 days. DO concentrations was estimated after 3 days in these incubated bottles and the mean was calculated (D2).

Chemical Oxygen Demand (COD)

10ml of sample in 100ml conical flask was taken in triplicate. A blank was run for each using COD free water (Sample is diluted when COD is more than 16 mgl^{-1}). The flasks were kept in boiling water bath for 1 hour after adding 1ml of 0.1 N $KMnO_4$ solutions. After cooling for 10 minutes, to each flask, 1ml of KI solution and 2ml of H_2SO_4 were added. Then the sample was titrated with 0.1N sodium thiosulphate and 1ml of starch was added when the solution becomes pale yellow. Titration was continued until the colour disappeared completely. Finally the titre values were noted.

Results and Discussion

For the present study effluent sample was collected from dye industry. From the sample fungal species were isolated and recorded.

Fungal flora

Totally 13 species of fungi belongs to 8 genus from the effluent were recorded (Table.1). Among the genus *Aspergillus* was recorded as dominant genus with 5 species such as *A.niger, A.flavus, A,nidulans, A.luchensis* and *A.terreus.* The genus, *Trichoderma* was recorded two species (Table 1). The *Trichoderma* such as *Trichoderma sp* and *T. viride* were recorded. Among remaining genus such as *Penicillium, Helminthosporium, Cladosporium, Fusarium, Geotrichum* and *Rhizopus* were recorded single specieseach (Table 1).

Physico-chemical characteristics of effluent pH

The pH of the effluent was acidic in nature. It was recorded 7.2 initially and the fluctuation of pH was recorded during experimental period (Table 2).

Free CO_2

The free CO_2 level was 32 mg l^{-1} initially (Table 2). It was brought down from 15th day onwards in both free and immobilized *Aspergillus* treated

effluentAlkalinity

The carbondioxide alkalinity was completely absent in the effluent. Where as bicarbonate alkalinity was recorded. Initial bicarbonate level in the effluent was 108 mg l^{-1} (Table 2). It was reduced to nearly 80% in both free and immobilized *Aspergillus* treated effluent when compared to control.

Dissolved Oxygen

After treatment of effluent on 15th day the DO level was raised to 2.2mg l^{-1} in and 1.72 mg l^{-1} (Table 2) in free and immobilized *Aspergillus* treated effluent respectively.

Nitrate, Nitrite and Ammonia

The level of Nitrate, Nitrite and Ammonia were reorded 90, 48 and 41 mg l^{-1} initially (Table 2). The all the nitrogen was reduced nearly 50 in both free and immobilized *Aspergillus* treated effluent on 15th day (Table 2).

Total, inorganic and Organic phosphates

The levels of all forms phosphates were recorded initially in the effluent. Among the phosphates, the total phosphate level was recorded as maximum than the inorganic and organic phosphate. It was reduced on 15th day in both free and immobilized *Aspergillus* treated effluent (Table 2).

Calcium

The level of calcium was recorded 83 mg l^{-1} initially (Table 2). It was brought down from 5th day onwards. On 15th day it was recorded nearly 45% removal in immobilized *Aspergillus* treated effluent.

Magnesium

The level of magnesium hardness was recorded initially 56 mg $l^{-1.}$ There was similar reduction as in calcium level on 15th day in both free and immobilized *Aspergillus* treated effluent (Table 2).

Chloride

Chloride level was recorded initially as 49 mg l^{-1} (Table 2). On 15th day the calcium was 44 mg l^{-1} in immobilized Aspergillus treated effluent. Similarly 40 mg l^{-1} recorded in free cells of Aspergillus treated effluent.

BOD and COD

The level of BOD was recorded maximum as 340 mg l^{-1} initially (Table 2). It was reduced nearly 65% in the effluent treated with Aspergillus when compared tocontrol. The high amount of COD was recorded initially (620 mg l^{-1}) (Table 2). It was reduced in their level in Aspergillus treated effluent. In general all the form pollutants removed in Aspergillus treated effluent. When compared to control. Similarly immobilized Aspergillus treated effluent showed more removal than free cells of Aspergillus.

The abundant variation in the types of all living organisms taken together in any geophysical area is called biodiversity. With reference to large organisms (plant, animals etc.) the distinctive morphological and anatomical features visible to the naked eye enable usto identify the different genera and species and. therefore; we can easily assess the extent of their diversity. However, most fungi are not visible to the naked eye and they need to be studied under the microscope. The culturable fungi can be identified provided they sporulate but the biotrophs which are notamenable to culturing in growth media go unnoticed. It is, therefore, imperative that there is a huge gap in our knowledge of the diversity of all microbes including fungi. Fungi are better known than bacteria, viruses and other smaller forms of life such as viroids. There are about 72,000 named species and new species are being added at the rate of about 1500 each year. The total number of fungal species both known and unknown are estimated at 250,000 (Hawksworth & Kirsop, 1988; Heywood, 1995). The 72,000 known species exist in different culture collection centres in the form of several subspecies, pathovars and strains, the total cultures going up to 170,000 (Bennett &Faison, 1997).

The mycoflora of unique ecological niches have some common features and it will be very rewarding to explore fungal diversity in habitats such as thermophilic environments, e.g., hot springs, thermal vents, sun-heated soils, compost pits, self-heated coal refuse piles, steam line discharge sites, etc. The thermophilic fungi and bacteria grow at temperatures between 40-60^{0}C are the sources of thermostable enzymes. Other ecological niches to be explored are western ghats, the Himalayan ranges, marine ecosystems, mangroves, coral reefs, sand dunes, industrial effluent contaminated soils, refineries, activated sludge, insects and several other natural sources. Endophytes of plants and animals are verymeagerly understood.

Hence, the present study was undertaken to know the fungal species in industrial effluent. For the present investigation effluent sample was collected from leather industry. The fungal species were isolated and identified from the effluent sample were recorded (Table.1).

Totally 13 species of fungi belongs to 8 genus from

theeffluent (Table 1). Among the genus Aspergillus was recorded as dominant genus with 5 species such as, A. niger, A. flavus, *A.* nidulans, A. luchensis and A. terreus.. Similar work was done by Carroll andWicklow (1992). They isolated 17 species of fungi inthe household wastewater in which Aspergillus was thedominant group of fungi.

In this present study two species of Trichoderma such as Trichoderma sp and T. viride. The present results was inconformity the earlier reports of Watts *et. al*.,(1999). They isolated and recorded three species of Trichoderma from automobile industrial effluents. In this present study Penicillium Helminthosporium, Cladosporium, Fusarium, Geotrichum and Rhizopus were recorded single species each. These types of fungal species always occur in industrial effluent (Bennett & Faison, 1997).

The ability of fungi to transform a wide variety ofhazardous chemicals has aroused interest in using them in bioremediation (Alexander, 1994). The white rot fungi are unique among eukaryotes for having evolved nonspecific methods for the degradation of lignin; curiously they do not use lignin as a carbon source for their growth (Kirk *et al.,* 1976). Lignin degradation is, therefore, essentially a secondary metabolic process, not required for the main growth process. Lamar *et al.,* (1993) compared the abilities of three lignin-degrading fungi, Phanerochaete chrysosporium, P. sordida and Tramates hirsuta to degrade PCP (Pentachlorophenyl) and creosote in soil. Inoculation of soil with 10% (wt/wt) Phanerochaetesordida resulted in the greatest decrease of PCP and creosote. P. sordida was also most useful in the degradation of PAHs (Polycyclic aromatic hydrocarbons) from soil. Davis et al.,, (1993) showed that P. sordida was capable of degrading efficiently the three ring PAHs, but less efficiently the four-ring PAHs.

The present study was carried out to treat the leather industry effluent by using the dominant species of Aspergillus. In this study all the pollutants were reduced in their level in both unsterilized and sterilized effluent due to the inoculation of Aspergillus. The similar work was done by Thanh and Simard (1973). They reported treatment of wastewater by fungal species. While maximizing reduction of phosphate, ammonia, and organic matter. The studies were conducted in batch 500-ml baffled culture shake flasks containing 150 ml of sterile wastewater. Reported phosphate removal ranged from 12 to 100%, total nitrogen removal from 22 to 93%, ammonia nitrogen from 27 to 90%, and COD removal from 0 to 72%.

Hiremath *et al.,* (1985) performed a similar study except they tested seven fungal species isolated from a wastewater stabilization pond. The major goal of the study was to maximize biomass production of fungi asa food source for animal or human consumption. The trials were conducted in 2-L conical flasks containing

1.5 L of sterile fresh wastewater. Flasks were inoculated with pure cultures of fungi and incubated at room temperature for 10 days. The culture flasks were gently agitated twice a day. The study reported BOD removal between 53 and 72%, phosphate removal from34 to 77%, and ammonia nitrogen removal between 49 and 77%. Due to the experimental design, the cultures were most likely completely anaerobic for the entire 10-days incubation.

Both studies were performed under non-ideal conditions with process optimization of the parameters reported. Although the data showed fungi will treatwastewater, it does not give any indication of the maximum removal efficiencies. However, both studies indicated promising results for removal of nitrogen andphosphorus. Additional evidence, although not conclusive, that fungi are capable of wastewatertreatment was found during the study of onsite sphagnum peat wastewater treatment system. Brooks (1988) found excellent removal of BOD, organic nitrogen, and ammonia nitrogen (90 to 95% and 95 to 99%, respectively). Based on standard bacterial andfungi enumeration techniques, the ratio of fungi to bacteria was 8:1 during winter and 4:1 during the summer. This led to hypothesize that fungi had a large role in removal of nitrogen from the wastewater. However, due to the use of standard enumeration techniques it is very likely the populations were under estimated due to the fastidious nature of environmental microbes. Furthermore, a direct comparison ratio of fungi to bacterial colony forming units is not appropriate. This ratio employs the flawed assumption that each bacteria and fungi is equal in degradation rate and range (BOD, ammonia nitrogen, and organic nitrogen) of the wastewater. A different experimental design and enumeration is needed before comparisons can be made.

Whenever bioremediation figures as the topic of discussion, bacterial agents come into focus and fungi are much less studied. One should realize, however, thegreater potential of fungi by virtue of

their aggressive growth, greater biomass production and extensive hyphal reach in soil. More research will be focused in future on using the diverse fungal flora forbioremediation. The work on the fungal diversity in the chemical industrial effluents and promising fungi Aspergillus flavus that can degrade various pollutants in these effluents.

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Table 1: Physicochemical characteristics of effluent

S.No	Parameters	Initial	5th Day			10th Day			15th Day		
			Control	Treated		Control	Treated		Control	Treated	
				FC	IMC		FC	IMC		FC	IMC
1.	pH	7.2	6.8	6.8	6.7	6.8	6.6	6.7	6.8	6.6	6.7
2.	Free CO_2	32	30	28	24	30	22	20	28	12	10
3.	Alkalinity	108	106	78	96	106	62	48	104	20	21
4.	Dissolved Oxygen	1.35	1.72	1.74	1.37	1.72	2.2	1.37	2.2	2.2	1.72
5.	Nitrate	90	90	82	70	90	68	59	88	42	52
6.	Nitrite	48	48	46	40	46	36	32	44	26	28
7.	Ammonia	41	41	32	30	40	26	22	39	16	16
8.	Total Phosphorus	147	146	136	130	144	126	168	142	80	78
9.	Inorganic	83	82	78	23	80	72	62	78	42	42
10.	Organic	64	64	58	57	62	54	44	60	38	30
11.	Calcium	83	82	76	68	80	70	58	78	50	49
12.	Magnesium	56	55	50	50	54	42	45	54	22	37
13.	Chloride	49	44	48	44	49	46	42	48	44	40
14.	BOD	340	320	250	260	310	220	180	310	160	110
15.	COD	620	610	520	500	600	420	380	580	300	220