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Removal of colour in distillery effluent (spent wash) by *Phanerochaete chrysosporium* and *pseudomonsas fluorescens*

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Abstract: Distillery producing alcohol from molasses is considered to be one of the most polluting agro-based industries. Especially the problem of spent wash disposal is accentuated by its high temperature, deep colour and acidic nature with obnoxious odour. The awful colour of distillery waste is due to molasses and charred sugar like caramels, melanoidins and decomposition product like hydroxyl methyl furfural. As the physical and chemical methods of effluent treatment are cost ineffective, the emphasis has shifted to biological method to decolorize the effluent. In the present study effect of treatment with microorganisms *viz*., *Phanerochaete chrysosporium* and *Pseudomonas fluorescens* was tested on effluent for colour removal. *P. chrysosporium* showed 22.3% colour removal and 14.14% COD reduction over that of *P. fluorescens.* However, sequential treatment by *P. chrysosporium* and *P. fluorescens* showed relatively better results in COD and colour removal by 79.55 and 92.53% than any of the individual microorganisms. A high correlation was observed between the physico-chemical parameters studied. Increase in EC and decrease in pH seems to be a function of reduction in COD, TSS, TDS and colour. Mineralization of organic matter resulted in formation of organic acids and inorganic compounds to bring down the pH. The study clearly demonstrated that sequential microbial treatment can enhance substrate biodegradation due to the improvement in bioavailability of substrate through various metabolic activities of microorganisms and thus con be gainfully exploited for spent wash decolourization.

Key words: Distillery, Waste, Molasses, Effluent treatment, Melanoidins, Decolourization, Microorganisms

Introduction

With the advent of industrialization, sugar byproduct industry achieved tremendous growth. The fermentation industry including distilleries, breweries and malteries are posing serious environmental threat throughout the world. In India alone, there are more than 180 distilleries, breweries and malteries established, among which 35 are located in Uttar Pradesh that produces a great deal of pollution load owing to its acidic pH, high biochemical oxygen demand (BOD), chemical oxygen demand (COD) and awful colour (Binkley and Wolform, 1983). They generate large volume of coloured waste water known as spent wash or stillage with high organic load. India produces around 6500 million liters of alcohol in about 200 distilleries every year with an output of 130 billion litres of waste water. Biological treatment of spent wash, especially anaerobic process is extensively used for reduction of BOD/COD but there appears to be practically no colour removal by anaerobic treatment (Rao *et al.,* 1979; Rao and Viraraghavan, 1985). Various methods for the colour removal of distillery effluent have been adopted such as physicochemical and microbial treatment(Singh *et al*., 2007) studied the physicochemical characteristics of anaerobically digested distillery spent obtained from Jubiliant Organosys, Gajraula (UP). Sowmeyan and Swaminathan (2008) reported the 82.5% COD removal and 67.6% reduction in colour by treating the high strength distillery effluent with lime and ozone. They have also reported that $FeCl_3$ and $AlCl_3$ treatment, reduced 93% colour and 76% total organic carbon.

In recent years, there has been growth in the potential use of biotechnological approach to convert or utilize plant residue and industrial waste (Idaka *et al.,* 1978). There have also been several attempts to use microbial methods to decolourize effluents. A wide variety of microorganisms have been implicated in decolourization (Modi *et al.,* 1998). Biological oxidation is the most widely used technique to remove BOD and chlorinated organics because of its effectiveness and low cost. Leach *et al.* (1977) reported biodegradation of seven compounds representing the major categories of toxicants in aerated lagoons. Fungi, bacteria, actinomycetes and algae are among the various microorganisms that participate in the aerobic treatment methods. Pant and Adholeya (2009) reported nitrogen removal from bio-methanated spent wash using hydroponic treatment followed by decolourization by fungi. Sirianuntapiboon *et al*. (1999) reported the decolourization of microbial spent wash by *Acetobacter aceti* in presence of sugar especially glucose and fructose. Other methods is operation of a laboratory scale membrane bioreactor (MBR) in the continuous mode for distillery wastewater (spent wash) treatment using submerged 30 μm nylon mesh filters. The inoculum was municipal activated sludge, which was first acclimatized in a fed-batch reactor over a period of 102 days, before initiating continuous operation. Critical flux for this wastewater-filter combination was measured by flux stepping method, and the MBR was operated under sub critical flux conditions. Organic loading rates ranging from 3 to 5.71 kg m^{-3} day^{-1} were investigated and the performance was analyzed in terms of COD removal and biomass growth in the reactor. Up to 41% COD removal was obtained over 245 days of reactor operation. The molecular weight profiles of the untreated and treated effluent suggested that the degradation of low molecular weight compounds occurred in the reactor while the high molecular weight compounds comprising the colour imparting melanoidins remained unaffected. Up to 100% suspended solid retention (average 87%) was obtained and the system could be operated up to 2 weeks without significant flux drop (Satyawali and Balakrishnan, 2008; Pant and Adholeya, 2007).

The present study aims to access the color removal efficiency of spent wash by using fungi (*Phanerochaete chrysosporium*) and bacteria (*Pseudomonas fluorescens*) separately and in sequential treatment.

Materials and Methods

Culture: The two cultures used in this investigation were obtained from microbial collection obtained from IMTECH, Chandigarh. The cultures used were *Phanerochaete chrysosporium*-BKM1768 (a white rot fungus), *Pseudomonas fluorescens*-AM181176 (Gram negative, non motile and non spore former). *P. chrysosporium* was grown on malt extract medium (malt extract-20 g L^{-1}, glucose-20 g L^{-1}, peptone-1 g L^{-1}, agar-20 g L^{-1}; pH was adjusted to 5.8). *P. fluorescens* was grown in nutrient broth (peptone- 5 g L^{-1}, meat extract- 1g L^{-1}, yeast extract-2g L^{-1}, NaCl-5g L^{-1}; pH was adjusted to 7.0).The interval of subculturing for *P.chrysosporium* and *P. fluorescens* was 7 days and 3 days respectively.

Sampling of distillery effluent: The effluent samples from the distillery Kesar Enterprises, Baheri (UP) were collected at the main outlet point where combined effluents from the factory are being discharged into nala (1 km away from the distillery plant). Water samples at the point of discharge were collected in clean plastic container from the main outlet. The sampling was done twice on 14th September 1999, and 14th February 2000. Immediately after collecting the water samples, the BOD was fixed by adding $MnSO_4$ and the samples were brought to the laboratory and kept in the refrigerator at 4°C till used for analysis.

Treatment by individual microorganisms -

Fungi: One hundred and fifty ml of effluent sample was transferred into 2 sets of three 250 ml Erlenmeyer flasks. Glucose 2.5% (w/v), KH_2PO_4 0.250% (w/v) and $MgSO_4.7H_2O$ 0.125% (w/v) were added in each flask. One set of three flasks were now inoculated with *P. chrysosporium* and the other three flasks of second set were taken as control having no fungi. All the flasks were shaken for 10 days at 25°C and 150 rpm. Estimation of colour, COD, pH, TSS (Total suspended solids), TDS (Total dissolved solids), and EC (Electrical conductivity) was done at 0, 5 and 10 days interval after innoculation.

Bacteria: One hundred and fifty ml of effluent sample was transferred into 2 sets of three 250 ml Erlenmeyer flasks. Glucose 2.5% (w/v), KH_2PO_4 0.250% (w/v) and $MgSO_4.7H_2O$ 0.125% (w/v) were added in each flask. One set of three flasks were now inoculated with *P. fluorescens* and the other three flasks of second set were taken as control having no bacteria. All the flasks were shaken for 10 days at 25°C and 150 rpm. Estimation of colour, COD, pH, TSS, TDS and EC was done at 0, 5 and 10 days intervals after inoculation. One year was taken for microbial treatment.

Sequential treatment by fungi and bacteria: Two sets of three 250 ml sterilized Erlenmeyer flasks were filled with 150 ml of effluent sample. In all the flasks glucose, KH_2PO_4 and $MgSO_4.7H_2O$ were added. The first set of 3 flasks, were inoculated with fungi while rest of the flasks were taken as control. All the flasks were shaken at 25°C and 150 rpm for 5 days. COD, colour, pH and EC were measured at 0 and 5 days after inoculation. After removing the fungal mycelia by centrifuging at 5000 rpm for 10 minutes in one set of flask, the pH was adjusted to 6.5 using 0.5 M NaOH and inoculated with *P. fluorescens.* Thereafter, the flasks were kept on rotatory shaker at 25°C and 150 rpm for 30 minutes. Colour, COD, pH, EC, TSS and TDS were measured. Finally, the flasks were shaken at 150 rpm for five days. Again colour, COD, pH and EC were measured.

Analytical methods: Electrical conductivity (EC) of the effluent was measured using a pocket type digital EC meter (Hanna Instruments Co.) calibrated at 20°C. The reading was taken in milli siemens (mS m^{-1}). The pH of the effluent sample was measured by a pH meter using glass electrode.

For total suspended solids (TSS), 100 ml of the sample was centrifuged at 2000 rpm for 10 minute. The supernatant was removed and the residue was washed three times by resuspending it in distilled water and recollecting by centrifugation. The residue was finally transferred quantitatively to pre-weighed dish (X_1 g). The dish was weighed again after drying (X_2g) to a constant weight. The dish was weighed again after drying (X_2g) to a constant weight at 105°C. TSS was calculated by using the following formula.

$$\text{TSS (ppm)} = \frac{(X_2 - X_1) \times 1000 \times 1000}{\text{ml of sample}}$$

The TDS was calculated as the difference between the total solids (TS) and total suspended solids (TSS);

TDS (ppm) = TS (ppm) – TSS (ppm)

COD was calculated according to the method given by APHA (1989). Colour unit was measured using a DU-70 Spectrophotometer (Beckmann, USA). The sample was centrifuged at 10000 rpm for 30 minutes to remove all the suspended matter. The pH was adjusted to 7.6 with 2 M NaOH (CppA standard method) and then used for the measurement of absorbance at 465 nm. The absorbance values were transformed into colour unit (CU) using the following relationship.

$$CU = 500 \times \frac{A_1}{A_1}$$

where, A_1 = Absorbance of 500 CU platinum cobalt standard solution (A_{465} = 0.132) and A_2 = Absorbance of the effluent sample.

Results and Discussion

Treatment by white rot fungi (*P. chrysosporium*) and bacteria (*P. fluorescens*) resulted in change in physico-chemical characteristics of the distillery effluent (Table 1, 2, 3). Both the organisms resulted in significant reduction in colour, COD, TSS, TDS and pH within 10 days of incubation. However, relatively greater decolourization was observed with *P. chrysosporium* (85.97 and 84.06%) and COD reduction (62.9 and 56.41%) in diluted and non-diluted conditions compared to 75.75% and 62.53% decolourization and 55.00 and 50.72% COD reduction in diluted and non-diluted conditions by *P. fluorescens* (Table 1, 2, 3). In sterilized conditions the corresponding values for colour removal and COD reduction were relatively lower than that were observed in non-sterilized condition incubated with *P. chrysosporium* and *P. fluorescens* (Table 1, 2, 3 and Fig. 1). Fahy *et al.* (1997) reported that *P. chrysosporium* decolourized molasses spent wash supplemented with glucose by 85% after 10 days incubation. Decolourization by white rot fungi primarily depends on the strain of fungus used and origin of bleach plant effluent (Bergbouer *et al.,* 1991).

The three strains of *Pseudomonas aeruginosa* decolourized the anareobically digested spent wash in presence of basal salts and glucose (Mohana *et al*., 2007). Dahiya *et al*. (2001a) also reported the decolourization of distillery effluent by *P. fluorescens* with the help of cellulose carrier coated with collagen (decolourized cell). Reuse of decolourized cell reduced the decolourization efficiency.

P. chrysosporium JAG-40 decolourized synthetic and natural melanoidin present in spent wash upto 80% Dahiya *et al*. (2001b).

Sirianuntapiboon *et al*. (1995) reported that the decolourization of melanoidin pigment by *Rhizoctonia* sps. D-90 by adsoprtion mechanism. The pigment was accumulated in the cytoplasm and around the cell membrane as melanoidin complex, which was gradually decolourized by intracellular enzyme. The decolourization was less in sterilized spent wash than in non-sterile solution. Adikane *et al*. (2006) reported 69% decolourization of molasses spent wash by using 10% (w/v) soil in absence of any additional carbon or nitrogen source. Similarly, Thakkar *et al*. (2006) reported decolourization in stationary and submerged cultivation conditions of molasses medium by *P. chrysosporium*.

Conversely, the same pace was maintained in increase of EC by treatment with *P. chrysosporium* and *P. fluorescens.* Basically EC is a numerical expression of the ability of an aqueous solution to carry out the electric current. This ability depends on the presence of ions; their total concentration, mobility, valency and temperature of measurement. Solution of most inorganic acids, bases and salts are relatively good conductors. Conversely, molecules of organic compounds that do not dissociate in aqueous solution conduct current very poorly. Our findings suggested that treatment of effluent by *P. chrysosporium* and *P. fluorescens,* the major colorant melanoidins and caramels are dissociated and enhances the electrical conductivity. These resultsfurther support to our previous report of formation of inorganic compound as the result of mineralization action of microorganisms (Singh *et al.*, 2007). However, the detailed study of the probable formation of ionic compounds and its involvement to increase the electric current is yet to be examined.

The maximum decrease in pH was shown by *P. chrysosporium*. 22.16% in diluted and 32.73% in non-diluted under sterilized condition, while only 19.58% in diluted and 20.11% in non-diluted condition by *P. fluorescens.* In case of non-sterilized effluent almost similar results were obtained with *P. chrysosporium* and *P. fluorescens* at the end of 10 days incubation. Such marked decrease may be probably due to the formation of organic acids from the metabolisms of the immobilized fungus. However, Livernoche *et al.* (1983) reported that decolourization was not due

Table - 1: Changes in pH, EC, TDS, TSS, COD, and colour by treatment with *P. chrysosporium* in 10 days

Parameters	10 times dilution				Without dilution			
	Control		Treated		Control		Treated	
	Sterilized	Non sterilized	Sterilized	Non sterilized	Sterilized	Non sterilized	Sterilized	Non sterilized
pH	3.50±0.05	3.22±0.04	3.02±0.127	2.52±.260	5.42±0.2	4.75±0.21	3.72±0.07	3.26±0.1
EC (mS m^{-1})	9.1±0.07	8.4±0.07	9.8±0.14	10.0±0.01	18.0±0.7	19.3±0.07	18.3±0.14	19.8±0.1
TSS (mg L^{-1})	13097±0.71.41	10292±414.14	625.0±14.14	1090±7.07	42030±10.60	32000±70.71	22000±82.16	10000±166.87
TDS (mg L^{-1})	47263±162.6	25628±142.19	10220±107.23	7880±71.42	76232±196.87	70050±131.52	20800±104.7	16875±65.76
COD (mg L^{-1})	67060±102.5	48000±162.5	27000±63.76	21000±31.89	84168±28.34	72000±260.92	38792±113.84	28567±107.52
Colour	1231.42±14.79	512.71±19.8	212.92±10.92	187.7±7.86	8325.78±130.56	5895±142.9	4334.82±70.00	1628±17.28

Table - 2: Changes in pH, EC, TDS, TSS, COD, and colour of by treatment with *P. flurorescens* in 10 days

Parameters	10 times dilution				Without dilution			
	Control		Treated		Control		Treated	
	Sterilized	Non sterilized	Sterilized	Non sterilized	Sterilized	Non sterilized	Sterilized	Non sterilized
pH	3.50±0.05	3.22±0.04	3.12±0.070	2.98±.0.014	5.42±0.2	4.75±0.21	3.92±0.1	3.38±0.14
EC (mS m^{-1})	9.1±0.07	8.4±0.07	9.3±0.010	8.9±0.010	18.0±0.7	19.3±0.07	18.4±0.7	19.6±0.1
TSS (mg L^{-1})	13097±0.71.41	10292±414.14	7896±47.32	1487±47.32	42030±10.60	32000±70.71	27630±43.13	24651±63.63
TDS (mg L^{-1})	47263±162.6	25628±142.19	18986±207.1	12531±181.32	76232±196.87	70050±131.52	31970±41.30	29237±230.5
COD (mg L^{-1})	67060±102.5	48000±162.5	32756±143.2	28983±160.5	84168±28.34	72000±260.92	43851±205.7	37832±92.63
Colour	1231.42±14.79	512.71±19.8	568.28±39.54	312.2±37.33	8325.78±130.56	5895±142.9	4772.72±87.42	3828.8±225.8

Table - 3: Changes in pH, EC, TDS, TSS, COD, and color by sequential treatment with *P. chrysosporium* and *P. flurorescens* in 10 days

Parameters	10 times dilution				Without dilution			
	Control		Treated		Control		Treated	
	Sterilized	Non sterilized	Sterilized	Non sterilized	Sterilized	Non sterilized	Sterilized	Non sterilized
pH	3.50±0.05	3.22±0.04	3.00±0.14	2.40±0.1	5.42±0.2	4.75±0.21	3.52±0.2	3.55±0.2
EC (mS m^{-1})	9.1±0.07	8.4±0.07	9.9±0.2	10.2±0.1	18.0±0.7	19.3±0.07	18.8±0.1	19.9±0.2
TSS (mg L^{-1})	13097±0.71.41	10292±414.14	5895±47.64	877±63.63	42030±10.60	32000±70.71	2300±162.6	5000±86.97
TDS (mg L^{-1})	47263±162.6	25628±142.19	8732±27.57	4310±58.9	76232±196.87	70050±131.52	16800±127.2	11192±31.11
COD (mg L^{-1})	67060±102.5	48000±162.5	10870±212.83	8860±105.76	84168±28.34	72000±260.92	23832±156.8	17582±144.6
Colour	1231.42±14.79	512.71±19.8	200.31±15.10	106.43±35.28	8325.78130.56	5895±142.9	1003.23±37.28	762.86±25.97

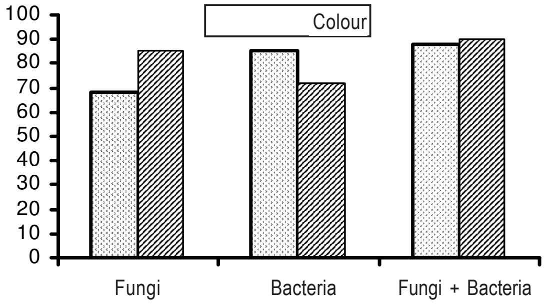

Fig. 1: Percentage reduction in colour and COD of the spent wash treated with fungi *P. chrysosporium* and *P. flurorescens* in isolation and in sequence

to the lowering pH, although the colour of the effluent happens to be pH dependent. Benito *et al.* (1997) supported this idea after observing the precipitation of dissolved solids which renders the light colour to the effluent. Although, the addition of NaOH redissolved the precipitate and resumed the effluent colour back to normal. Based on this, it was presumed that pH adjustment was unwarranted and unnecessary for microbial decolourization of the effluent.

Other parameters, such as TSS and TDS in sterilized and non-sterilized condition were also reduced due to *P. chrysosporium* and *P. fluorescens* treatment. Similar observation has been made by Rai and Saxena (2000). This clearly depicts that *P. chrysosporium* is one of the most efficient white rot fungi to degrade a wide range of organic pollutants, especially melanoidins, caramels and furfural. Various reports reveal that this fungus is capable of decolourizing and reducing COD of distillery effluent. Reduction in COD is due to degradation of chloro-lignin to CO_2 and chloride which decolourizes the distillery effluent by destroying the coloured bodies and chromophoric structures and removing total organic chloride converting it into inorganic chloride (Jokela *et al.*, 1993; Lafond and Ferguson, 1991).

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