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Decolorization Study of Biomethanated Effluent by Pregrown Biomass of a Fungal Natural Isolate of *Aspergillus Oryzae JSA-1*.

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Abstract. Fungi have been attracting a growing interest for the biotreatment (removal or destruction) of wastewater ingredients such as metals, inorganic nutrients and organic compounds.Filamentous fungi show their decolorizing activity due to decomposition by an intracellular enzyme system via production of active oxygen from hydrogen peroxide and/ or the adsorption of coloring components by mycelia, especially for the decolorization of melanoidin.The adsorption of melanoidin is the first step of melanoidin decomposition mechanism in microorganisms and in case of *Aspergillus oryzae Y2-32*, due to lack of a melanoidin decolorizing enzyme, the mechanism of decolorization does not continue further.The study on decolorization of biomethated effluent(BME) indicated that the culture of a natural isolate, *Aspergillus oryzae JSA-1*, decolorized BME effectively by adsorption of color to the cell wall of mycelia. The fungal cell wall is composed of polymers such as polysaccharides, proteins and lipids and is located outside the plasma membrane of the cells. The functional components are important in nutrient transport, metabolism of non-permeable substrates, and cell wall modifications. It has the major function of providing a protective, rigid envelope for the cell. Standardization of the process of pre-grown biomass based decolorization to achieve maximum decolorization was carried out by 1) Biomass based decolorization of BME using biomass harvested from media free of BME, media containing 30% BME and media containing 200 mg% melanoidin and by 2) Use of synthetic chitin as an adsorbing material in biomass based decolorization of BME by *Aspergillus oryzae JSA-1*. The results indicated that when the biomass was harvested from the culture grown in presence of BME and melanoidin in glycerol peptone medium (GPM), the biomass based percent decolorization of BME decreased i.e.34.13 ± 0.56% and 36.56 ± 0.92% respectively, in comparison to the culture grown in GPM free of BME or melanoidin i.e. 62.89 ± 1.87 % in 30 minutes of incubation under shake flask condition. Addition of chitin from 1g% to 10 g% in the solutions containing melanoidin, caramel and alkaline degradation products (ADP) and effluent could not show decolorization effectively. The study proved the fact that presence of chitin in the cell wall of fungal culture *Aspergillus oryzae JSA-1*might not be the major factor in the function of adsorption of color to the mycelial cell wall.

Keywords: bioremediation, biomethanated distillery waste, decolorization, biosorption, chitin, fungal cell wall

1. Introduction

Common methods of decolorization of distillery effluent are physico-chemical and biological methods. Physico-chemical methods are cost intensive and generate a large quantity of sludge as well as hazardous pollutants while biological one is cost effective, ecologically safe and produces biogas which has great utility. The untreated distillery effluent is acidic in nature with pH 3.5 to 4.0 which does not have any toxic hazardous chemicals but has a potential as a liquid fertilizer after reducing color and COD as it contains high levels of organic carbon, N, K, S, Ca and Mg apart from small amounts of micronutrients viz. Zn, Fe, Cu and Mn [1]. Biomethanation and biological activated sludge reduce considerable level of COD and BOD but the color of the effluent still remains. The presence of brown color in effluent is mainly due to coloring compounds such as caramel, melanoidin, alkaline degradation products and polyphenols formed during manufacturing of sugar from sugar cane juice [2]. Microbial decolorization is an environmental friendly

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technique for removingcolor from distillery spent wash [3]. Fungi are found to decolorize it via adsorption of coloring compounds by mycelia and/ or via production of active oxygen from hydrogen peroxide [4]. Decolorization of molasses pigment could be seen with *Mycelia sterilia D-90* by around 93% [5] and with *Aspergillus fumigatus G-2-6* by around 75% [6]. It was found that *Aspergillus oryzae JSA-1,* the natural isolate from soil could decolorize the undiluted biomethanated effluenteffectively by simple adsorption and proved to possess a very high potential in bioremediation of different BME samples. Fungi are recognized for their superior aptitudes to produce a large variety of extracellular proteins, organic acids and other metabolites, and for their capacities to adapt to severe environmental constraints. For example, *Aspergillus niger* is the prototypical fungus for the production of citric acid, homologous proteins (especially enzymes) and heterologous proteins. Moreover, *Phanerochaete chrysosporium* is the model of white-rot fungi for the production of peroxidases. Beyond the production of such relevant metabolites; fungi have been attracting a growing interest for the biotreatment (removal or destruction) of wastewater ingredients such as metals, inorganic nutrients and organic compounds [7]. In earlier study, chemical analysis of cell walls of *Aspergillus oryzae JSA-1* was compared with that of cell walls of other species of Genus *Aspergillus* such as *Aspergillus nidulans*, *Aspergillus niger*. The pre-grown biomass based decolorization of BME was found out by using biomass of these species and compared with the pre-grown biomass based decolorization of BME by using biomass of *Aspergillus oryzae JSA-1*.The biomass based percent decolorization of BME was found by column chromatography for these three species which was found to be different in all three species such as 98.45 $\pm$ 0.02% in *Aspergillus oryzae JSA-1,* 56.32 $\pm$ 0.132% in *Aspergillus nidulans*, and 76.4 $\pm$ 2.342% in *Aspergillus niger*. This might be due to the differences in the cell wall composition of the fungal cultures [8]. The study of immobilization of fungal biomass indicated that the ability of the culture to decolorize the BME was decreased in immobilized form (approx. around 74 to 76%) than in free form (approx. around 88 to 90 percent of cells. The results were similar to the earlier reports of *Aspergillus fumigatus G-2-6* which showed poor melanoidin decolorization (45%) on immobilization in comparison to 60% with the free mycelia. While in *Aspergillus niger UM2*, the immobilized culture could maintain its potent decolorization ability with the supply of external nutrients [9]. The common wall constituents found in each division of fungi are Chitin, β - (1-3), β-(1-6) Glucan, Chitosan, Xylomannoproteins, α (1-3) Glucan, Galactomannoproteins, α (1-3) Glucan, Polyglucuronic acid & Glucuronomannoproteins Polyphosphate [10].

2. Materials and Methods

2.1. Biomethanated Effluent Sample

Biomethanated effluent (BME) sample was obtained from anaerobic treatment plant set up at molasses distillery in Neera (Maharashtra state, India). The sample was centrifuged at 10,000 rpm for 30 minutes and refrigerated at 4^0 C to avoid further oxidation.

2.2. Chemicals

All the chemicals used for the experiments were of analytical grade and were purchased from Hi media Laboratories Limited, India and Sigma Aldrich Pvt. LTD., USA.

2.3. Microorganism

Soil samples were collected from the nearby vicinity of biomethanation plants located in Pune District, in India. These soil samples were screened for growth of micro-organism showing activity of decolorization of biomethanated distillery effluent. On primary screening twenty strains showed visual decolorization activity on solid medium containing biomethanated distillery effluent. Therefore as the secondary screening, the decolorizationactivities of these strains in liquid medium with biomethanated distillery effluent, under shaking conditions were examined. Out of these twenty strains, one was found to give maximum decolorization of biomethanated distillery effluent i.e. up to 68 %. This strain was identified and named *Aspergillus oryzaeJSA-1* and was chosen for subsequent experiments of decolorization. The culture was sub cultured and maintained on potato dextrose agar at 4^0C in the refrigerator.

2.4. Study of Decolorization in Media Containing Biomethanated Distillery Effluent

Basal medium, GPM(g/100ml) Glycerol, 5; Peptone, 0.5; KH2PO4, 0.1; MgSO4.7H2O, 0.05 containing 30% effluent was autoclaved and inoculated with 10^7 spores of the culture and incubated on rotary shaker (150 rpm) at 30^0C for 10 days. Percent decolorization was determined by reading the absorbance at 475 nm before and after fungal treatment.

2.5. Biomass Based Decolorization of BME Using Biomass Harvested from Media Free of BME, Media Containing 30% BME and Media Containing 200 mg% Melanoidin

The spores (10^7 spores/ 100 ml) of *Aspergillus oryzae JSA-1* were inoculated in the three flasks containing sterile GPM free of BME, GPM containing 30% BME and GPM containing 200 mg% melanoidin for 6 days on rotary shaker at optimum conditions of growth. The biomass from each flask was harvested by vacuum filtration through four layers of cheese cloth and washed extensively with double distilled water. 20 ml of undiluted effluent (pH 4.5) was inoculated with 2g of each type of biomass separately and incubated for 24 hours on rotary shaker at 150 rpm. The percent decolorization of BME was found out at 30 minutes, 1 hour, 2 hours, 4 hours and 24 hours of incubation at 475 nm (Table 1).

2.6. Use of Synthetic Chitin as an Adsorbing Material in Biomass Based Decolorization of BME by Aspergillus oryzae JSA-1

To 20 ml of GPM containing 200 mg% synthetic melanoidin, 200 mg% synthetic caramel, 200 mg% synthetic ADP and 30%BME at (pH 4.5), synthetic chitin was added in 1g%, 3g%, 5g% and 10g% quantities separately in 100 ml Erlenmeyer's flasks and incubated for 24 hours on rotary shaker at 150 rpm. The uninoculated media were used as controls. The percent decolorization was found out at 30 minutes, 1 hour, 2hours, 4 hours and 24 hours of incubation at 330 nm, 283 nm, 264 nm and 475 nm for melanoidin, caramel, ADP and BME respectively (Table 2).

2.7. Comparison in Using Pre Grown Fresh Live (wet)Biomass,Pre Grown Fresh Autoclaved (wet) Biomass, Pre Grown Fresh Dry Biomass, Pre Grown Used Dry Biomass of *Aspergillus Oryzae JSA-1* in Biomass Based Decolorization of BME

The biomass of *Aspergillus oryzae JSA-1* was harvested as described earlier. Live wet biomass, autoclaved wet biomass (121^0C for 30 minutes), fresh Sun dried dry biomass and used (used previously for decolorization of BME) Sun dried dry biomass were used in this decolorization study of BME. 20 ml of BME media were inoculated with 4gm of biomass of each type in 100 ml Erlenmeyer's flasks and incubated for 24 hours on rotary shaker (150 rpm) at 30^0C. Percent decolorization was determined for each flask at 30 minutes, 1 hour, 2hours, 4 hours and 24 hours of incubation (Table 3).

3. Results and Discussion

3.1. Biomass Based Decolorization of BME Using Biomass Harvested from Media Free of BME, Media Containing 30% BME and Media Containing 200 mg% Melanoidin

The results of biomass based decolorization of BME using biomass harvested from spore grown culture of *Aspergillus oryzae JSA-1* grown for six days in glycerol peptone media (GPM) free of BME, GPM containing 30% BME and GPM containing 200 mg% melanoidin are shown in Table 1.

Table 1: Biomass based decolorization of BME using biomass harvested from media free of BME, media containing 30% BME and media containing 200 mg% melanoidin

Sr. No.	Biomass harvesting medium	% Decolorization				
		0.5 hr	1 hr	2 hr	4 hr	24 hr
1	GPM	62.89 ±1.87	63.49 ±1.23	66.4 ±0.98	66.9 ±0.45	67.9 ±0.67
2	GPM with 30% BME	34.13 ±0.56	35.95 ±0.42	37.8±0.39	38.1 ± 0.87	39.2 ±0.34
3	GPM with 200mg% melanoidin	36.56 ±0.92	37.72 ±0.47	39.3 ±0.56	39.9 ±0.37	42.6 ±0.78

The results indicated that when the biomass was harvested from the culture grown in presence of BME and melanoidin in GPM, the biomass based percent decolorization of BME decreased i.e.34.13 ± 0.56% and 36.56 ± 0.92% respectively, in comparison to the culture grown in GPM free of BME or melanoidin i.e.

62.89 ± 1.87 % in 30 minutes of incubation under shake flask condition. There was only 4-6 % increase in the percent decolorization when the flasks were incubated further up to 24 hours in all the three experiments.

3.2. Use of Synthetic Chitin as an Adsorbent in Decolorization of Synthetic Colorants and BME

The results of the use of synthetic chitin as an adsorbent in decolorization of synthetic colorants and BME are shown in Table 2.

Table 2: Synthetic chitin as an adsorbent in decolorization of synthetic colorants and BME

Sr. No.	Solution for decolorization in distilled water (pH 4.5)	Chitin (g %)	% Decolorization				
			0.5 hr	1 hr	2 hr	4 hr	24 hr
1	200mg% melanoidin	1	0.8 ±0.02	1.3 ±0.06	1.5 ±0.03	1.7 ±0.08	1.8 ±0.04
		3	1.8 ±0.04	2.5 ±0.05	3.1 ±0.06	3.4 ±0.05	3.5 ±0.02
		5	2.9 ±0.02	3.5±0.05	3.9 ±0.08	5.3 ±0.03	5.8 ±0.09
		10	4.7 ±0.07	5.7 ±0.06	6.1 ±0.02	6.5 ±0.03	6.9 ±0.05
2	200mg% caramel	1	0.9 ±0.05	1.1 ±0.04	1.5 ±0.06	1.8 ±0.03	1.9 ±0.04
		3	1.1 ±0.02	1.9 ±0.07	2.2 ±0.06	2.4 ±0.03	2.5 ±0.05
		5	2.7 ±0.06	3.8 ±0.09	4.9 ±0.04	6.2 ±0.02	6.7 ±0.03
		10	5.5±0.03	6.5 ±0.05	7.8 ±0.04	7.9 ±0.04	8.2 ±0.02
3	200mg% ADP	1	0.8 ±0.06	1.6 ±0.02	1.9 ±0.07	2.1 ±0.06	2.3 ±0.09
		3	1.1 ±0.02	1.7 ±0.07	2.9 ±0.06	3.2 ±0.05	3.5 ±0.03
		5	2.9 ±0.04	3.5 ±0.05	4.9 ±0.03	5.3 ±0.04	5.7 ±0.05
		10	5.7 ±0. 96	6.1 ±0. 60	6.8 ±0.06	7.2 ±0.09	7.3 ±0.08
4	30% BME	1	3.2 ±0.86	3.7 ±1.02	4.8 ±0.98	5.1 ±0. 90	5.9 ±0.40
		3	6.6 ±0.34	7.6 ±0.22	9.8 ±0. 40	11.5 ±0.73	12.2 ±0.52
		5	8.3 ±0.65	9.8 ±0. 20	11.2 ±1.07	13.9 ±0.85	14.4±0.14
		10	9.6 ±1.19	11.9 ±0.86	17.8 ±1.03	19.1 ±1.11	20.18±0.5

The results showed that addition of chitin from 1g% to 10 g% in the solutions containing melanoidin, caramel, ADP and effluent could not show decolorization effectively. When 1g% chitin was added to melanoidin solution, decolorization was 1.8± 0.04 %which increased to 6.9 ± 0.05 %when the chitin was increased to 10 g% after 24 hours of incubation. The caramel was found to be decolorized by 1.9 ± 0.04% by addition of 1g% chitin which increased to 8.2 ± 0.02% after 24 hours when 10 g% chitin was added to caramel solution. Similarly when 1 g% chitin was added to ADP solution and incubated for 24 hours, the decolorization of ADP was found to be 2.3 ± 0.09% which increased to 7.3 ± 0.08% when the concentration of chitin was increased from 1 g% to 10 g%. The percent decolorization of BME was found to be increased from 5.87 ± 0.40% to 20.18 ± 0.50% when the concentration of chitin was increased from 1g 5 to 10 g%. The results indicated that even the addition of chitin up to 10 g% in the solutions of colorants and effluent could not show decolorization more than 20 %. This proved the fact that presence of chitin in the cell wall of fungal culture *Aspergillus oryzae JSA-1*might not be the major factor in the function of adsorption of color to the mycelial cell wall.

3.3. Comparison in Using Pre Grown Fresh Live (wet) Biomass,Pre Grown Fresh Autoclaved (wet) Biomass, Pre Grown Fresh Dry Biomass, Pre Grown Used Dry Biomass of *Aspergillus Oryzae JSA-1* in Biomass Based Decolorization of BME

The results of biomass based decolorization are given in Table 3, when different forms of biomass were inoculated in undiluted BME and incubated for 24 hours under shake flask condition.

It was seen that the percent decolorization was maximum (66.2 ± 1.02%) when the biomass was freshly harvested and in wet as well as live form in 30 minutes of incubation which was decreased to 54.9 ± 0.54 % when the biomass was freshly harvested and in wet but autoclaved (121^0C for 30 minutes) form. The percent decolorization was further decreased to 30.5 ± 0.62 % when the biomass was freshly harvested and in dried (sun dried) powder form. The percent decolorization was found to be lowest (26.8 ± 0.43%) in the

experiment when the biomass previously used for decolorization was reused in dry powder form for decolorization of BME. There was only 5-10 % increase in the % decolorization when the flasks were incubated further up to 24 hours in all the experiments. It was seen from the results that the pre-grown fresh live (wet) mycelial biomass could decolorize the effluent by around 66.2% in 30 minutes while 68.46% in 24 hours (2.2% increase). Pre-grown fresh dry mycelial biomass powder could decolorize the effluent by around 30.5% in 30 min while 55.56% in 24 hours (25% increase). This showed that as the time increases, the structure of the dry cells get opened up and adsorption of the color increases.

Table 3: Biomass based decolorization in different forms of biomass (wet/dry; live/autoclaved; fresh/used)

Sr. No.	Biomass	% Decolorization / hours				
		0.5	1	2	4	24
1	**Pre grown fresh live (wet) mycelial biomass**	66.2 ± 1.02	67.5 ± 1.60	67.9 ± 1.06	68.1 ± 0.87	68.46 ± 0.69
2	**Pre grown fresh autoclaved (wet) mycelial biomass**	54.9 ± 0.54	55.19 ± 0.37	62.3 ± 0.89	63.9 ± 0.96	64.78 ± 1.04
3	**Pre grown fresh dry mycelial biomass (powder)**	30.5 ± 0.62	35.48 ± 0.53	45.2 ± 0.48	54.6 ± 1.07	55.56 ± 0.79
4	**Pre grown used dry mycelial biomass (powder)**	26.8 ± 0.43	29.26 ± 0.09	31.3 ± 0.26	32.9 ± 0.87	33.38 ± 0.12

4. Conclusion

The biomass grown in absence of BME or synthetic melanoidin in GPM could show more decolorization efficiency than grown in presence of BME or synthetic melanoidin. Use of synthetic chitin for decolorization of synthetic colorants and BME was found to be ineffective.The study ofBiomass based decolorization in different forms of biomass showed that the structure of the dry cells get opened up and adsorption of the color increases as the time increases.

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6. References

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