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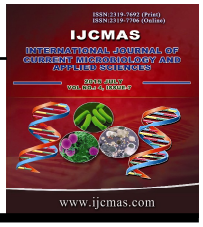

Original Research Article

Study of Bioremediation of Biomethanated Distillery Effluent by *Aspergillus oryzae* JSA-1, Using Electron Micrography and Column Chromatography Techniques

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A B S T R A C T

Filamentous fungi show their decolorizing activity in number of ways which are 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 decolorization of melanoidin by Coriolus species No. 20 and Coriolus versicolor Ps 4a was found to be due to intracellular enzymatic degradation of melanoidin by an inducible enzyme and a constitutive enzyme. Decolorization by the constitutive enzyme is only quarter the level than the decolorization by inducible enzyme. 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. In the present study the effluent decolorizing ability of Aspergillus oryzae JSA-1 was studied by more sophisticated experiments such as electron micrographs of the culture grown in effluent containing and effluent free medium. Biomass based decolorization studies were carried out by successive column chromatography of biomethanated effluent (BME) through the biomass of Aspergillus oryzae JSA-1. The electron micrographic study proved that the fungus decolorizes the BME mainly by adsorption of colorants on the surface of the cells. From the study of column chromatography using fungal biomass as a matrix, it was concluded that the biomass of Aspergillus oryzae JSA-1 could effectively decolorize BME samples by bioadsorption phenomenon as well as could reduce important pollution parameters COD and TDS efficiently.

Introduction

Nearly 319 distilleries in India produce ethanol from sugarcane molasses; generating approximately 45 billion litres of dark brown concentrated wastewater spent wash. This molasses spent wash (MSW) contains coloured pigments and polyphenols,

responsible for its dark brown color, high-suspended solids, high concentration of Chemical Oxygen Demand (COD) and Biological Oxygen Demand (BOD). Coloring compounds are more difficult to treat because of their synthetic origin and complex aromatic molecular structures. Such structures resist fading on exposure to water, light or oxidizing agents, and this render them more stable and less amenable to biodegradation. 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 (Sweeney and Graetz, 1991). 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 (Dhamankar and Patil, 2001). Microbial decolorization is an environmental friendly technique for removing color from distillery spent wash (Ghosh *et al.,* 2003). Fungi are found to decolorize it via adsorption of coloring compounds by mycelia and/ or via production of active oxygen from hydrogen peroxide (Sirianuntapiboon *et al.,* 1988). Decolorization of molasses pigment could be seen with *Mycelia sterilia D-90* by around 93% (Sirianuntapiboon *et al.,* 1988) and with *Aspergillus fumigatus G-2-6* by around 75% (Ohmomo *et al.,* 1987). It was found that *Aspergillus oryzae JSA-1,* the natural isolate from soil could decolorize the undiluted biomethanated effluent effectively by simple adsorption and proved to possess a very high potential in bioremediation of different BME samples. Therefore, the effluent decolorizing ability of the culture *Aspergillus oryzae JSA-1* was studied by more sophisticated experiments such as electron micrographs of the culture grown in effluent containing and effluent free medium. Biomass based decolorization studies were carried out by successive column chromatography of BME through the biomass of *Aspergillus oryzae JSA-1*.

Materials and Methods

Biomethanated effluent sample

Biomethanated effluent (BME) samples were obtained from anaerobic treatment plants set up at molasses distilleries in Neera, Pravara, Rahuri and Sanjeevani (Maharashtra state, India). The samples were centrifuged at 10,000 rpm for 30 minutes and refrigerated at 4^0 C to avoid further oxidation.

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.

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-organisms 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 decolorization activities 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 named *Aspergillus oryzae JSA-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.

Study of decolorization in media containing biomethanated distillery effluent

Basal medium (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.

Transmission electron micrographs of mycelia of *Aspergillus oryzae JSA-1*

The mechanism of color removal by the culture of Aspergillus oryzae JSA-1 was studied under Transmission Electron Microscope (TEM), during its growth in media used for decolorization containing biomethanated effluent (BME). The study was carried out by harvesting the mycelia grown from spores of Aspergillus oryzae JSA-1, in glycerol peptone medium (GPM) with optimum conditions for the growth of the culture. 100 ml aliquot of sterile GPM was inoculated with the spore suspension (10^7 spores /100 ml) of the fungal culture and the flask was incubated under shake flask condition (150 rpm, 30^0C) for 10 days. Mycelia were harvested after 10 days incubation and were used as control for TEM study (sample A). The mycelia were also harvested at two different stages of cultivation grown in GPM containing BME, from spores of Aspergillus oryzae JSA-1, to study the process of decolorization. In two 250 ml Erlenmeyer's flasks, 100 ml aliquots of sterile medium (GPM) with 30 % BME at pH 6 were inoculated with the spore suspension (10^7 spores /100 ml) of the fungal culture and these flasks were incubated under shake flask condition (150 rpm, 30^0C). Mycelia were harvested from one flask after four days growth of the culture for TEM study (sample B). The second flask was incubated further up to 10 days under same conditions and the mycelia were harvested after 10 days growth of the culture and used for TEM study (sample C). To study the pre-grown biomass based decolorization by using TEM, the sterile medium (GPM) was inoculated with spore suspension (10^7 spores/ 100ml) of Aspergillus oryzae JSA-1 and incubated at 30^oC on the incubator shaker (Pooja Labs, India) at 150 rpm for four to six days. The mycelium biomass was harvested by vacuum filtration through four layers of cheese cloth and washed extensively with double distilled water. The freshly grown biomass (4 g wet weight) of Aspergillus oryzae JSA-1, was inoculated in 20 ml of BME at pH 4.5 (optimum pH for biomass based decolorization and the flask was incubated under shake flask condition (150 rpm). Mycelia were harvested after 30 minutes of incubation for TEM study (sample D).

Preparation of samples for TEM study-

The harvested mycelial samples were prefixed in 3% glutaraldehyde for two to three hours and washed thrice with phosphate buffer (pH 7.2, 0.1 M). Then the samples were suspended in osmium tetra-oxide for two hours and washed thrice with the same buffer. Dehydration of the samples was then carried out with increasing concentrations of ethanol (50 %, 70 %, 90 %, and 100 % v/v) and fixed with propylene oxide. The dehydrated samples were cut in thin sections by using ultra microtome and stained initially with uranyl acetate for two hours and then with lead citrate for ten minutes. These stained sections were studied and photographed under TEM (Jeol, Japan) operated at suitable kilovolts (Plate 1- A, B, C, D).

Decolorization of BME by pre-grown mycelial biomass of *Aspergillus oryzae JSA-1* using Column Chromatography Technique

The study was done by packing the mycelia biomass of *Aspergillus oryzae JSA-1* in glass column and sample of BME was loaded from top to perform chromatography.

Glass column: Height - 10.5 cm, Diameter - 3.0 cm

Packing material: Biomass of *Aspergillus oryzae JSA-1*, Dry Weight – 3.4 gm

Buffer: Acetate buffer (0.1 M), pH 4.5

Sample used: Biomethanated effluent of 100% concentration, (pH 4.5)

Sample volume: 100 ml

Flow rate: 65ml/ hour

Result and Discussion

Transmission electron micrographs of mycelia of *Aspergillus oryzae JSA-1*

The electron micrographs of mycelial samples A, B, C and D are shown in Plate 1 A, B, C and D. In comparison with sample A, the mycelia were showing accumulation of the electron dense material around the cell wall in sample B while in sample C, the culture found to accumulate majority of the electron dense material around the cell wall and minute quantities were found to be absorbed in the cytoplasm due to ten days incubation of the culture in presence of the color pigment. Sample D showed high adsorption of the electron dense material around the cell wall with unaffected inner cytoplasmic contents. The accumulation of electron dense material around the cell wall was mainly due to the efficiency of adsorption of coloring pigments by the cell wall of the culture. TEM study suggested that the decolorization process by *Aspergillus oryzae JSA-1* occurred in two steps, as, initial adsorption of majority of coloring pigment on the mycelial cell wall followed by intracellular absorption of minute quantities of coloring pigments. This is in contrast with the earlier reports for electron micrographic studies carried out on melanoidin decolorizing activity by *Rhizoctonia species D 90* and *Aspergillus niger UM-2*, in which the decolorization was seen mainly by intracellular enzymatic degradation of melanoidin.

Decolorization of BME by pre-grown mycelial biomass of *Aspergillus oryzae JSA-1* using Column Chromatography Technique

Study on decolorization of biomethanated distillery effluent was carried out by column chromatography using mycelium biomass of

Aspergillus oryzae JSA-1 (Plate 2 and 3). The average percent reduction of color was around 72.33% after passing different undiluted effluent samples through first column which was increased to 92.90%, 97.53% and 98.90% in the effluent fractions of second, third and fourth column respectively (Plate 3).

Table.1 Percent reduction of different pollution parameters in BME sample after column chromatography

Parameters	**Initial**	**Final**	**% Reduction**
Color (O.D. 475)	1.19	0.010	99.16 ± 0.09
Melanoidin (w/v %)	**0.32**	**0.010**	**96.69 ± 0.05**
ADP (w/v %)	**1.25**	**0.049**	**96.12 ± 0.08**
Caramel (w/v %)	**1.9**	**0.07**	**96.30 ± 0.02**
COD (ppm)	20,400	1880	90.78 ± 0.22
TDS (ppm)	19,460	4750	75.59 ± 0.05
Phosphates (ppm)	**217.6**	**74.8**	**65.62 ± 0.05**
Sulphates (ppm)	**772.73**	**56.82**	**92.75 ± 0.12**
Chlorides (ppm)	**3678.86**	**1359.58**	**63.04 ± 0.13**
Sodium (ppm)	**345.4**	**62.8**	**81.82 ± 0.24**
Potassium (ppm)	**5030.9**	**3753.2**	**25.47 ± 0.06**
Calcium (ppm)	**320.00**	**< 8**	**97.81 ± 0.32**
Iron (ppm)	**3.67**	**0.155**	**95.77 ± 0.17**
Copper (ppm)	**0.097**	**0.0198**	**79.59 ± 0.08**

Plate.1A, B, C, D Mycelia harvested from A - Spore inoculated medium (free of BME) after 10 days incubation; B - Spore inoculated BME medium after 4 days incubation; C - Spore inoculated BME medium after 10 days incubation; D - BME inoculated with pre-grown biomass, after 30 minutes incubation

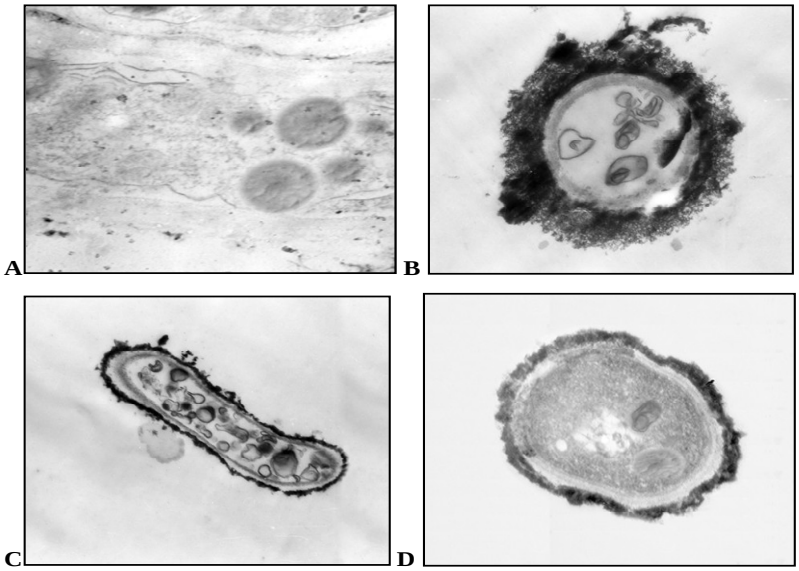


Plate.2 Glass columns packed with wet biomass of *Aspergillus oryzae JSA-1* in column chromatography (0 - before passing the effluent through the packed biomass; 1, 2, 3, 4 - after passing the effluent serially from columns 1 to 4, through the packed biomass)

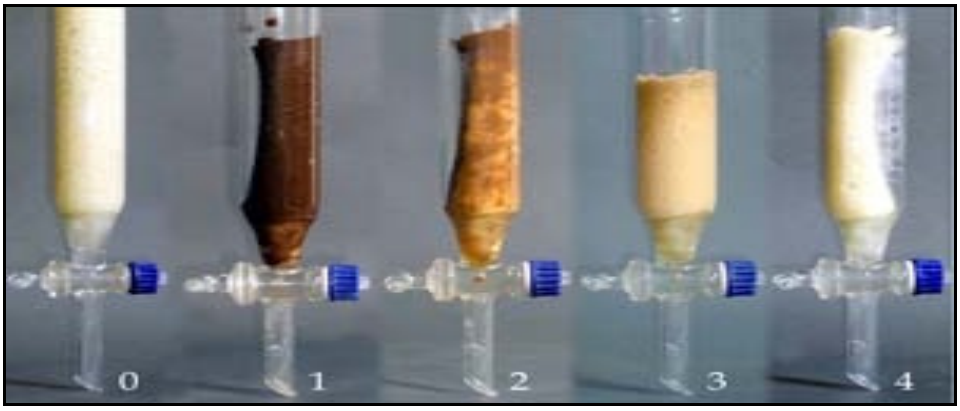
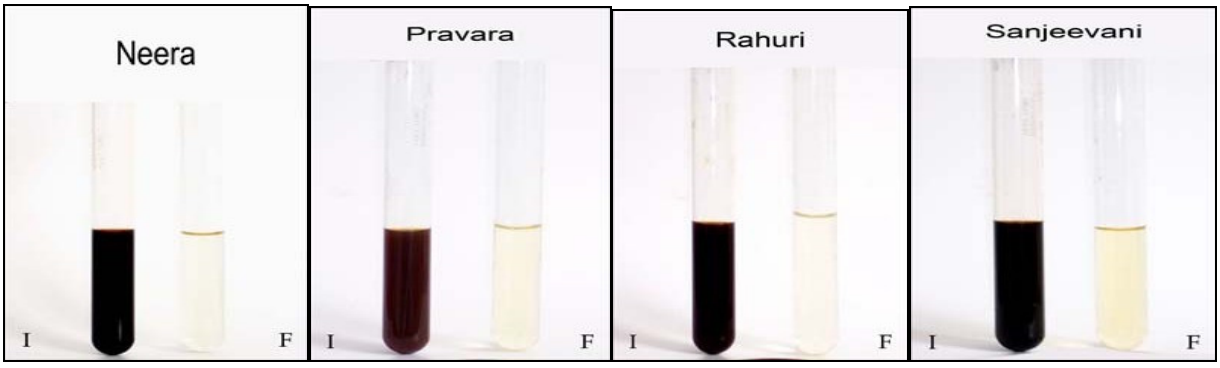


Plate.3 Color of effluent fractions after column chromatography (I- Effluent showing initial color; 1, 2, 3, 4 - Effluent fractions showing color after passing through the packed biomass in series of four columns)

Plate.4 Reduction of color in different effluent samples after passing through series of four columns in column chromatography technique packed with the wet biomass of Aspergillus oryzae JSA-1, A- Neera effluent, B – Pravara effluent, C- Rahuri effluent, D- Sanjeevani effluent (I: Initial color; F: Final color)



The average percent reduction of COD in the effluent fraction of first column was around 65.73% which was increased to 79.16%, 85.89% and 89.70% in the effluent fractions of second, third and fourth column respectively. The efficiency of *Aspergillus oryzae JSA-1* to reduce color and COD by column chromatography technique by passing different biomethanated effluent samples (100 ml) separately through series of four columns loaded with the biomass in the similar way has been shown in Plate 4.

The results of chemical analysis of the effluent fraction collected from last column of the four columns in series, in the column chromatography are shown in table 1. Melanoidin, ADP and caramel were found to be reduced by more than 96% and removal of total color was up to 99.16 ± 0.09%. Removal of COD was found to be up to 90.78 ± 0.22%. TDS was reduced to 75.59 ± 0.05 %. Levels of all other parameters were also reduced effectively. Phosphates, sulphates and chlorides showed 65.62 ± 0.05%, 92.75 ± 0.12% and 63.04 ± 0.13% reduction respectively. Sodium, potassium and calcium showed around 81.82 ± 0.24%, 25.47 ± 0.06% and 97.81± 0.32 % reduction respectively. The trace elements like iron and copper also showed 95.77 ± 0.17% and 79.59± 0.08% reduction respectively.

It could be concluded from the electron micrographic study of the fungal culture of *Aspergillus oryzae JSA-1*, that the fungus decolorizes the biomethanated effluent mainly by adsorption of colorants on the surface of the cells. From the study of column chromatography using fungal biomass as a matrix, it can be concluded that the fungal biomass of *Aspergillus oryzae JSA-1* could effectively decolorize biomethanated effluent samples by bioadsorption phenomenon as well as could reduce some important pollution parameters such as COD, sulphates, metals (iron, copper) and TDS efficiently.

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