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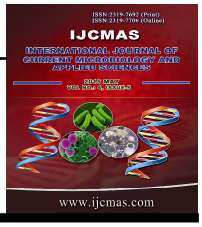

Original Research Article

Use of Fungal Culture in Free and in Immobilized Form to Decolorize Biomethanated Distillery Effluent

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Decolorization, Biomethanated distillery effluent, Bioremediation, Immobilization

A B S T R A C T

Wastewater is generated at various stages of alcohol production. Wastewater from the fermenter sludge, spent wash and spent lees are the main contributors to pollution. Different plant pigments present in sugar cane juice and sugar degradation reaction products formed during sugar manufacturing at high temperature impart color to the molasses or distillery waste. The effluent formed in the process of biomethanation, is still a highly colored liquid with high COD load (25,000- 60,000 mg/L) and which is difficult to treat by normal biological processes such as activated sludge or anaerobic lagooning. Its recalcitrance is due to the presence of brown polymers melanoidins which are formed by the maillard amino-carbonyl reaction, caramel and alkaline degradation products. These compounds have antioxidant properties which render them toxic to many microorganisms, such as those present in wastewater treatment processes. 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. Filamentous fungi show their decolorizing activity due to decomposition by an intracellular enzyme system via production of active oxygen from hydrogen peroxide and/ or due to the adsorption of coloring components by mycelia, especially for the decolorization of melanoidin. It was found that *Aspergillus oryzae JSA-1,* the natural isolate from soil could decolorize the undiluted biomethanated effluent **(**BME) effectively by simple adsorption and proved to possess a very high potential in bioremediation of different BME samples. Therefore some exploratory studies were carried out to boost up the decolorization of BME. The present study can put a light on efficiency of a natural fungal isolate of *Aspergillus* in immobilized form to bring about reduction in concentration of colorants from biomethanated effluent for its safe disposal in environment.

Introduction

The impact of colored waste water from any industry like distillery, paper and pulp, textile etc is a major factor in the environmental pollution and it is mainly associated with the dark brown or black color and high COD. According to a recent estimate, the alcohol production in India has reached the 2.7 million-liter mark. The proportion of wastewater, generally known as spent wash, is nearly 15 times the total alcohol production. This massive quantity, approximately 45 billion liters of effluent, if disposed untreated can cause considerable stress on the water sources leading to widespread damage to aquatic life. Distillery spent wash is dark brown colored high strength organic matter loaded waste having high magnitudes of BOD, COD, nitrates, phosphates and nitrogen; high conductivity due to high salt concentration and highly acidic in nature. Color of the distillery effluent posses a very high pollution threat to the soil, surface water, ground water and surrounding ecosystem after disposal. The treatment of distillery wastewater has gained worldwide attention as in some regions it is posing a serious threat to ground water quality. Microbial decolorization is seen as environment friendly technique for removing the coloring compounds from distillery wastewater.

Materials and Methods

Biomethanated effluent samples were obtained from anaerobic treatment plants set up at four different molasses distilleries in Maharashtra state (India).

1) Jubilant Organosys, Neera.

II) Padmashree Dr. Vikhe Patil S.S.K. Ltd., Pravaranagar.
III) Rahuri S.S.K. Ltd., Shrishivajinagar, Rahuri.
IV) Sanjeevani S.S.K. Ltd., Kopargaon.

These samples were centrifuged at 10,000 rpm for 30 min. and refrigerated at 4^0C for further studies. The four effluent samples were analyzed for different pollution parameters such as color, COD, Kjeldahl Nitrogen, phosphates, sulphates, chlorides, sodium, potassium, calcium, iron and copper (Table I) by standard methods of analysis for examination of water and wastewater [Greenbers, A. E., et al,1998,Greenberg et al 2005]]. All chemicals and reagents used were of analytical reagent grade.

Decolorization of biomethanated distillery effluent with fungal mycelium of *Aspergillus oryzae JSA-1* in free form

Medium used for decolorization (BME medium)

Glucose 5%; Peptone 0.5%; KH_2PO_4 0.1 %; $MgSO_4$, $7H_2O$ 0.05 % and biomethanated distillery effluent 30% (v/v) in distilled water (Dhamankar, V. S., *et al.*, 2001).

Optimization of the growth medium parameters for maximum decolorization of biomethanated distillery effluent *by Aspergillus oryzae JSA-1* in free form

The selected fungal strain *Aspergillus oryzae JSA-1*, was used for the study of optimization of the growth medium parameters for maximum decolorization of biomethanated distillery effluent. All experiments were carried out in triplicates.

Effect of pH

To study the effect of pH of the growth medium on the decolorization efficiency by JSA-1 isolate, 100 ml aliquots of BME medium were taken in 250 ml Erlenmeyer's flasks and initial pH of the medium was

varied from 4.5 to 9 with 1N NaOH and 1N HCl. After sterilization of the media at 121^0C for 15 minutes, they were inoculated with culture (10^7spores/100 ml medium) and incubated on rotary shaker (150 rpm) for 10 days at 30^0C. After 10 days these media were analyzed for percent decolorization of the effluent, dry weight mycelia of the culture and final pH of the medium.

Effect of carbon source

The effect of carbon source was studied by growing the culture in BME media devoid of carbon source (glucose) as well as replacing glucose with supplements of thirteen different sugars. 100 ml sterile aliquots of BME medium (without glucose) at pH 6 having maltose, glycerol, mannitol, xylose, sucrose, lactose, fructose, mannose, sorbitol, raffinose, arabinose and ribose 5 % w/v separately in 250 ml Erlenmeyer flasks were inoculated with the culture (10^7spores/100 ml medium) and incubated at 30^0Cunder shake flask condition (150 rpm) for 10 days and analyzed for percent decolorization of the effluent, dry weight mycelia of the culture and final pH of the medium.

Effect of concentration of Glycerol (Optimum carbon source)

The effect of different concentrations of glycerol in the medium as a carbon source was studied by varying glycerol concentrations from 2% to 8% w/v in the BME medium. 100 ml aliquots of medium with glycerol in the concentrations of 2% to 8% w/v, having pH 6 in 250 ml Erlenmeyer flasks were sterilized and inoculated with the culture (10^7spores/100 ml medium). These flasks were then incubated at 30^0C (150 rpm) for 10 days and analyzed for percent decolorization of the effluent, dry weight mycelia of the culture and final pH of the medium.

Effect of nitrogen source

Different types of inorganic and organic nitrogen sources were used to study the effect of nitrogen source on decolorization of effluent. This was done by replacing peptone with inorganic nitrogen sources such as NaNO3, NH_4Cl, and $(NH_4)_2SO_4$ while organic nitrogen sources such as urea, yeast extract, beef extract and malt extract (0.5 % w/v) separately in the BME medium containing glycerol as sole carbon source (5% w/v), having pH 6. The sterilized media were inoculated (10^7spores/100 ml medium) and incubated for 10 days at 30^0C (150 rpm) and analyzed for percent decolorization of the effluent and dry weight mycelia of the culture.

Decolorization of biomethanated distillery effluent with fungal mycelium of *Aspergillus oryzae JSA-1* in immobilized form

Medium used for decolorization study by immobilized culture (GYE medium)

Glycerol 6%; Yeast Extract 0.5%; KH_2PO_4 0.1 %; $MgSO_4$, $7H_2O$ 0.05 % and biomethanated distillery effluent 30% (v/v) in distilled water (Levernoche *et al.*, 1981).

Preparation of immobilized biomass

200 ml aliquots of sterile medium (Glycerol Peptone Medium), at pH 6 containing glass beads (approx. 2 cm) were inoculated with the spore suspension of *Aspergillus oryzae JSA-1* (10^7 spores /100 ml) of the fungal culture in 500 ml Erlenmeyer's flasks and these flasks were incubated for 10 days under shake flask condition (150 rpm, 30^0C). The purpose of adding glass beads in the flasks was to allow the mycelia to grow in suspension without forming mycelial pellets. The mycelial biomass was harvested

by vacuum filtration through four layers of cheese cloth and washed extensively with double distilled water and then with 0.001 M phosphate buffer (pH 6) to remove all traces of nutrients. Dewatered biomass (wet weight 10 g) was then suspended in 300 ml of 2 % sodium alginate solution. Homogeneous suspension was made by using homogenizer (Remi, India) and it was then extruded slowly trough syringe in to 1000 ml of 500 mM calcium chloride solution. The beads formed with immobilized biomass were kept in 2.5 % glutaraldehyde solution for 30 minutes and then resuspended in 500 mM of calcium chloride solution. The concentration of wet mycelia per gram of bead was maintained to 0.055 g throughout the experiment of decolorization.

Optimization of process parameters for decolorization of biomethanated distillery effluent by Aspergillus *oryzae JSA-1* in immobilized form

The process parameters such as pH, inoculum size of biomass, concentration of carbon source (glycerol) and concentration of nitrogen source (yeast extract), were optimized for decolorization of biomethanated distillery effluent by immobilized *Aspergillus oryzae JSA-1***,** in sterile glycerol yeast extract (GYE) medium.

Effect of pH

20 ml of sterile GYE media having different pH values such as 4.5, 5, 5.5, 6, 6.5, 7 and 7.5 were inoculated with 12 gm of biomass and incubated on rotary shaker (150 rpm) at 30^0C for 5 days (120 hrs). The percent decolorization of the culture filtrates was determined after every twenty four hours incubation followed by recording the readings spectrophotometrically at 475 nm. The uninoculated medium was used as control.

Effect of inoculum size

20 ml of sterile GYE medium having pH 6.5 were inoculated with different quantities of immobilized biomass such as 8g, 12g, 16g and 18g. The flasks were incubated on rotary shaker (150 rpm) at 30^0C for 24 hours. The percent decolorization of the culture filtrates was determined spectrophotometrically at 475 nm. The uninoculated medium was used as control.

Effect of glycerol concentration

20 ml of sterile GYE medium having different glycerol concentrations such as 0g, 0.25g, 0.5g, 1g, 2g, 4g, and 6g, having pH 6.5 were inoculated with 16g of immobilized biomass. The flasks were incubated on rotary shaker (150 rpm) at 30^0C for 24 hours. The percent decolorization of the culture filtrates was determined spectrophotometrically at 475 nm. The uninoculated medium was used as control.

Effect of yeast extract concentration

20 ml of glycerol free sterile medium having different yeast extract concentrations such as 0g, 0.1g, 0.2g, 0.3g, 0.4g, and 0.5g, having pH 6.5 were inoculated with 16g of immobilized biomass. The flasks were incubated on rotary shaker (150 rpm) at 30^0C for 24 hours. The percent decolorization of the culture filtrates was determined spectrophotometrically at 475 nm. The uninoculated medium was used as control. The optimum process parameters were used for the experiment of decolorization of BME by immobilized culture.

Results and Discussion

Optimization of the growth medium parameters for maximum decolorization of biomethanated distillery effluent *by Aspergillus oryzae JSA-1* in free form

Effect of pH

The effect of pH of the growth medium on decolorization of effluent has been shown in Figure 1. The results showed that initial pH of the medium had considerable effect on the decolorization efficiency by *JSA-1*. After 10 days the percent decolorization at pH 6 was maximum that is 57.5 and at pH 6.5 it was 56.5. There was decline in percent decolorization below pH 6 till 45.6% and above pH 6 till 24.8%. Growth in terms of dry weight mycelia was found to be highest at pH 6 which was found to be decreasing slightly till pH 8 and was affected greatly when pH was increased further. It was also noted that during the incubation period i. e. 10 days, pH values of the media were found to increase in the range of 0.2 to 0.5 units.

Effect of carbon source

The effect of carbon source on decolorization of effluent has been shown in table 1.

The results showed that the culture did not grow in the effluent medium without supplementation of additional carbon source unlike some fungal strains which could be grown and decolorized natural melanoidin of soil in absence of carbon and nitrogen source. It was seen that media containing glycerol and raffinose gave more than 70 % decolorization of effluent. Medium containing glycerol was found to give maximum decolorization as 72.9 % (due to maximum growth) while mannitol medium gave least decolorization as 37.72 %. Glycerol was selected as the optimum carbon source in the decolorizing medium for further studies. Glucose and glycerol were commonly required for melanoidin decolorization as reported in *Mycelia sterilia D-90* (Sirianuntapiboon *et al.,* 1888), *Aspergillus fumigatus G 2-6* (Ohmomo *et al.,* 1987), *Aspergillus oryzae Y-2-32* (Ohmomo *et al.,* 1988).

Effect of concentration of Glycerol (optimum carbon source)

The effect of concentration of glycerol on decolorization of effluent has been shown in figure 2. Up to 5% glycerol concentration, there was increase in the decolorization efficiency i.e.72.9% while after 5% there was no considerable change in the decolorization of BME by *JSA-1*.

Effect of nitrogen source

The effect of nitrogen source on decolorization of effluent has been shown in table 2.

Among inorganic nitrogen sources ammonium sulphate was found to give maximum decolorization i.e.70.50% while ammonium chloride and sodium nitrite were found to be good for the growth of the culture but gave less decolorization up to 59.81% and 44.66 % respectively. Peptone gave maximum decolorization among organic nitrogen sources i.e. 73.3% while yeast extract and beef extract found to give slightly less decolorization by around 10 % than peptone. Urea was found to be inefficient to give decolorization (30.12%) as compared to other nitrogen sources.

Effect of concentration of peptone (optimum nitrogen source)

When peptone was used as the optimum nitrogen source in different concentrations

in decolorization experiments, it was seen that 0.5% peptone concentration showed decolorization efficiency 72.3% while peptone concentration at 1%; there was slight increase in the decolorization by around 6% (Table 3). Growth of the culture and decolorization of effluent were found to be decreased with increasing peptone concentration above 1% which is similar to the results reported in *Phaenerochaete chrysosporium BKM –f 1767* (Roland, B. 1989) and *Aspergillus niger-UM2* (Dhamankar *et al.,* 2001).

Optimization of process parameters for decolorization of biomethanated distillery effluent by Aspergillus *oryzae JSA-1* in immobilized form

Effect of pH

The results of the experiment of pH optimization on decolorization of biomethanated effluent by immobilized culture are shown in figure 3.

The optimum pH for decolorization of BME by immobilized culture was found to be 6.5 showing percent decolorization as 62.5%. When the decolorization assays were carried out for every 24 hours it was found that the decolorization rate was maximum in initial period of 24 hours incubation, at all pH values which might be due to the adsorption of color pigments to the immobilized cells in the initial incubation phase i. e from 47.75% to 61.3 %. The decolorization rate showed slow increase up to 72 hours and remained almost constant upon further incubation.

Effect of inoculum size

The results of effect of inoculum size of immobilized biomass on decolorization of biomethanated effluent by immobilized culture are shown in Table 4. The immobilized culture showed increase in % decolorization of BME when the inoculum size of immobilized biomass was increased from 8g to 16g/20 ml of the culture medium with highest decolorization (71.5 %) of BME when inoculated with 16g of immobilized biomass/ 20 ml. Increase in the level of inoculum size further than 16g did not show increase in percent decolorization of BME.

Effect of glycerol concentration

Effect of glycerol concentration in the medium on decolorization of biomethanated effluent by immobilized culture is shown in Table 5.

Effect of yeast extracts concentration

It was found that the percent decolorization remained the same (approx. around 70 to 73%) in glycerol free medium and media containing different concentrations of glycerol from 0.25% to 6% w/v. When the culture was studied in spore inoculated glucose peptone medium for growth and decolorization, it did not show growth and decolorization in absence of any carbon source in the medium. The results indicated that decolorization of BME in 0% glycerol concentration was merely due to mycelial adsorption of color on the cell walls of fungal culture (Table 6). The results were similar to that of effect of glycerol concentration. There was no significant difference in the percent decolorization of BME even in the absence of yeast extract in the medium. The percent decolorization of BME remained almost the same (approx. around 74 to 76%) when the concentration of yeast extract was changed from 0.15 to 0.5%. This indicated that the process of decolorization of BME by immobilized culture did not require external supply of carbon and nitrogen source in the medium.

Table.1 Effect of carbon source on the decolorization of BME by *Aspergillus oryzae* JSA-1

Carbon Source (5%w/v)	% Decolorization	DWM*(g/100ml)	Final pH
Glucose	57.31 ± 0.23	1.84 ± 0.05	5.98
Maltose	59.40 ± 0.09	1.625 ± 0.04	6.20
Glycerol	**72.90 ± 0.17**	**1.859 ± 0.07**	**6.40**
Mannitol	37.72 ± 0.08	1.085 ± 0.03	6.10
Xylose	46.69 ± 0.12	1.485 ± 0.02	5.90
Sucrose	53.70 ± 0.07	1.753 ± 0.04	6.23
Lactose	62.13 ± 0.08	1.094 ± 0.06	6.60
Fructose	50.74 ± 0.15	1.864 ± 0.03	6.13
Mannose	46.68 ± 0.04	1.625 ± 0.02	5.57
Sorbitol	54.76 ± 0.01	1.971 ± 0.09	6.78
Raffinose	70.50 ± 0.06	1.571± 0.02	6.98
Arabinose	39.47± 0.11	0.812 ± 0.07	6.10
Ribose	69.80 ± 0.02	1.502 ± 0.09	6.80

Table.2 Effect of nitrogen source on the decolorization of BME by *Aspergillus oryzae* JSA-1

Nitrogen Source (0.5%w/v)	% Decolorization	DWM (g/100ml)
Inorganic		
NaNO3	44.66 ± 0.12	1.407 ± 0.07
NH4Cl	59.81± 0.05	1.302 ± 0.02
(NH4)2SO4	70.50 ± 0.23	1.295 ± 0.04
Organic		
Urea	30.12 ± 0.08	1.032 ± 0.08
Yeast Extract	62.72 ± 0.06	1.528 ± 0.07
Beef Extract	61.93± 0.13	1.503 ± 0.09
Peptone	**73.23 ± 0.14**	**1.906 ± 0.02**
Malt Extract	48.69 ± 0.08	1.320 ± 0.12

Table.3 Effect of concentration of peptone (optimum nitrogen source) on decolorization of BME by *Aspergillus oryzae* JSA-1

Conc. of peptone (% w/v)	% Decolorization	DWM (g/100ml)	Final pH
0.25	59.70 ± 0.07	0.754 ± 0.02	6.2
0.5	72.30 ± 0.05	1.600 ± 0.01	6.5
1.0	**78.60 ± 0.08**	**1.878 ± 0.05**	**6.8**
1.5	63.68 ± 0.03	0.745 ± 0.07	6.1
2.0	24.12 ± 0.02	0.525 ± 0.03	5.9
2.5	17.38 ± 0.04	0.239 ± 0.02	6.0

Table.4 Effect of inoculum size of immobilized biomass on decolorization of biomethanated effluent

Inoculum size (g)	% Decolorization
8	60.31 ± 1.23
12	68.19 ± 2.31
16	**71.50 ± 1.09**
18	72.0 ± 0.99

Table.5 Effect of glycerol concentration in the medium on decolorization of biomethanated effluent by immobilized culture

Glycerol concentration (% w/v)	% Decolorization
0	**72.5 ± 2.45**
0.25	72.8 ± 1.67
0.5	71.8 ± 0.98
1	71.3 ± 0.12
2	70.93 ± 1.11
4	70.8 ± 2.12
6	70.0 ± 1.78

Table.6 Effect of yeast extract concentration in the medium on decolorization of biomethanated effluent by immobilized culture

Yeast Extract Concentration (% w/v)	% Decolorization
0	**75.66 ± 1.21**
0.1	74.03 ± 1.50
0.2	75.07 ± 1.03
0.3	74.33 ± 0.96
0.4	74.63 ± 2.01
0.5	74.18 ± 1.65

Table.7 Optimized process parameters for decolorization of biomethanated distillery effluent by immobilized *Aspergillus oryzae* JSA-1

Sr. No.	Parameter	Optimized condition
1	pH	6.5
2	Incubation time	72 hrs
3	Inoculum size (g/20 ml)	16
4	Concentration of glycerol (g %)	NIL
5	Concentration of yeast extract (g %)	NIL

Fig.1 Effect of pH of the growth medium on the decolorization of BME by *Aspergillus oryzae* JSA-1

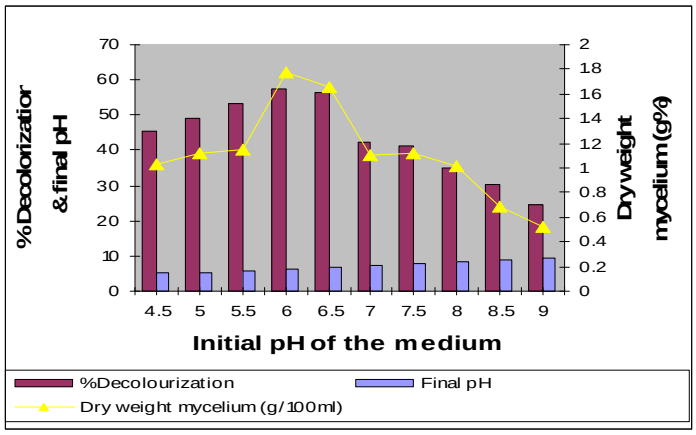


Fig.2 Effect of varied concentrations of different carbon sources on the decolorization of BME by *Aspergillus oryzae* JSA-1

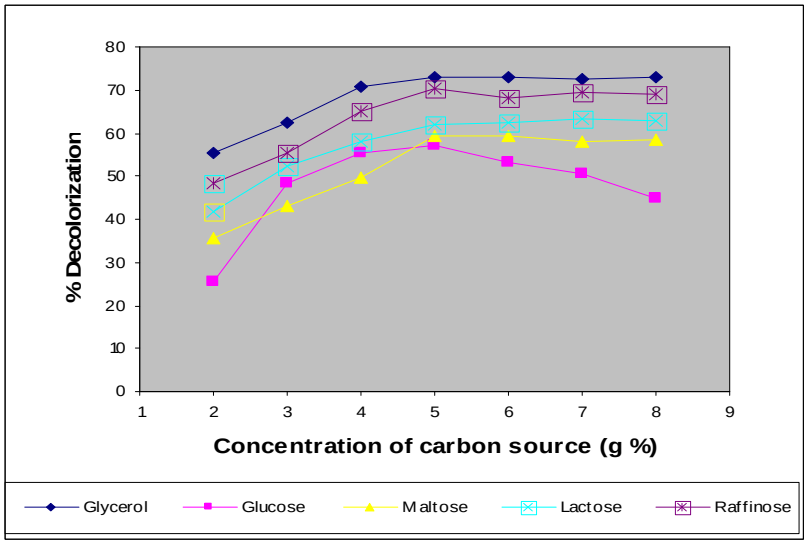
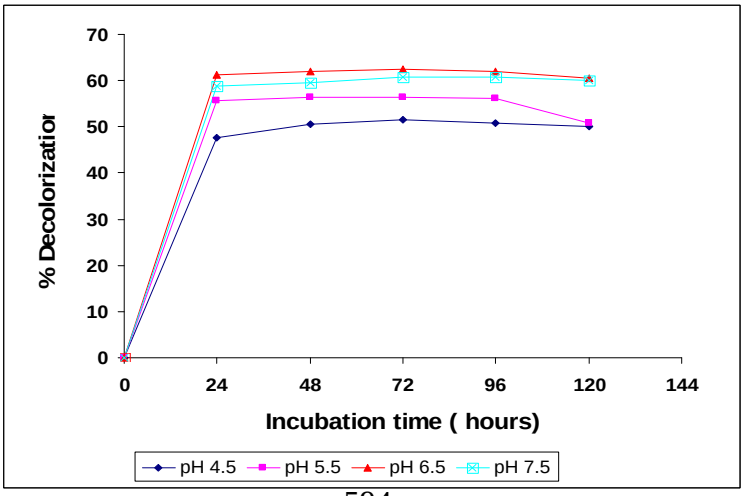


Fig.3 Effect of pH on decolorization of biomethanated effluent by immobilized culture



The reason for this might be the same as for glycerol concentration i.e. decolorization by the immobilized culture may be due to mere adsorption of color to the mycelia. The process parameters for the maximum decolorization of BME by immobilized *Aspergillus oryzae JSA-1*, were then optimized as shown in table 7.

Study on optimization of the growth medium parameters for maximum decolorization of biomethanated distillery effluent *by Aspergillus oryzae JSA-1*, showed that the requirement of carbon source was the growth limiting parameter in the medium and glycerol was found to be the optimum carbon source at 5g% concentration. Peptone was found to be the best nitrogen source at 0.5g% concentration, pH 6, temperature 30^0C and rotational speed 150 rpm were found to be other important optimum parameters.The results of this 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%) 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 (Ohmomo *et al.,* 1987). While in *Aspergillus niger UM2*, the immobilized culture could maintain its potent decolorization ability with the supply of external nutrients (Patil *et al.,* 2001). The study on immobization of *Aspergillus oryzae JSA-1* by sodium alginate could not increase the efficiency of the culture to decolorize the BME effectively but the process of decolorization of BME by immobilized culture was found to be economical due to no requirement of external supply of carbon and nitrogen source in the medium.

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