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SCREENING OF MICROORGANISMS FOR DECOLORIZATION OF TREATED DISTILLERY WASTEWATER*

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Abstract– The objective of this research work was to obtain a microorganism capable of decolorizing treated distillery wastewater (TDW). 21 isolated and procured microorganisms were screened for their percentage decolorization. The screening strategy was performed using three different culture media in two main steps. The primary screening was carried out in two stages. In the first stage 10 microorganisms had a lower than 25% decolorization of TDW (with 25% TDW concentration). In the second stage 8 microorganisms had more than a 48% decolorization of TDW. In the secondary screening all 3 different culture media, the effect of TDW concentration, pH and nitrogen source were studied. A fungus identified by morphology examination to be *Aspergillus fumigatus* U_{B2}, isolated from soil samples taken from the vicinity of the Bidestan Distillery and Food Products, Qazvin, Iran had maximum decolorization of 81%.

Keywords– Aspergillus niger, distillery wastewater decolorization, melanoidin, microorganism screening, molasses

1. INTRODUCTION

In the sugar industry, many by-products such as molasses, bagasse and fiber cake are produced, among which molasses is the most important. Molasses contains about 48 to 50% sugar and has a high commercial value due to its use as a carbon source in various fermentation processes, and also as cattle feed and biofertilizer [1]. The use of molasses as a raw material in distilleries produces wastewater with a high COD content of about 90000 mgl^{-1} [2] and toxic substances such as phenols, which can be decreased to a certain extent by methane fermentation and activated sludge treatments [1] but, the dark color remains as a problem which requires a pretreatment before its safe disposal into the environment. Low pH and a dark brown color are characteristics of this wastewater [2]. The major problem in treating distilleries wastewater (DW) is its color, which contains nearly 2% (w/w) of a dark brown recalcitrant pigment, melanoidin. Conventional biological treatments can degrade melanoidin only up to 6 or 7% [3].

Melanoidin is known as a natural browning polymer. It is produced by the Maillard reaction between amino- and carbonyl groups of organic matters and is closely related to humic substances in the natural environment [4]. The disposal of distillery wastewater into the environment is hazardous. Its highly colored components also lead to a decrease in sunlight penetration in rivers, lakes or lagoons, which in turn, decreases both photosynthetic activity and dissolved oxygen concentrations causing harm to aquatic life. Disposal on land is equally hazardous, causing a decrease in soil alkalinity and also in soil manganese availability [5], inhibition of seed germination and the retardation of vegetation growth.

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Several methods have been examined for decolorization of distillery wastewater. These can be classified as physicochemical and biological methods. Physicochemical methods are not economic and remove colors, toxicants, suspended solids and chemical oxygen demand. But BOD and low molecular weight compounds are not removed efficiently [6]. The biological color removal process is particularly attractive, since in addition to color and COD, it also reduces BOD and color [7].

Biotechnological advances in the field of effluent treatment have resulted in a number of efficient technologies; however, no biological method is available for the treatment of distillery effluent for color removal. Various microorganisms were screened for decolorization of distillery wastewater and some bacteria and fungi have been shown to degrade melanoidin. Certain fungi such as *Aspergillus fumigatus* [8], *Aspergillus niger* [9], *Coriolus versicolor* [10], *Phanerochate chrysosporium* [11] and certain bacteria such as *Pseudomonas fluorescens* [1], *Oscillatoria boryana* [3], and *Bacillus* sp. [12] were applied for the decolorization of distillery wastewater. Although most of the microorganisms cited above are capable of decolorizing distillery wastewater, an economically suitable microorganism is yet to be introduced.

In Iran, there are approximately 30 distilleries using beet molasses (with the exception of one that uses cane molasses) [13]. Only a few of these distilleries use anaerobic digestion for their effluent treatment to decrease COD content. In order to investigate the possibility of decolorizing distillery wastewater after anaerobic digestion, a project was initiated and supported by the Iranian Ministry of Industries and Mines. The present research outcome was part of a project on the identification of microorganisms and systems to improve decolorization and effluent treatment during ethanol production from molasses in Iran.

In this paper, we report on the isolation and identification of a fungus capable of decolorizing anaeorobically-digested and aerobically-treated distillery wastewater (TDW).

2. MATERIALS AND METHODS

a) Sources of microorganisms

21 microorganisms were used in this study. *Phanerochaete chrysosporeum* 5270 was obtained from the Iranian Research Organization (IROST) Iran. *Geotrichum candidum* MTCC 5265 and *Trametes versicolor* MTCC 138 was kindly provided by the Chemical Engineering Department, IIT Madras, India. 10 unknown microorganisms (named U_1-U_{10}) were isolated from materials and Energy Research Center soil samples and 8 microorganisms (named U_{B1}-U_{B8}) were isolated from soil samples taken from the vicinity of the Bidestan Distillery and Food Products, Qazvin, Iran. The isolation of microorganisms was conducted solely by using the method introduced by Kumar *et al*. [14] except for temperature. The method is as follows: Soil samples were collected as mentioned above. Two grams of soil samples were added to test tubes containing 20 ml of 25% diluted, digested spent wash. Three different enrichment media were used: in the first, digested spent wash was the sole carbon and nitrogen source; in the second, TDW was the sole carbon source and additional nitrogen (as ammonium sulphate) was added, and in the third, the digested TDW was the sole nitrogen source, while additional carbon (as glucose) was added. Tubes were incubated at 30^{o} C for 7 days under aerobic conditions to enrich for melanoidin- degrading cultures. Test tubes showing some decolorization were subcultured successively several times. The isolation of microbial cultures was carried out using the spread plate and streak plate techniques on gar medium containing the following: 1% glucose, 0.5% yeast extract, 0.1% KH_2PO_4, 0.05% $MgSO_4$ $7H_20$ and 2% agar at pH 7.5 value. All microorganisms were fungi and were maintained on PDA (Potato - Dextrose - Agar) slants.

b) Treated distillery wastewater (TDW)

TDW was obtained from the Bidestan Distillery in Qazvin, Iran after anaerobic (UASB) and aerobic (activated sludge) treatment to decrease BOD content to approximately 100-150 gl^{-1}. The TDW used had a BOD of 100-150 mg/l, COD of 200-4000 mg/l and pH of 7.5. The TDW was centrifuged at 4200 xg for 10 min before use.

c) Culture media preparation

Two synthetic and one molasses media were prepared. Two synthetic media were selected on the basis of one having an inorganic nitrogen containing salt ($NaNO_3$) and the other having a complex medium containing nitrogen and ammonium sulfate. The presence and absence of potassium chloride was another reason for choosing two different synthetic media because of the high potassium content of molasses. Therefore media 1 consisted of gl^{-1}: Glucose 50; KH_2PO_4 1.0; KCl 0.5; $NaNO_3$ 2.0; $MgSO_4.7H_2O$ 0.5; $FeSO_4$ 0.001 and pH was 5.5., and media 2 consisted of gl^{-1}: glucose 50; KH_2PO_4 1.0; $MgSO_4.7H_2O$ 0.5; Yeast extract 1.0; $(NH_4)_2SO_4$ 1.0 and pH was 5.5. Molasses media was prepared by the addition of 50 g molasses in one liter of distilled water and pH was adjusted to 5.5. The composition of two synthetic media is also listed in Table 1.

Table 1. The composition and conditions of media 1 and media 2

Media	Glucose (g/l)	KH_2PO_4 (g/l)	KCl (g/l)	$NaNO_3$ (g/l)	$MgSO_4.7H_2O$ (g/l)	$FeSO_4$ (g/l)	Yeast extract (g/l)	$NH_2(SO4)_2$	pH
Media 1	50	1.0	0.5	2.0	0.5	.001	-	-	5.5
Media 2	50	1.0	-	-	0.5	-	1.0	1.0	5.5

To investigate the percentage decolorization, 5×10^7 spores l^{-1} of microorganisms (for *T. versicolr*, a few chops of slant was used) were added to 100 ml of inoculum culture in 500-ml shaking flasks at 30°C on a rotary shaker at 160 rpm. After incubation for 3 days, 20 ml of culture grown in the form of pellets were added to 50 ml of diluted enriched TDW in 350-ml flasks at 30°C on a rotary shaker with 160 rpm each with two duplicates. Diluted enriched TDW was the effluent of the bidestan distillery diluted by three times its volume using distilled water. To each liter the following were added in gl^{-1}: Glucose 20: $NaNO_3$ 2.0: KH_2PO_4 1.0: $MgSO_4.7H_2O$ 0.5 and pH was 6.

d) Primary screening

1. Stage one: 5 ml of sterile water was added to a slant and all the suspended microorganisms added to media 1. After three days of cultivation in media 1, 20 ml of culture was centrifuged at 4200x g for 10 minutes, discarding the supernatant and adding the precipitated cells to the enriched TDW. After incubation in enriched TDW, the percentage decolorization was determined every 24 hours. Microorganisms having more than 25 percentage decolorization were selected for stage two.

2. Stage two: Selected microorganisms in stage one (for their higher decoloriztion ability) were incubated again in media 1 (for repeatability), media 2 and molasses media, and their percentage decolorizations compared. Microorganisms with a percentage decolorization higher than 55 were selected for secondary screening.

e) Secondary screening

The effects of TDW concentration, initial pH and the nitrogen source of the enriched 25% (v/v) TDW on percentage decolorization of the selected microorganisms were carried out and the best decolorizing microorganism was selected.

f) Decolorization assay

Samples were centrifuged at 4200x g for 10 min. The supernatant was used for the decolorization assay. The precipitated cells were washed three times with distilled water and dried in an oven at 95°C overnight to a constant weight. The amount of decolorization in each sample was measured by absorbance at 475 nm using a Pye Unicam Spectophotometer [15]. The decolorization was expressed as a percentage of the original absorbance at the start, T_o.

$$\text{Percentage Decolorization} = \frac{\text{Initialabsorbance (475 nm) } T_o - \text{Finalabsorbance (475 nm)}}{\text{Initialabsorbance (475 nm) } T_O} \times 100$$

g) Morphological examination

Morphological examination was performed using a light microscope equiped with a micrometer eyepiece with 400x magnifications [16].

3. RESULTS AND DISCUSSION

After successive purification, isolated microorganisms were of filamentous, as well as unicellular fungi.

a) Primary screening

1. Stage one: In this stage media 1 was used. 20 ml of this media containing cultivated microorganisms were centrifuged and the precipitated cells were added to diluted enriched TDW with 25% (v/v) concentration. At the end of this stage, microorganisms named U_{B1}, U_{B3}, U_{B5}, *G. Candidum*, U_2, U_3, U_4, U_5, U_9 and U_{10} had very low percentage decolorization (less than 25). Other microorganisms named U_{B2}, U_{B4}, U_{B6}, U_{B7}, U_{B8}, *T. versicolor*, *P. chrysosporeum*, U_1, U_6, U_7 and U_8, had percentages of decolorization higher than 25 and consequently, were selected for stage two (results not shown).

***2. Stage two*:** Percentage decolorization, suitable media and the time of maximum percentage decolorization are shown in Table 3. The results show that the U_{B2} microorganism had the highest percentage decolorization (81.2). U_6 with 75, *T. versicolor* with 74.6 and U_8 with a 73.7 percentage decolorization were next to U_{B2} from the color removal point-of-view. All of these 4 microorganisms were of the filamentous type. The results of decolorization of TDW by the U_{B2} microorganism grown on 3 different media are shown in Figure 1. As shown in this figure, media 1, having $NaNO_3$ as the nitrogen source, was a better medium compared to media 2 and molasses. As shown in Fig. 1, this microorganism had 81.2 percent decolorization by incubation in media 1 after 48 h. This microorganism also had 75.9 and 75.2 percent decolorization by incubation in media 2 and in molasses media, respectively (Fig. 1). U_{B2} had a higher percentage decolorization of TDW in all three media (i.e. media 1, media 2 and molasses media), compared to the rest of the isolated and centrifuged miroorganisms studied (the only result of percentage decolorization of U_{B2} in three different media is shown in Fig. 1).

U_{B2} and U_6 were very similar from the morphological point-of-view and were identified to be *Aspergillus fumigatus* by morphological examinations. The two microorganisms grew rapidly at 30 °C covering the surface of slant media in 4 days, with surface character strictly velvety white at first, becoming green with the development of conidial heads. Conidial heads were almost globular, often with short columns. Conidiophores were short and smooth with up to 300 μm in length and 5 to 6 μm in diameter. Vesicles diameter were up to 20 to 25 μm in diameter with sterigmata in one series. Conidia were green in color with diameter up to 2.0 to 2.5 μm. A microscopic view of the *Aspergillus fumigatus* showing the mycelia and conidia heads is shown in Fig. 2.

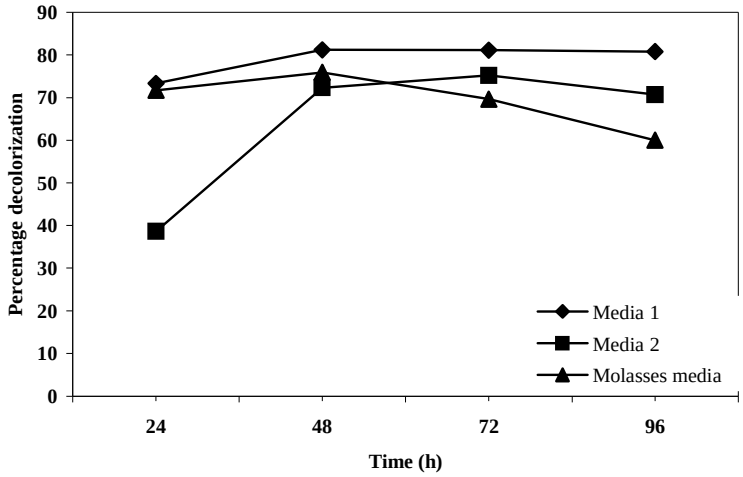

Fig. 1. Decolorization efficiency of UB_2 microorganism after incubation in molasses media, media 1 and media 2

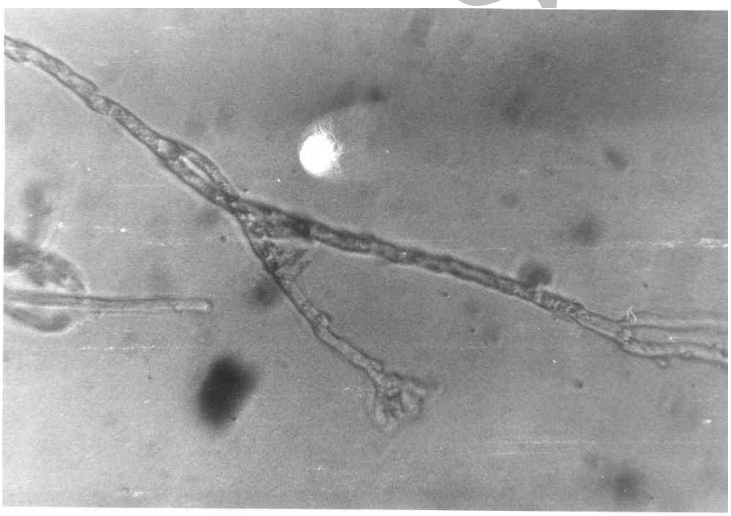

Fig. 2. Light microscope photomicrograph of *A. fumigatus* U_{B2} with 400× magnification

b) Secondary screening

After investigation of the results of primary screening (Table 2), U_1, U_{B2}, U_6, *T. versicolor*, U_8 and *P. chrysosporeum*, with more than 55 percent decolorization, were selected for secondary screening. For further studies, *T. versicolor* and U_{B2} were incubated in media 1 as they had a higher percentage of decolorization in this inoculum culture, and U_1, U_6, U_8 and *P. chrysosporeum* were incubated in media 2 for the same reason.

1. Effect of TDW concentration: To investigate the effect of higher TDW concentration on percentage decolorization, the 6 selected microorganisms were cultivated in enriched TDW with 50% (v/v) concentration. As shown in Fig. 3, U_{B2}, U_6 and *T. versicolor* had high percentages of decolorization of 75.5, 74.5 and 63.4, respectively, while U_1, U_8 and *P. chrysosporeum* have lower percentages of decolorization of 30.5, 25.6 and 24.6, respectively. Comparing the results obtained with those presented in Table 2, it can be observed that the percentage decolorization of U_6 was not affected by increasing the TDW concentration (25% to 50%), however the other 2 microorganisms (U_{B2} and *T. versicolor)* showed a

nearly 5 to 10% decrease in their efficiencies. The other 3 microorganisms showed a sharp decrease in their percentage decolorization. Although the concentration of TDW was doubled, the reduction in percentage decolorization was negligible by both U_{B2} and U_6 strains. This can be an indication of the promotion in activity of these strains due to the increased TDW concentration.

Table 2. Suitable primary media for maximum percentage decolorization of each microorganism

Microorganism	Primary inoculum culture	Maximum percentage decolorization	Time (h)	(% Decolorization/g biomass/h)
U_1	2	66.8	48	0.23
U_6	2	75	96	0.13
U_7	1	48.5	48	0.17
U_8	2	73.7	72	0.18
U_{B2}	1	81.2	48	0.27
U_{B4}	1	54.7	96	0.11
U_{B6}	1	54	96	0.09
U_{B7}	2	53.7	96	0.1
U_{B8}	2	46.9	48	0.19
T. versicolor	1	74.6	120	0.11
P. chrysosporeum	2	56.3	120	0.08

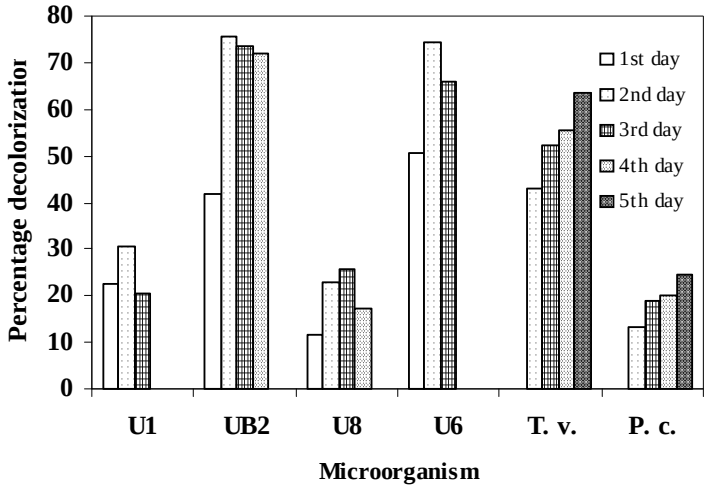

Fig. 3. PD of microorganisms of T. v. (*T. versicolor*), UB_2, U_6, U_1, U_8, P. c. (*P. Chrysosporeum*) in diluted TDW (50 % (v/v))

2. Effect of pH: The initial pH of the enriched TDW (with 25% (v/v) concentration) was adjusted first to 4 and then 6. The effect of pH on percentage decolorization is shown in Fig. 4. The results show that U_{B2}, U_8, *T. versicolor* and U_1 have a better percentage decolorization with an initial pH of 6, while *P. chrysosporeum* and U_6 had a better percentage decolorization with an initial pH of 4. On the other hand, U_6 and U_{B2} had very close percentages of decolorization (76-81) and differ only by 4-5 percent in their ability to decolorize at two different initial pH values. The pH of U_{B2} was later optimized with two other variables (carbon source and cell mass concentration) using response surface methodology [17] and determined to be 5.6.

3. Effect of nitrogen source: The effect of four different nitrogen sources ($NaNO_3$, $(NH_4)_2SO_4$, yeast extract and peptone) on percentage decolorization of TDW was examined by 6 selected microorganisms. The amount of nitrogen sources added was 2 gl^{-1}, with a TDW concentration of 25% and pH=6. The results are shown in Table 3. *A. fumigatus* U_{B2} showed the highest activity when $NaNO_3$ was used as a nitrogen source in the medium. This was also later confirmed after UV irradiation of *A. fumigatus* U_{B2}

spores [18]. From the results obtained it was determined that *Aspergillus niger* fumigatus U_{B2} was better for decolorizaing TDW. The conditions for growth and decolorization media was later partially optimized using three sequential Plackett-Burman experimental designs [18]. The maximum percentage decolorization obtained was 84% and COD reduction was 82%. pH was reduced to 3-3.5 during decolorization and the cell mass produced was 7 g/l (dry weight) [18].

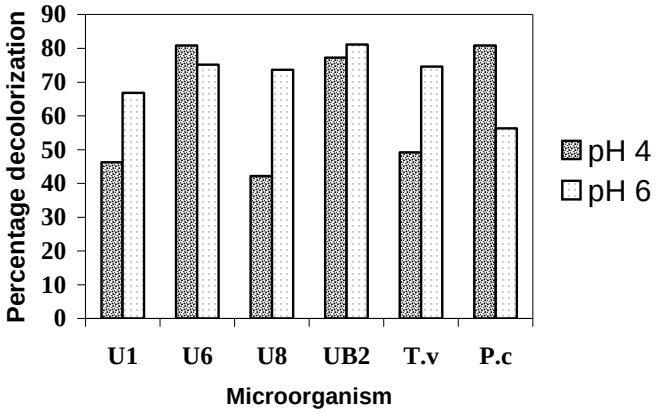

Fig. 4. Effect of pH on percentage decolorization by U_1, U_6, U_8, T. V. (*T. versicolor*) and P. C. (*P. Chrysosporium)*

Table 3. Effect of nitrogen source on decolorization by selected microorganisms

Microorganism	Best nitrogen source	Maximum percentage decolorization	Time (h)	(% Decolorization/g biomass/h)
U_1	$(NH_4)_2 SO_4$	73.3	48	0.26
U_6	Yeast extract	76.1	96	0.13
U_8	$NaNO_3$	73.7	72	0.18
U_{B2}	$NaNO_3$	81	48	0.27
T. versicolor	$(NH_4)_2SO_4$	75.3	120	0.11
P. chrysosporeum	$(NH_4)_2SO_4$	60.7	120	0.09

4. CONCLUSION

From 21 microorganisms studied for the decolorization of TDW, only 11 strains showed considerable percentages of decolorization. Out of them, 6 fungi had higher than 55 percent decolorization. The isolated Ascomycetes that were identified as *Aspergillus fumigatus* U_{B2} were incubated in media 1 with $NaNO_3$ as the nitrogen source for 3 days and cultivation in enriched 25% TDW had 81 percent decolorization in 48 h.

Although U_{B2} and U_6 were both identified as *A. fumigatus*, strain U_{B2} isolated from soil samples taken from the vicinity of the Bidestan Distillery was more efficient in decolorizing TDW, probably due to its acquaintance with melanoidin for a longer period of time. Use of this fungus may be practical as a biological decolorizer for distillery wastewater. The ability of this microorganism was later confirmed by the continuous mode of decolorization, by decolorizing more than 22 replacements in sequenced batch reactor [19].

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