

# Simultaneous detoxification and decolorization of molasses spent wash by the immobilized white-rot fungus *Flavodon flavus* isolated from a marine habitat

Chandralata Raghukumar\*, C. Mohandass, Shilpa Kamat and M.S. Shailaja

National Institute of Oceanography, Dona Paula, Goa 403 004, India

---

## Abstract

The wastewaters of molasses-based alcohol distilleries contain brown colored melanoidin pigments that are one of the major pollutants. We reported decolorization of such intensely brown colored molasses spent wash (MSW) by *Flavodon flavus*, a white-rot basidiomycete fungus isolated from a marine habitat. We have further attempted to improve the process of decolorization of MSW by this fungus by immobilization. Polyurethane foam-immobilized-fungus decolorized 10% diluted MSW by 60% and 73 % by day 5 and 7 respectively. The immobilized fungus could be effectively used for a minimum of 3 cycles repeatedly to decolorize MSW. Besides decolorization, the fungus also removed the toxicity of MSW. Toxicity bioassay of the fungus-treated molasses spent wash using an estuarine fish *Oreochromis mossambicus* showed no liver damage in contrast to untreated effluent, which showed moderate liver damage. We detected benzo(a)pyrene, a polycyclic aromatic hydrocarbon (PAH) in the MSW and this appears to be one of the causes of toxicity of the MSW. The concentration of PAH in the MSW decreased by 68% by day 5 on treatment with the fungus. This is the first report where decolorization of MSW is accompanied by simultaneous detoxification and decrease in PAH content of the MSW. A comparison of gel filtration chromatography of MSW, before and after treatment with the immobilized *F. flavus* showed disappearance of the most of the colored fractions in the fungus-treated MSW. A possible mechanism of decolorization of MSW is via the action of glucose oxidase accompanied by production of hydrogen peroxide that may ultimately act as a bleaching agent.

**Keywords:** Molasses spent wash, decolorization, detoxification, immobilization, white-rot fungus, comet assay

---

\*Corresponding author: Email: [lata@nio.org](mailto:lata@nio.org)  
Phone: 91-0832-2450480; Fax: 91-0832-2450603

## Introduction

The wastewater from molasses-based alcohol distilleries is known as molasses spent wash (MSW). When released in aquatic system, it cuts off light due to its intense brown color and affects photosynthesis. The high organic load of the effluent causes eutrophication. When disposed in soil, MSW acidifies soil and affects agricultural crops. About 2.7 billion litre of alcohol is produced by about 285 distilleries in India (1). Molasses spent wash in such effluents is nearly 15 times in volume of the total alcohol production. This huge quantity, about 40 billion litres of MSW effluent, if disposed untreated in water courses can cause great stress on the aquatic life. Alcohol serves as a basic chemical for a large number of chemical industries and therefore, demand for alcohol will see a great increase in future and so also the distilleries producing alcohol. This will eventually lead to an increased pollution by intensely brown colored MSW. The brown color of MSW is due to the presence of melanoidin pigments which are the products of the “Maillard reaction” between sugars and amino compounds produced on heating (2).

Anaerobic digestion of MSW-containing effluents is one of the treatments followed by distilleries and the resulting dark brown sludge is used as a fertilizer. The effluent after such treatment has a reduced chemical oxygen demand (COD) and a lower biological oxygen demand (BOD). However, this treatment does not remove the dark brown color of the effluent, which is a major pollution problem. The effluent therefore is released after diluting with fresh water which is a very dear commodity to industries. In order to remove color from such effluents, several species of terrestrial fungi have been tried (3 and 4). These fungi have been reported to remove about 70 to 80 % of the color (5). We have earlier reported decolorization of MSW by using free mycelia of *Flavodon flavus*, a basidiomycete isolated by us from decomposing leaves of a seagrass from a coral lagoon in India (6). In this paper, we report an improvement of this process by using immobilized fungus and also simultaneous detoxification of the effluent

by the fungus. In addition, we report for the first time the presence of polycyclic aromatic hydrocarbon in MSW and its removal by the fungus.

## **Materials and Methods**

### *Growth conditions*

The basidiomycete fungus *Flavodon flavus* was isolated from decomposing seagrass leaves from a coral lagoon in one of the Lakshadweep Islands in the Arabian sea (7). The isolate of *Flavodon flavus* was deposited in the ARS Patent Culture Collection, USDA, Illinois, on June 19, 2000 under the accession number NRRL 30302.

### *Decolorization of MSW*

The raw MSW was obtained from a local distillery unit manufacturing alcohol from sugarcane molasses. The raw MSW was dark brown in color and according to the data provided by the distillery, had a pH of 4.3 and contained B.O.D of 42,000 mg L<sup>-1</sup> and C.O.D of 80,000 mg L<sup>-1</sup>. Primary and secondary treatment of the effluent reduced BOD and COD and increased the pH to 8.2 but there was very little reduction in the color intensity of the effluent after these two treatments.

For raising the inoculum, the fungus was grown in the low nitrogen medium (LNM) containing 2.4 mM nitrogen in the form of ammonium tartrate and 10 % glucose (8).

### *Immobilization of the fungus*

Polyurethane foam cubes of 1 cm<sup>2</sup> were boiled in water, washed repeatedly with distilled water and were wet sterilized in an autoclave at 121°C. Sterilized polyurethane foam cubes were soaked with low nitrogen medium and inoculated with macerated mycelium of the fungus grown in low nitrogen medium. Within 7 days the foam cubes were covered with the fungal mycelium. The excess

medium was drained off and 10% diluted sterile molasses spent wash was added to the cubes. Low nitrogen medium was used for diluting the MSW to the desired concentrations. The cultures were aseptically oxygenated on alternate days with 100% oxygen using a sterilized glass Pasteur pipette attached to a sterile tubing (containing sterile cotton pad) to an oxygen cylinder. Aliquots (0.5 to 1.0 ml) of culture supernatants from experimental and heat-killed cultures were appropriately diluted and changes in the absorbance maximum at 475 nm (3) were measured using a Shimadzu 2001 Visible spectrophotometer (Shimadzu, Japan). The results were calculated as the difference in decolorization between heat-killed cultures and experimental cultures on the day of measurements and expressed as percentage decolorization. Foam cubes without fungal growth and heat-killed immobilized fungus were used as controls. Decolorization of MSW by free and immobilized fungus was compared in several experiments. Decolorization of different concentrations of MSW by immobilized fungus was also compared.

#### *Reusing immobilized fungus for decolorization*

Same batch of immobilized fungus was used for decolorization of different cycles of 10% diluted MSW and the percentage of decolorization was measured after 5 days of incubation with each fresh batch of MSW.

#### *Viability test of the fungus immobilized in foam cubes*

The polyurethane foam cubes (moistened with LN medium) containing the immobilized fungus were maintained for nearly 75 days in sterile conditions. At the end of this period, viability test was conducted by transferring these cubes into fresh medium to observe growth.

### *Estimation of amylase and glucose oxidase*

Amylase was estimated using 1% starch and iodine and potassium iodide (9). Glucose oxidase was estimated with peroxidase and chromogenic oxygen acceptor O-dianisidine which results in formation of a colored product which is measured at 460 nm (10).

### *Toxicity test*

Increased level of serum sorbitol dehydrogenase (SSDH) is a specific indicator of chemically-induced liver damage in fish. SSDH activity was determined in an estuarine fish *Oreochromis mossambicus* wherein, the rate of conversion of fructose to sorbitol by SSDH was followed by measuring the oxidation of the cofactor NADH (11). The fish was exposed to untreated and fungus-treated MSW in aquarium tanks for 4 days. Six replicate fish were maintained for each treatment. Serum sorbitol dehydrogenase assay was conducted at pH 7.4 in Tris buffer (0.2 M) using 0.2 ml of the composite serum (n=6) obtained by cardiac puncture. The reduction of D-fructose (400 mM) to sorbitol by NADH (0.4 mM) was followed at 366 nm with a Jasco Model V530 spectrophotometer. One unit of SSDH activity refers to a change in absorbance of  $0.001 \text{ min}^{-1}$  in 3 ml reaction mixture.

Toxicity test was also done using Comet assay, a technique to detect DNA damage in individual cells (12). Isolated hepatic cells of *Oreochromis mossambicus* were exposed *in vitro* to fungus-treated and untreated MSW for 3 hours at 37° C. The exposed cells were subsequently subjected to single cell gel electrophoresis (Comet assay). Briefly, the assay involved embedding the isolated cells in agarose gel on microscopic slides, lysing by detergent at high salt concentrations, treatment with alkali to denature the DNA and then electrophoresis under alkaline conditions. The slides were next stained with ethidium bromide and examined under a Olympus BX 60 epifluorescence

microscope (Tokyo, Japan) using green excitation filter. Cells with damaged DNA appear like comets. Twenty five cells were scored at random per slide to determine the percentage of comets formed. Three such replicate slides were scanned for each treatment for the presence of comets.

#### *Estimation of polycyclic aromatic hydrocarbons in the MSW*

As the pigments in molasses spent wash are products of Maillard reaction between sugars and amino compounds produced on heating, it was suspected that the heating process would also produce pyrogenic compounds like polycyclic aromatic hydrocarbons (13). Therefore, polycyclic aromatic hydrocarbons (PAHs) present in the MSW (raw and fungus-treated) were extracted into hexane and their concentrations measured fluorimetrically using an excitation  $\lambda$  of 310 nm and emission  $\lambda$  of 360 nm (14). Chrysene (Sigma Chemicals, USA) was used as the standard.

#### *Gel filtration of MSW*

The raw molasses spent wash, diluted to 10% was used for gel filtration chromatography using a column of Sephadex G-50. The column was equilibrated with 0.01 M phosphate buffer (pH 7.0) and the MSW was eluted with the same buffer at a flow rate of 1ml min<sup>-1</sup>. Fractions of 3 ml were collected and the color intensity was measured at 475 nm. A 1% solution of blue dextran was passed through the column to calculate the void volume. The MSW decolorized by the immobilized fungus was similarly passed through Sephadex G-50 column.

## **Results**

*Flavodon flavus* immobilized in polyurethane foam cubes, removed up to 60% of the color from MSW used at 10% concentration within 5 days (Fig. 1). Further about 73% of the color was removed by day 7. No decolorization of MSW was observed in polyurethane foam cubes used without the fungal inoculum, thereby

ruling out color removal by adsorption. In the heat-killed immobilized fungus treatment, no decolorization of MSW was noticed throughout the experiment. Maximum decolorization was achieved when MSW was used at 10% concentration with live, immobilized fungus (Fig. 2). The same batch of immobilized fungus could be used effectively for three consecutive cycles of decolorization of fresh MSW used at 10% concentration. (Fig. 3). In another trial, MSW treated for 5 days with the fungus with 50 % of the color removed, was further treated with a new batch of immobilized fungus. This resulted in an additional 30% reduction in color. Therefore, in effect upto 78-80% of the color could be removed by employing different strategies of using the immobilized fungus.

Almost similar percentage of decolorization was achieved within day 3 to 5 by using free or immobilized fungus in several trials (Fig. 4). This was further confirmed by absence of statistical significant difference between the two treatments. The fungus remained viable in the polyurethane foam for a minimum period of up to 75 days.

In the estuarine fish *Oreochromis mossambicus* exposed to 10% diluted, untreated MSW for 4 days, the serum sorbitol dehydrogenase activity was  $121.7 \pm 5.3 \text{ U ml}^{-1}$  serum. On the other hand, with fungus-treated MSW at the same dilution, the activity was  $2.6 \pm 2.6 \text{ U ml}^{-1}$  serum. Thus, treatment of the MSW has effected a near total (ca. 98%) removal of the damage-causing factor.

In the presence of untreated MSW, 85% of the hepatic cells of *Oreochromis mossambicus* showed damaged nuclei as comets whereas in the presence of fungus-treated MSW only 9% of the cells showed the presence of comets indicating a near total removal of toxic factor after the fungal treatment.

The total PAH concentration in the untreated MSW was  $3.8 \mu\text{g ml}^{-1}$  and this was reduced by nearly 68% ( $1.2 \mu\text{g ml}^{-1}$ ) after treatment with the white-rot

fungus. Fluorescence measured at 380/430 nm excitation and emission wave lengths which is specific for the PAH, benzo(a)pyrene, a known environmental carcinogen, showed ~30% reduction in MSW treated with the fungus. Removal of PAH was further confirmed when 60-70 % of phenanthrene and chrysene (polycyclic aromatic hydrocarbons) used at 8 and 12 ppm concentrations in the low nitrogen medium were removed within 6 days by this fungus from the culture medium (unpublished results).

The high molecular weight fractions, which got eluted initially during gel filtration of the untreated MSW, showed much higher absorbance at 475 nm than the low molecular weight fractions that got eluted later (Fig. 5). In MSW treated with immobilized fungus (after 5 days) reduction in color of high molecular weight fractions was proportionately greater than the low molecular weight fractions (Fig. 5).

The efficiency of decolorization by free mycelia increased when the culture medium (malt extract broth) was supplemented with 1% starch. Although amylase activity did not show much difference, there was an increase in glucose oxidase activity (Table 1). The increase in the glucose oxidase activity correlated with increasing decolorization of MSW (Table 1).

## **Discussion**

The use of immobilized cells in bioremediation of pollutants has been investigated as an alternate technology for environmental applications. Advantages of using immobilized microorganisms for pollutant degradation are well known (15). Besides being economically cheaper and easier to handle, the immobilized fungus is reusable for several batches. *Flavodon flavus*, which is highly potent in decolorization of MSW can be effectively used by immobilizing it in polyurethane foam, yielding values similar to its free mycelium. This substrate has also been used for decolorization of bleach plant effluent of paper and pulp



mill by *Rhizopus oryzae* (16). *Flavodon flavus* requires oxygenation for effective decolorization of MSW (6). The porous nature of polyurethane foam may help in better diffusion of oxygen. Moreover, the same fungal biomass immobilized in polyurethane foam cubes could be used up to 3 consecutive cycles of decolorization. Further, the fact that the fungus remains viable in foam for a long time makes the process easier to handle. *Phanerochaete chrysosporium* immobilized in alginate beads decolorized 6.25% diluted MSW by 60% on day 8 (4). On the contrary, *F. flavus* immobilized in foam cubes removed about 60% of the color even by day 5 when 10 % MSW was used. Therefore, polyurethane foam is ideally suited as a carrier for immobilization of this fungus.

The white-rot basidiomycetous fungus *F. flavus* isolated by us produces extracellular enzymes, manganese-dependent peroxidase (MNP), lignin peroxidase (LIP) and laccase in low nitrogen medium (8). It has also been shown to decolorize a number of synthetic dyes in low nitrogen medium prepared with distilled water or with artificial sea water and in malt extract broth (8). It has further been shown to decolorize bleach plant effluent of paper mill (17). A positive correlation was observed between concentrations of MNP in the extracellular culture filtrate of the fungus grown in low nitrogen medium and decolorization of synthetic dyes Poly R and remazol brilliant blue R and bleach plant effluent. On the other hand a negative correlation existed between MNP concentrations and decolorization of MSW in the same medium (18). However, we found levels of glucose oxidase activity correlating with decolorization of MSW (Table 1)). Therefore, we propose that hydrogen peroxide produced as a result of glucose oxidase activity might act as a bleaching agent on MSW. This is further confirmed by the fact that decolorization is lesser when the fungus is grown in malt extract broth (in the absence of glucose). On addition of starch to this medium, amylase activity is stimulated releasing reducing sugars and these are oxidized further by glucose oxidase producing hydrogen peroxide as a byproduct. This results in greater decolorization of MSW (Table 1). Production of

H<sub>2</sub>O<sub>2</sub> is reported in several white-rot fungi and the amount produced varies depending on the substrates used and other culture conditions (19). Hydrogen peroxide is implicated in lignin and cellulose degradation by white and brown-rot fungi (20). The lignin degrading enzymes manganese peroxidase and lignin peroxidase require H<sub>2</sub>O<sub>2</sub> as co-substrates.

Gel filtration of the MSW treated with fungus confirmed that majority of color imparting fractions were removed. It appears that the high molecular weight fractions are being decomposed faster. Similar results were reported with the white-rot fungus *P. chrysosporium* wherein larger molecular weight fractions were decolorized more rapidly than smaller molecular weight fractions (4).

Besides removing color from MSW, this fungus when used as free mycelium brought down levels of chemical oxygen demand (COD) and total phenolics in MSW by 50% (6). In addition, the toxicity of the MSW was totally removed on treatment with the fungus. This toxic factor is apparently due to the presence of PAHs which were reduced by 68% and ~30% reduction of benzo(a)pyrene on treatment with the fungus. Presence of PAHs in MSW and simultaneous decolorization and detoxification of MSW is being reported here for the first time. The cumulative decrease in PAHs and phenolics might be responsible for reducing the toxicity of the fungus-treated MSW.

## References

- [1] Joshi HC. Bio-energy potential of distillery effluents. <http://www.undp.org.in/programme/GEF> 2001
- [2] Wedzicha BL, Kaputo MT. Melanoidins from glucose and glycine: composition, characteristics and reactivity towards sulphite ion. *Food Chem* 1992;43:359-67.
- [3] Srianuntapiboon S, Somchai P, Ohmomo S, Attampasampunna P. Screening of filamentous fungi having the ability to decolorize molasses pigment. *Agric Biol Chem* 1988; 52:387-92.
- [4] Fahy V, Fitzgibbon FJ, McMullan G, Singh D, Marchant R. Decolorization of molasses spent wash by *Phanerochaete chrysosporium*. *Biotechnol Lett* 1997;19:97-99.
- [5] Raghukumar C. Bioremediation of coloured pollutants by terrestrial versus facultative marine fungi. In: Hyde KD, editor. *Fungi in marine environments*. Fungal Diversity Research Series 2002;7:317-44.
- [6] Raghukumar C, Rivonkar G. Decolorization of molasses spent wash by the white-rot fungus *Flavodon flavus*, isolated from a marine habitat. *Appl Microbiol Biotechnol* 2001;55:510-14.
- [7] Raghukumar C, Raghukumar S, Chinnaraj A, Chandramohan D, D'Souza TM, Reddy CA. Laccase and other lignocellulose modifying enzymes of marine fungi isolated from the coast of India. *Bot Mar* 1994;37:515-22.
- [8] Raghukumar C, D'Souza TM, Thorn RG, Reddy CA. Lignin-modifying enzymes of *Flavodon flavus*, a basidiomycete isolated from a coastal marine environment. *Appl Environ Microbiol* 1999;65:2103-11.
- [9] Medda S, Chandra, AK. New strains of *Bacillus licheniformis* and *B. coagulans* producing thermostable  $\alpha$ -amylase active at alkaline pH. *J Appl Bacteriol* 1980;48:47-48.

- [10] Tsuge H, Natsuaki O, Ohashi K. Purification, properties and molecular features of glucose oxidase from *Aspergillus niger*. J Biochem 1975;78: 835-43.
- [11] Dixon DG, Hodson PV, Kaiser KLE. Serum sorbitol dehydrogenase activity as an indicator of chemically induced liver damage in rainbow trout. Environ Toxic Chem 1987;6:685-96.
- [12] Singh NP, McCoy MT, Tice RR, Schneider EL. A simple technique for quantitation of low levels of DNA damage in individual cells. Exp. Cell Res 1988; 175: 184-91.
- [13] Cerniglia CE. Biodegradation of polycyclic aromatic hydrocarbons. Biodegradation 1992;3:351-68.
- [14] Keizer PD, Gordon DC, Jr. Detection of trace amounts of oil in seawater by fluorescence spectroscopy. J Fish Res Bd Can 1973;30:1039-46.
- [15] Cassidy MB, Lee H, Trevors JT. Environmental applications of immobilized microbial cells: a review. J Ind Microbiol 1996;16:79-101.
- [16] Nagarathnamma R, Bajpai P. Decolorization and detoxification of extraction-stage effluent from chlorine bleaching of Kraft pulp by *Rhizopus oryzae*. Appl Environ Microbiol 1999;65:1078-82.
- [17] Raghukumar C, Chandramohan D, Michel FC Jr, Reddy CA. Degradation of lignin and decolorization of paper mill bleach plant effluent (BPE) by marine fungi. Biotechnol Lett 1996;18:105-08.
- [18] Raghukumar C. Fungi from marine habitat: application in bioremediation. Mycol Res 2000;104:1222-26.
- [19] Zhao J, Janse BJH. Comparison of H<sub>2</sub>O<sub>2</sub> –producing enzymes in selected white-rot fungi. FEMS Microbiol Lett 1996;139:215-21.
- [20] Ander P. The cellulose-oxidizing enzyme CBQ and Cho as related to lignin and cellulose degradation- a review. FEMS Microbiol Rev 1994;13: 297-312.

Table 1. Involvement of glucose oxidase in decolorization of MSW by free mycelia.

<b>Media used</b>	<b>% Decolorization</b>	<b>Amylase (U L<sup>-1</sup>)</b>	<b>Glucose oxidase (U L<sup>-1</sup>)</b>
Malt extract broth+ 10% MSW	53	26	147
Malt extract broth+1% starch+ 10% MSW	93	26	289
Low nitrogen medium without MSW			
Day 3	-	Nd	79
Day 5	-	8.4	119
Day 7	-	nd	165
Day 9	-	nd	188
Low nitrogen medium + 10% MSW			
Day 3	53	0.6	119
Day 5	78	1.5	371
Day 7	85	5.3	409
Day 9	89	8.4	441

Nd= not detected. Standard deviation was less than 5% in all the readings.

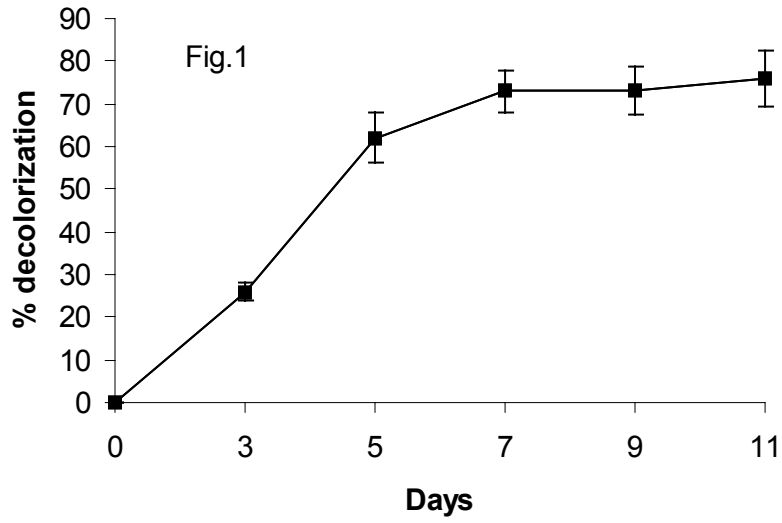


Fig. 1. Progress of decolorization of molasses spent wash used at 10% concentration using the immobilized fungus *F. flavus*.

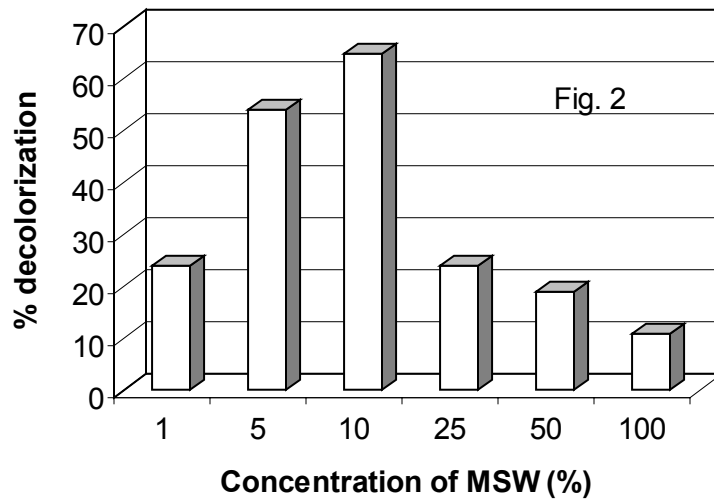
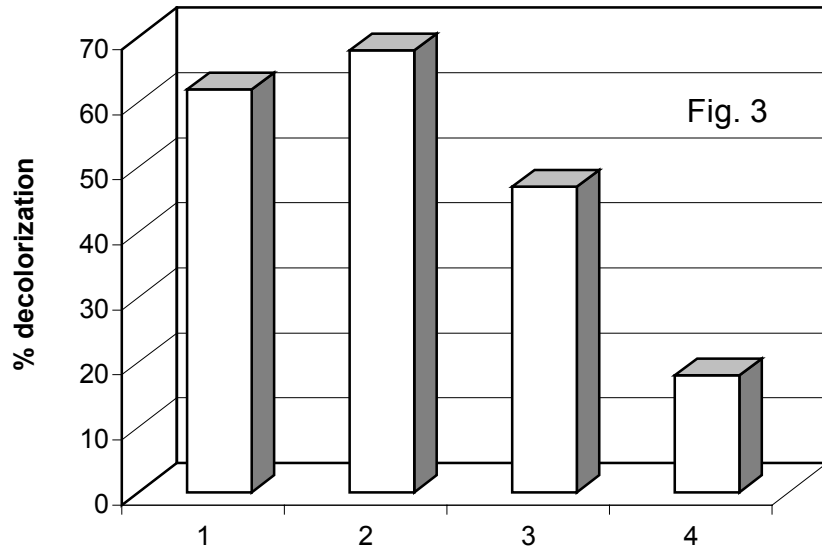


Fig. 2. Percentage decolorization (on day 5) of MSW used at different concentrations by the immobilized fungus.



**Decolorization by reusing the immobilized fungus in cycles**

Fig. 3. Successive decolorization of several batches of fresh MSW used at 10% concentration by reusing the same batch of the immobilized fungus. Color was measured on day 5 after each treatment.

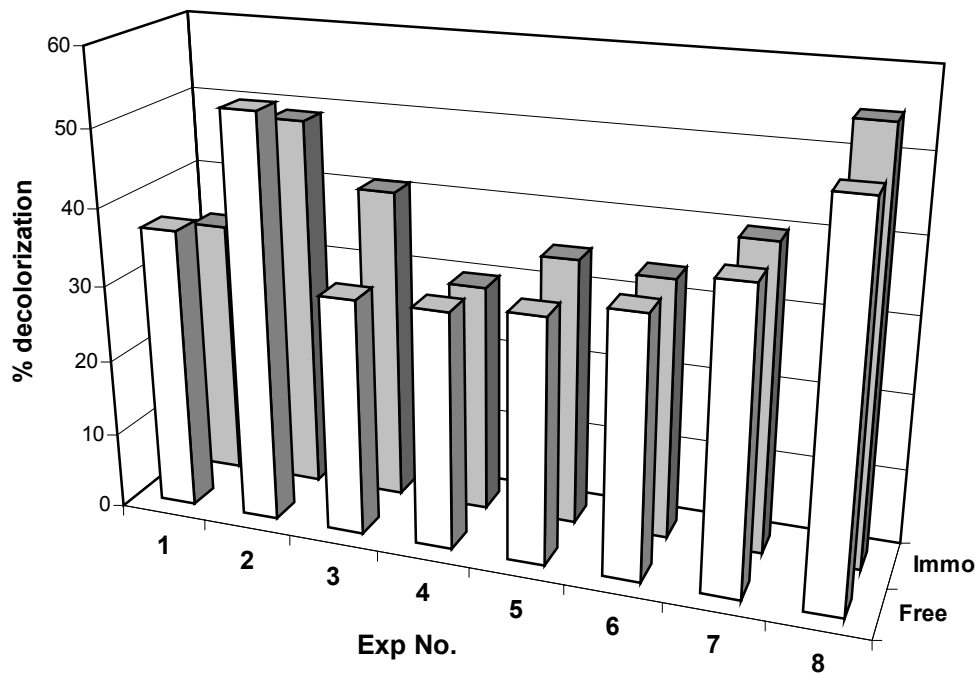


Fig. 4. A comparison of MSW decolorization achieved after 3 days by free versus immobilized fungus during several trials. MSW was used at 10% concentration for all the experiments.

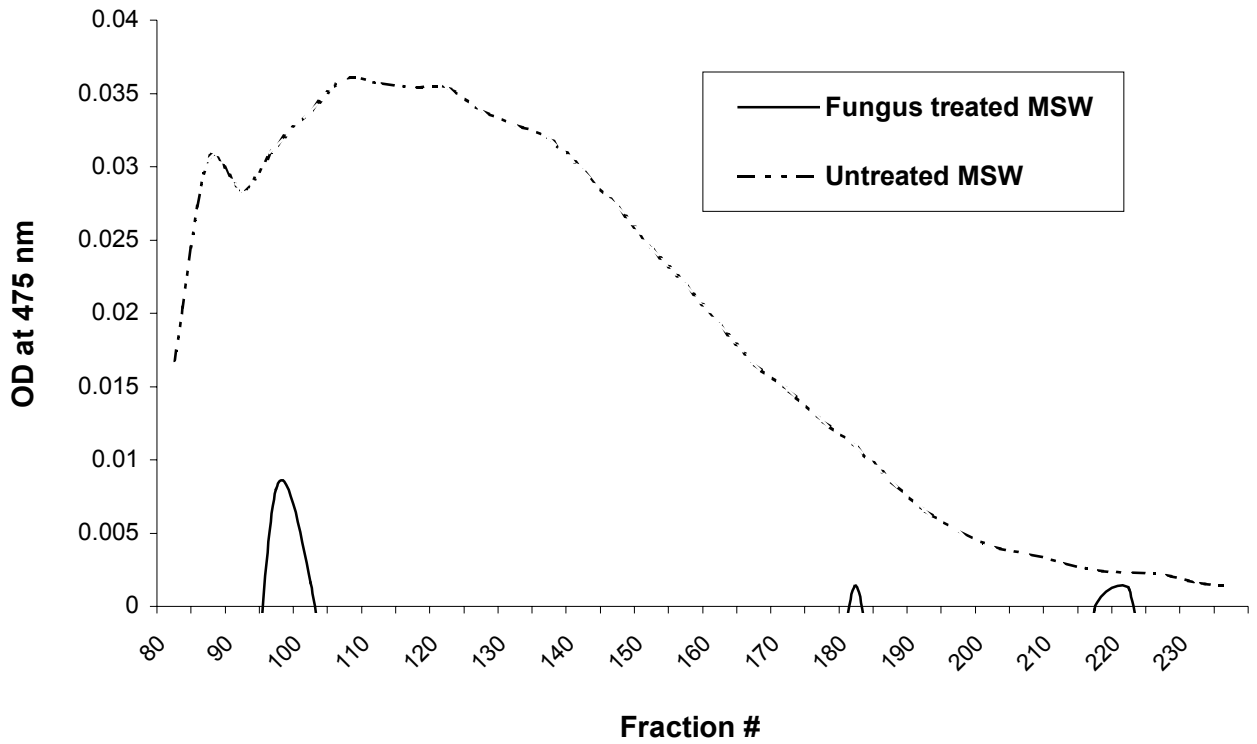


Fig. 5. Gel filtration chromatography of MSW on a Sephadex G-50 column. Solid line represent 10% diluted MSW decolorized by the live immobilized fungus and broken line represents fractionation of untreated, 10% diluted MSW. The color absorbance of the fractions was read at 475 nm. MSW treated with the fungus for 5 days was used for gel filtration.