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ORIGINAL PAPER

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Decolorization of molasses spent wash by the white-rot fungus *Flavodon flavus*, isolated from a marine habitat

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Abstract *Flavodon flavus* (Klotzsch) Ryvarden, a basidiomycete (NIOCC strain 312) isolated from decomposing leaves of a sea grass, decolorized pigments in molasses spent wash (MSW) by 80% after 8 days of incubation, when used at concentrations of 10% and 50%. Decolorizing activity was also present in media prepared with half-strength seawater (equivalent to 15 ppt salinity). Decolorizing activity was seen in low-nitrogen medium, nutrient-rich medium and in sugarcane bagasse medium. The percentage decolorization of MSW was highest when glucose or sucrose was used as the carbon source in the low-nitrogen medium. The production of lignin-modifying enzymes, manganese-dependent peroxidase (MNP) and laccase decreased in a medium containing MSW. MNP production and MSW decolorization were inversely correlated, suggesting no role for MNP in MSW decolorization. The decolorization of MSW was not effective when *F. flavus* was immobilized in calcium alginate beads. Decolorization was achieved best in oxygenated cultures. Besides color, total phenolics and chemical oxygen demand were reduced by 50% in MSW treated with *F. flavus*, suggesting its potential in the bioremediation of effluents.

Introduction

Distilleries in India use sugarcane molasses as the raw material for the production of ethanol. The effluents from such distilleries contain large amounts of molasses spent wash (MSW) which is highly colored due to the presence of melanoidin pigments (Wedzicha and Kaputo 1992). Melanoidin pigments are the product of the "Maillard reaction" between sugars and amino compounds produced on heating (Fitzgibbon et al. 1995). MSW pollutes aquatic ecosystems due to its intense brown color which cuts off light, prevents photosynthesis and causes anaerobic conditions. When MSW is disposed in soil, it acidifies the soil and thereby affects agricultural crops. 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); but it is still dark brown in color and is a major problem for the distilleries. Color removal in such effluents using terrestrial fungi has been reported (Sirianuntapiboon et al. 1988). The possibility of using obligate and facultative marine fungi which occur in coastal environments has not been explored for the bioremediation of saline soil and wastewaters. We report here decolorization of MSW by *Flavodon flavus*, a basidiomycete fungus recently isolated by us from decomposing leaves of a seagrass from a coral lagoon in India (Raghukumar et al. 1999).

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Materials and methods

Growth conditions

Several fungal isolates were obtained from various sources such as mangrove, seagrass and algal detritus, sediments and driftwood, as described earlier in detail (Raghukumar et al. 1994). Low-nitrogen (LN) medium supplemented with the synthetic polymeric dye Poly R-478 (Sigma Chemicals, St. Louis, Mo.) and streptomycin–penicillin solution was used for the initial isolation of fungi (Raghukumar et al. 1994). One of the fungi, isolate 312 obtained from decomposing seagrass leaves, showed strong Poly-R decolorizing activity at both acidic and alkaline pH, indicating a lignolytic enzyme activity (Raghukumar et al. 1994). The fungus was identified to be *F. flavus*, as described earlier (Raghukumar et al. 1999). Isolate 312, *F. flavus*, was deposited in the ARS Patent Collection, USDA, Ill., on June 19, 2000, under accession number NRRL 30302.

The effect of salinity on the growth of isolate 312 was determined by mycelial dry weight, both in malt extract (ME) broth containing ME (17 g) and mycological peptone (3 g) in 1 l of water and in LN medium containing 2.4 nM nitrogen in the form of ammonium tartrate. As per the experimental requirement, deionized water or half-strength artificial seawater (Instant Ocean; Aquarium Systems, Mentor, Ohio) was used for preparing the

above-mentioned media. The contents of three replicate flasks were vacuum-filtered through tarred Whatman GF/C filter papers, rinsed with 100 ml of deionized water, dried to a constant weight and the net mycelial weight was calculated (Raghukumar et al. 1999).

Decolorization of MSW

The raw MSW obtained from a local distillery unit, manufacturing alcohol from sugarcane molasses, was dark brown in color. According to the data provided by the unit, the MSW had a pH of 4.3, a BOD of 42,000 mg l^{-1} and a COD of 80,000 mg l^{-1}. As per the data provided by the distillery unit, the primary treated effluent had a BOD of 3,700 mg l^{-1}, a COD of 37,000 mg l^{-1} and a pH of 7.3. The secondary treatment reduced the BOD to 98 mg l^{-1} and COD to 2,800 mg l^{-1} with pH of 8.2. However, there was very little reduction in the color intensity of the effluent after these two treatments. Thus, the intense brown color of the effluent is a major problem in this distillery too, where sugarcane molasses is used as a raw material.

Decolorization of MSW was carried out in static cultures, using ME broth, LN medium prepared either with deionized water or with half-strength, naturally aged seawater and a medium containing powdered sugarcane bagasse (1% w/v) suspended either in deionized water or in half-strength seawater. MSW at final concentrations of 10% and 50% was added to 5-day-old cultures of *F. flavus* in the above mentioned media. The cultures were aseptically oxygenated on alternate days with 100% oxygen, using a sterilized glass Pasteur pipette attached to an oxygen cylinder via sterile tubing. Aerosystem Plus (Aerozyme International, USA), a bio-organic catalyst which is known to increase dissolved oxygen content in liquid cultures, was added at final concentrations of 10 ppm and 20 ppm and its effect on decolorization of MSW (without any additional oxygenation) by *F. flavus* was observed. Heat-killed cultures and uninoculated media supplemented with the same concentrations of MSW served as controls. Aliquots (0.5–1.0 ml) of culture supernatants from experimental and heat-killed cultures were appropriately diluted and changes in the absorbance maxima at 475 nm (Sirianuntapiboon et al. 1988) were measured using a Shimadzu UV-1201 V spectrophotometer (Shimadzu, Japan). The results were calculated as the difference in decolorization between readings on day 0 and those on the day of measurement and were expressed as percentage decolorization.

Effect of MSW on the production of lignin-modifying enzymes

The lignin-modifying enzymes, manganese-dependent peroxidase (MNP), lignin peroxidase (LIP) and laccase were assayed in the extracellular culture fluid of isolate 312 grown in LN medium with and without the addition of MSW at a concentration of 10%. MNP activity was determined by monitoring the oxidation of Mn^{2+} to Mn^{3+} (Paszczynski et al. 1988). LIP activity was determined by measuring the rate of H_2O_2-dependent oxidation of veratryl alcohol to veratraldehyde (Tien and Kirk 1988). In each case, activity was expressed in units of enzyme per liter (U l^{-1}). One unit of enzyme was defined as 1 µmol of Mn(II) oxidized min^{-1} and 1 µmol of veratryl alcohol oxidized min^{-1} for MNP and LIP respectively. Laccase activity was determined using the substrate 2,2′-azino-bis(3-ethylbenzthiazoline)-6-sulfonic acid in glycine-HCl buffer at pH 3.0 (Niku-Paavola et al. 1988). The enzyme units were expressed as nanokatals l^{-1}; and 1 katal was defined as 1 mol of product formed s^{-1}.

Analysis of total phenolics in the raw MSW

Total phenols were extracted from 10% diluted MSW before and after incubation with *F. flavus*, using a 1:1 mixture of ethyl acetate and acetone (Fitzgibbon et al. 1995). The extract was concentrated in a rotary evaporator at 35 °C (Fitzgibbon et al. 1995). Total phenolics were estimated using the Folin-Dennis reagent (Swain and Hillis 1959). Catechol (Sigma Chemicals, St. Louis, Mo.) was used as a reference standard.

COD was monitored by standard oxidation, followed by titration (APHA 1975). Dissolved oxygen in cultures was estimated using a modified Winkler's method (Parsons et al. 1984).

Results

F. flavus grew in ME and LN media prepared with half-strength artificial seawater and yielded 66 mg and 44 mg mycelial dry weight per 10 ml of medium, respectively. The corresponding growth yields in media prepared with distilled water were 52 mg and 23 mg mycelial dry weight per 10 ml of media, indicating the salt tolerance of this organism.

F. flavus removed up to 50% of the color from raw MSW (used at 10% concentration) within 2 days in LN media prepared either with distilled water or with half-strength seawater, in ME broth and in sugarcane bagasse medium (Figs. 1, 2). Further, about 70–80% of the color was removed by day 8 in these media. When the concentration of MSW was increased to 50%, about 40–50% decolorization was achieved within 2 days (Fig. 3). After

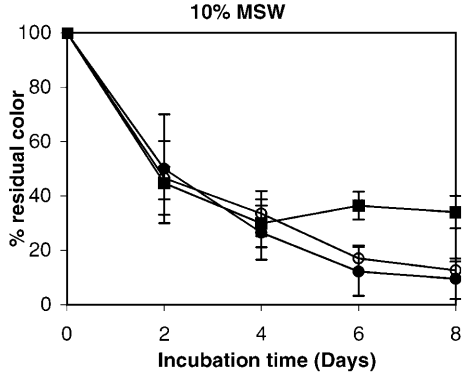

Fig. 1 Decolorization of molasses spent wash (MSW) used at a concentration of 10%, in malt-extract medium (■), low-nitrogen medium (●) and low-nitrogen medium with seawater (○)

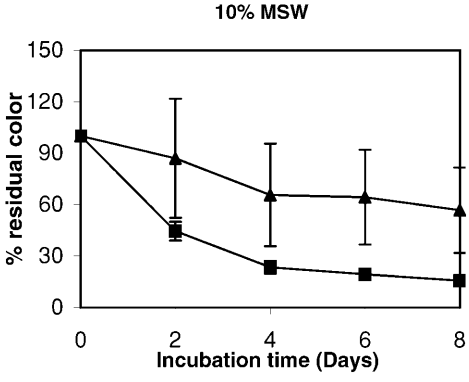

Fig. 2 Decolorization of MSW used at a concentration of 10%, in sugarcane bagasse medium in distilled water (■) and sugar cane bagasse medium prepared with seawater (▲)

Table 1 Decolorization of different stages of molasses distillery waste by *Flavodon flavus* in low-nitrogen (LN) medium. *Raw MSW* Untreated molasses spent wash, *primary treated MSW* comes after anaerobic sludge digestion, *secondary treated MSW* comes after clarification of the primary effluent. The different concentrations of MSW were obtained by dilution with LN medium. Percentage decolorization refers to the decrease in color (as measured at 475 nm) obtained after 4 days of incubation of MSW with *F. flavus*

Effluent	Concentration used (%)	Decolorization (%)
Raw MSW (pH 4.3)	10	70
Raw MSW (pH 4.3)	50	60
Raw MSW without isolate 312 and oxygenated	10	0
Primary treated MSW (pH 7.3)	50	39
Secondary treated MSW (pH 8.2)	50	56
Secondary treated MSW (pH 8.2)	100	9

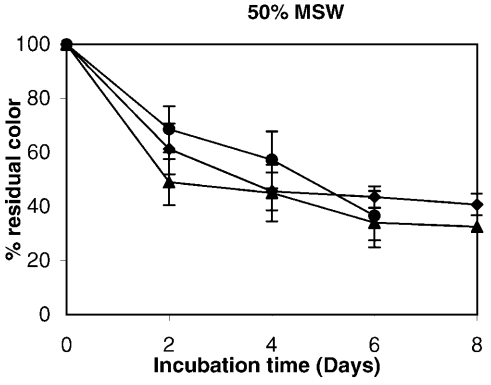

Fig. 3 Decolorization of MSW used at a concentration of 50%, in low-nitrogen medium (▲), low-nitrogen medium with seawater (●) and with a double quantity of fungal biomass (◆). This was achieved by pooling the fungal mycelium from two flasks and adding raw MSW to the culture

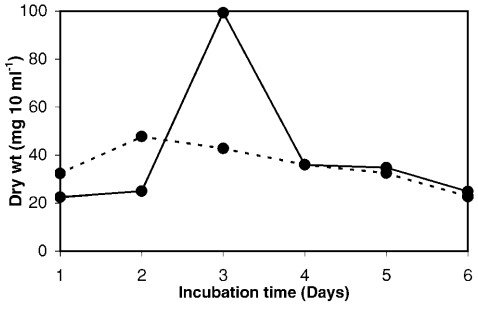

Fig. 4 Fungal dry weight in a 10-ml aliquot of medium. *Solid line* without MSW, *broken line* with MSW

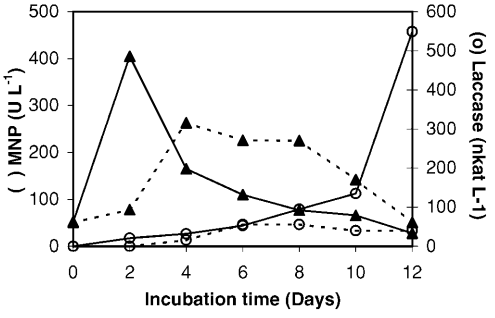

Fig. 5 Production of manganese-dependent peroxidase (*MNP*, ▲) and laccase (○) in low-nitrogen medium without MSW (*solid line*) and with MSW (*broken line*)

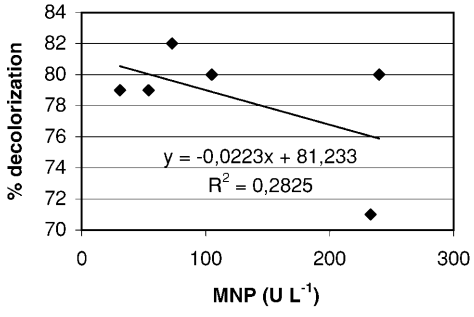

Fig. 6 Negative correlation between MNP production and decolorization of MSW.

doubling the fungal biomass used for decolorization, no further increase in decolorization was observed (Fig. 3). About 40–50% color removal of the primary and secondary treated MSW was observed by day 4 (Table 1), but no further decrease in the color of these effluents was noticed upon longer incubation. Oxygenation of 10% diluted MSW without the fungal culture did not show any decolorization up to 10 days (Table 1).

A two- to three-fold decrease in mycelial dry weight and production of MNP and laccase was observed in the presence of raw MSW (Figs. 4, 5). A negative correlation was observed between percentage decolorization and MNP production in cultures supplemented with MSW (Fig. 6).

Total phenolics and COD were reduced by 50% in the MSW decolorized by the fungal isolate. Decolorization of MSW occurred best when the cultures were flushed with oxygen (Table 2). Aeration using compressed air did not show any improvement over the control treatment, which did not receive any oxygen. Results with Aerosystem Plus (at concentrations of 10 ppm and 20 ppm) were also not comparable with that for pure oxygen. The dissolved oxygen concentration in the medium was obviously highest in oxygenated cultures (Table 2).

Decolorization of MSW was best when glucose or sucrose was used as carbon source in the LN medium. Fun-

Table 2 Effect of different treatments on decolorization of raw MSW by *F. flavus*

Treatment	Decolorization on day 10 (%)	Dissolved oxygen (mg l^{-1})
Heat-killed mycelia[a]	0	–
Living culture without extra oxygenation or aeration	43	1.3
Cultures with oxygenation[b]	79	5.9
Cultures with aeration[c]	44	1.9
Cultures with Aerosystem Plus[d](10 ppm)	41	3.5
Cultures with Aerosystem Plus(20 ppm)	42	4.0

[a] Fungal mycelium was autoclaved for 15 min
[b] Cultures were flushed with 100% oxygen aseptically after inoculation and every other day thereafter
[c] Cultures were flushed with air aseptically after inoculation and every other day thereafter
[d] Commercially available biocatalyst Aerosystem Plus(Aerozyme International, USA), which is recommended for increasing dissolved oxygen in wastewater, was added at the time of inoculation and the cultures were neither aerated nor oxygenated further

Table 3 Decolorization of raw MSW in the presence of various carbon sources, used at 10% concentration in LN medium. Decolorization of raw MSW was measured after 6 days of incubation in shallow stationery cultures. – No data

Carbon source	Decolorization (%)	Standard deviation (*n*=3)	Fungal dry weight	Standard deviation (*n*=3)
Sucrose	64	3.1	239.3	35.9
Glucose	63	5.5	149.0	25.0
Mannose	56	1.0	–	–
Mannitol	55	11.5	154.3	20.3
Xylose	52	5.5	28.7	2.9
Sorbose	48	1.9	24.7	10.0
Arabinose	47	5.9	67.3	12.9
Fructose	43	6.3	102.0	15.7
Glycerol	43	2.4	36.7	12.6

gal biomass and decolorization of MSW in the presence of various sugars were however not correlated (Table 3).

Discussion

Although *F. flavus* is not an obligate marine basidiomycetous fungus, it was isolated from a large number of samples of seagrass detritus from a coral lagoon (Raghukumar et al. 1994). It showed better growth in the presence of sea salts and thus appears to be well adapted to the marine environment. This is the first report of decolorization of MSW achieved in the presence of sea salts by a basidiomycetous fungus isolated from coastal marine environments. This was not unexpected, because we observed good growth and moderate production of lignin-modifying enzymes by this fungus in the presence of seawater (Raghukumar et al. 1999). Decolorization of MSW was observed in a nutrient-rich ME broth, in LN medium and in sugarcane bagasse medium. Thus, decolorization of MSW under such a wide range of nutrient conditions gives an added advantage for a biotechnological application. Also, sugarcane bagasse appears to be a cheap alternative source for growing this fungus on a large scale.

About 85% color removal after 10 days was reported in *Phanerochaete chrysosporium* grown in a medium containing 6.25% MSW (Fahy et al. 1997). Using another white-rot fungus, *Coriolus versicolor*, only about 4% decolorization of MSW was achieved (Fitzgibbon et al. 1995). In contrast, strain Ps4a of *C. versicolor* (*Trametes versicolor*) yielded about 79% color removal of molasses pigment by day 4 (Aoshima et al. 1985). About 70% decolorization of dialyzed melanoidin preparation by day 4 was achieved at 45 °C by the thermophilic fungus *Aspergillus fumigatus* (Ohmomo et al. 1987). Our results showed about 50% color removal by day 2, whether the concentration of MSW used in the culture medium was 10% or 50%. This is the first report of a high degree of decolorization achieved within a short time, using a fungus isolated from a marine habitat. However, comparing the decolorization activity of different fungi is not appropriate, as the conditions employed by various workers are not the same.

The MSW decolorizing activity was maximum by day 4, when the mycelial growth also reached its peak. However, after doubling the fungal biomass no further increase in decolorization was noticed, indicating that fungal biomass alone was not the key factor in colorremoval. This was further confirmed by the fact that decolorization of raw MSW in the presence of various carbon sources was not correlated with the growth of *F. flavus*.

The addition of MSW decreased the production of MNP and laccase by *F. flavus*. However, the addition of Kraft paper mill bleach effluent increased the production of MNP and laccase in this culture (Raghukumar 2000). Also, a positive correlation was obtained between MNP production and decolorization of synthetic dyes by *F. flavus* (Raghukumar 2000), whereas in the present study a negative correlation was observed between MNP production and MSW decolorization by the same isolate. The lignin-modifying enzyme MNP of *F. flavus* does not

appear to be involved in the decolorization of MSW, in contrast to that of *T. versicolor*, where an extracellular MNP was reported to be involved in the decolorization of melanoidins (Dehorter and Blondeau 1993). Melanoidin-decolorizing activity in the white-rot fungus *C. versicolor* Ps4a was attributed to two intracellular enzymes, namely a sugar-dependent and a sugar-independent enzyme, which were induced by the molasses pigment in this fungus (Aoshima et al. 1985). The enzyme system responsible for the decolorization of MSW in *F. flavus* needs to be studied in detail.

Besides removing color from molasses pigment, this fungus brought down levels of COD and total phenolics in raw MSW by 50%. Almost similar results were obtained with *A. fumigatus* (Ohmomo et al. 1987) and *Geotrichum candidum* (Fitzgibbon et al. 1995). Our culture was much more effective in decolorizing raw MSW than was the molasses wastewater collected either after anaerobic treatment or after aerobic treatment (Raghukumar 1999). This may be due to changes in the chemical structure of melanoidin pigments during anaerobic and aerobic treatment. It might be easier to implement a biological decolorization process on a large scale with *F. flavus*, because the fungus is effective both in decolorizing raw MSW and in reducing COD and phenolics, provided the high oxygen demand of the fungus is met and the treatment is carried out in shallow ponds or reactors. The color removal of raw MSW using this fungus could be integrated into the effluent treatment system before it is subjected to primary and secondary treatment. Since treatment with the fungus reduces COD levels, a further reduction in COD could be brought about during primary and secondary treatment. Consequently, a combination of different treatment processes including a decolorization step could result in an effective bioremediation of the molasses wastewaters

In conclusion, it appears that *F. flavus* (NIOCC strain 312) has the potential for the bioremediation of raw MSW by virtue of its high decolorizing activity in both simple and complex media under both saline and non-saline conditions (Raghukumar 1999) and its effectiveness in reducing the COD and total phenolics.

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