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Decolorization and Bioremediation of Molasses Wastewater by White-Rot Fungi in a Semi-Solid-State Condition

S. KAHRAMAN[a], O. YEŞILADA[b]

[a]*Department of Science, Faculty of Education,* [b]*Department of Biology, Science and Art Faculty, Inonu University, 44069 Malatya, Turkey*

e-mail sskahraman@inonu.edu.tr, oyesilada@inonu.edu.tr

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ABSTRACT. Molasses wastewater (vinasse; the by-product of distillation of fermented sugar) was decolorized and its chemical oxygen demand (COD) was reduced in static cultivation using the fungi *Coriolus versicolor*, *Funalia trogii*, *Phanerochaete chrysosporium* and *Pleurotus pulmonarius* ('*Pleurotus sajor-caju*'). The effect of cotton stalk on decolorizing and COD removing capability of four fungi was determined. In the entire concentration range tested (10–30 %), wastewater was effectively decolorized by *C. versicolor* and *F. trogii*. Cotton stalk addition stimulated the decolorization activity of all fungi. The utilization of cotton stalk represents several advantages due to its function as an attachment place and as a source of nutrients; its use also reduces process costs.

The fermentation processes using molasses as carbon and energy sources (yeast, amino acid production *etc*.) generate wastewater (vinasse) with a high organic load. For each L of ethanol produced 10–15 L of vinasse are generated (Kumar *et al.* 1997). The environmental impact of vinasse is very high due to its organic matter content and dark color. Its color is due to brown polymer melanoidin (a recalcitrant polymer formed by the amino–carbonyl condensation reaction) (Cabrera *et al.* 1984; Kumar *et al*. 1997*a*,*b*) present. Color is a major pollutant when it is discharged into rivers and lakes and it leads to the reduction of sunlight penetration in natural water bodies that in turn decreases both photosynthetic activity and dissolved oxygen concentration (FitzGibbon *et al*. 1995; Kumar *et al*. 1997*a*,*b*; Gonzales Benito *et al*. 1999). This can therefore create anaerobic conditions that kill aerobic marine organisms. Its high chemical oxygen demand (COD) means that the discharge of vinasse into rivers and lakes may result in their eutrophication and then hypereutrophication. Treatment of vinasse and decrease of its color by conventional wastewater methods is difficult. New methods have to be developed with the application of specific microorganisms.

White-rot fungi can degrade lignin, various xenobiotics and industrial wastewaters (Vyas *et al*. 1994; Novotný *et al.* 1997; Kahraman and Yeşilada 2001; Sam and Yeşilada 2001; Baldrian and Gabriel 2002; Eichlerová *et al*. 2002; Rodríguez Couto *et al.* 2002; Verma and Madamwar 2002; Zilly *et al*. 2002). Studies performed on vinasse with white-rot fungi demonstrated their effectiveness in the decolorization of this wastewater (Ohmomo *et al*. 1985, 1988; FitzGibbon *et al*. 1995; Yeşilada and Fiskin 1995; Yeşilada *et al*. 1995; Pena Miranda *et al*. 1996; Fahy *et al*. 1997; Garcia Garcia *et al*. 1997; Gonzales Benito *et al*. 1997; Kumar *et al*. 1997*a*,*b*; Miyata *et al*. 1998; Nakajima-Kambe *et al*. 1999). We previously showed the possibility of stimulating the decolorization activity and COD reduction capacity of white-rot fungi during the cultivation in olive-oil mill wastewater by cotton stalks (Kahraman and Yeşilada 1999). To our knowledge, molasses wastewater decolorization and bioremediation by white-rot fungi in semi-solid state (SSS) has not been reported. SSS conditions are defined as growth of microorganisms on solid materials in the presence of small quantities of free water.

In most studies on microbial vinasse decolorization by microorganisms, additional sources of carbon (glucose, sucrose, *etc*.) and nitrogen (peptone) are needed. However, these substances are not cost-effective compared to cheaper sources, such as cotton stalks available in vast quantities in all cotton growing countries and forming the largest proportion of local agricultural waste. The aim of this work was to use the cotton stalks for improving the COD and color removal ability of white-rot fungi during the cultivation in vinasse.

MATERIALS AND METHODS

Microorganisms. The white rot fungi *Coriolus* (*Trametes*) *versicolor* ATCC 200801, *Funalia trogii* ATCC 200800, *Phanerochaete chrysosporium* ME446 and *Pleurotus pulmonarius* (FR.)QUÉL. ('*Pleurotus*

sajor-caju') (*Department of Biology, Hacettepe University*, Ankara, Turkey) were used. They were maintained at 4 °C after subculturing at 30 °C, every 2–3 weeks on Sabouraud dextrose agar (SDA).

Inoculum preparation. The fungi were cultured at 30 °C on slant SDA. After 1 week, conidial suspensions were prepared according to Yeşilada *et al.* (1998) and used to cultivate the inoculum. Ten mL of each suspension were transferred into a 250-mL flask with 100 mL Sabouraud dextrose broth (SDB) and 2 mL vinasse. After a 4-d incubation, the cultures were homogenized (*Kinetic Polytron* homogenizer) and used to inoculate fresh media.

Preparation of growth media and culture conditions. Filtered and sterilized (12 min, 120 °C) vinasse was used as growth medium. It was diluted according to individual experimental procedure. All cultures were grown in 250-mL flasks containing 30 mL media. One g of spent cotton stalks was added to these media as indicated. All of these media were inoculated with homogenized stock cultures (2 mL) and incubated statically (8 d, 30 °C).

Assays. Decolorization was determined as the relative decrease of absorbance A_{475} (Yeşilada and Fiskin 1995) and expressed as the percentage of the decrease in absorbance at 475 nm related to initial A_{475}. The determination of chemical oxygen demand (COD), total solid, volatile solid, ash, total nitrogen and elements was done according to *Standard Methods* (1979). Total and reducing sugars were estimated using the anthrone and 3,5-dinitrosalicylic acid methods, respectively (Miller 1959; Rosenberg 1980). The dry fungal mass was estimated by filtering the contents of each flask (30 mL) through pre-weighed *Whatman* no. 1 filter paper and drying it to constant mass at 50 °C. The results are the means of 3 replicates.

RESULTS AND DISCUSSION

Some characteristics of vinasse wastewater are shown in Table I. We used 4 white-rot fungi and a waste product – cotton stalks – as inducer. Tests of their decolorizing ability with distilled water containing 10, 20 and 30 % vinnase in the presence and absence of cotton stalks showed that most of the fungi were able to decolorize this wastewater even without adding cotton stalks. *C. versicolor* decolorized 48 % of 30 % diluted vinasse without the presence of additional source; decolorization activity was further increased by cotton stalk addition. The vinasse color was then reduced down to 71 % (depending on vinasse concentration). When cotton stalks were added, vinasse decolorization increased from 33 to 71 %; the highest decolorizing activity was obtained with *C. versicolor* with cotton stalks added to 10 % diluted vinasse. This fungus also showed high decolorization activity (63 %) in the case of cotton stalks added to 30 % diluted vinasse. The efficiency of color removal appeared to be dependent on the cotton stalk addition (Table II).

Table I. The chemical composition of vinasse

Quantity	Value	Quantity	Value
pH	6.51	Ash, mg/L	29.5
Color, A_{475}	17.4	Fe^{2+}, mg/L	20
COD, g/L	72	Mn^{2+}, mg/L	70
Total sugar, g/L	37.8	Zn^{2+}, mg/L	30
Reducing sugar, g/L	27.8	NO_3^-, mg/L	1720
Total solid, g/L	88.8	NH_4^+, mg/L	9
Suspended solid, g/L	8.82	Cl^-, mmol/L	71
Volatile solid, g/L	59.3	PO_4^{3-}, mg/L	86

Although all the fungi used in our study were able to reduce COD values of vinasse, addition of cotton stalks further stimulated COD reduction ability of most of the fungi. Cotton stalks can thus be considered as a cheap alternative to fuel the bioremediation. Vinasse concentration had also a positive effect on biomass production, at its higher concentration the biomass production remarkably increased which can be explained by higher concentration of both organic and inorganic compounds added (complete analysis of vinasse showed that it contains amino acids, some sugars *etc*.; Koutinas *et al.* 1991; Yeşilada and Fiskin 1995). On the other hand, cotton stalk addition did not significantly stimulate the growth of our fungi.

Vinasse decolorizing activity of white-rot fungi was reported in the presence of high amounts of additional carbon and nitrogen sources. Gonzales Benito *et al*. (1997) found that the best operational conditions for the biological treatment of the anaerobically/aerobically treated vinasse, with a COD value of 4 g/L, were obtained upon addition of 3 g/L sucrose and 1 g/L KH_2PO_4; *T. versicolor* could then remove 82 %

color and 77 % COD with agitation. For optimal decolorization of vinasse (65 %), with a COD value of 0.1 g/L (pretreated an anaerobic/aerobic process), by *C. versicolor* in shaken flasks needed the addition of 0.5 % glucose and 50 ppm peptone (Ohmono *et al*. 1985). *P. chrysosporium* decolorized 85 % of vinasse during cultivation in highly diluted molasses spent wash (6.5 %) supplemented with 25 g/L glucose (Fahy *et al*. 1997). Increasing the wastewater concentration in media more than 6.25 % resulted in a significant decrease in the decolorizing ability. Only 3 % color removal could be obtained during the cultivation in 25 % diluted wastewater supplemented with 25 g/L glucose. In another experiment, *Aspergillus niger* was used to treat a similar kind of wastewater and the maximum yields achieved were 69 % for color and 75 % for COD removal. However, the biodegradation with this fungus needed 10 g/L sucrose and addition of magnesium and ammonium (Pena Miranda *et al*. 1996).

Table II. The effect of cotton stalks (CS) on COD reduction and color removal from media with vinasse (10–30 %)

Fungus	CS[a]	COD removal, %			Color removal, %		
		10	20	30	10	20	30
C. versicolor	–	46 ± 7.2	54 ± 2.3	52 ± 10	33 ± 9.3	40 ± 10	48 ± 2.6
	+	53 ± 2.0	56 ± 2.5	49 ± 8.5	71 ± 3.0	62 ± 7.8	63 ± 6.4
F. trogii	–	41 ± 14	40 ± 9.4	36 ± 8.7	10 ± 2.6	35 ± 5.0	30 ± 13
	+	58 ± 2.1	55 ± 1.2	62 ± 6.5	29 ± 5.3	43 ± 4.6	57 ± 7.1
P. chrysosporium	–	54 ± 0.5	44 ± 5.5	39 ± 7.2	0	0	47 ± 8.5
	+	39 ± 7.6	50 ± 6.4	57 ± 2.0	30 ± 6.6	24 ± 5.0	37 ± 3.3
P. pulmonarius	–	31 ± 10	22 ± 11	30 ± 5.0	24 ± 3.0	6 ± 0.4	19 ± 4.5
	+	24 ± 3.5	42 ± 3.0	34 ± 2.1	35 ± 12	57 ± 10	43 ± 4.0

[a](–) – without CS, (+) – CS added.

Bacteria also needed additional sources for their decolorizing abilities. Ohmomo *et al*. (1988) showed that *Lactobacillus hilgardii* decolorized only 28 % of melanoidin solution containing 1 % glucose under optimum culture conditions. Similarly, two bacterial cultures decolorized 12.5 % diluted, anaerobically digested vinasse, containing 3 % glucose and 0.5 % peptone or yeast extract (Kumar *et al.* 1997*a*). Maximum decolorization of 37 and 33 %, and COD reduction of 41 and 39 % were achieved. A facultative anaerobic culture achieved 31 % decolorization and 57 % COD reduction from digested wastewater supplemented with 10 g/L glucose after 7 d (Kumar *et al*. 1997*b*). *Bacillus* sp. decolorized 36 % of molasses pigment solution containing 3 g/L peptone and 2 g/L yeast extract within 20 d. All the above inducers and carbon sources are expensive which excludes their practical application in large-scale biotechnological processes.

Our study shows that cotton stalks stimulate effectively the color and COD-removal ability of most of the fungi used in the absence of synthetic carbon and nitrogen sources. Their utilization represents several advantages – improvement of biodegradation by white-rot fungi, double function as an attachment place and as a source of nutrients, and reduction in process costs. Furthermore, this type of support provides the fungus a similar environment to its natural habitat and offers the possibility of re-using an agricultural waste.

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