Decolorization of Molasses Waste Water from an Alcoholic Fermentation Process with *Phanerochaete Chrysosporium* - Involvement of Ligninase

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ABSTRACT: *Waste water from a molasses alcoholic fermentation plant (MWW) was treated biologically with Phanerochaete chrysosporium. The ability of this white-rot fungus to degrade the dark colored pigments present in MWW and the consequent decrease the effluent's color was examined. The Optimum concentration of MWW for color removal was determined and set at the ratio of 1:10. The color changes during incubation of the diluted MWW at 37úC, show a smooth decreasing trend, without any fluctuation , the highest degree of the decolorization (77%) was obtained in less than five days' of incubation (98 hours) at 37úC. Observatios show that the fungus grows in a proper and uniform manner and an integrated cellular mass is formed at the end of the incubation time . The degradative ability of the fungus on the colored substances seems to follow the pattern of the proper growth of the cellular mass. The only ingredient added to the MWW was the basal salt solution (With some modification) which is required for P. chrysosporium growth . Additional carbon source in the form of glucose or sucrose was not required. No pH adjustment was necessary. Based on the results obtained from several studies conducted by others, the main bioagent in the ligninolytic behavior of P. chrysosporium is the enzymes which the expression of the activities lead to hydrogen peroxide production with the simultaneouse accumulation of veratryl alcohol in the culture medium. In this study addition of veratryl and hydrogen peroxide to the decolorization medium showed to have no inducible effect. Moreover, the ligninase activity was detected in the MWW culture medium at its highest level of decolorization (77%) was found to be 224 UL-I.*

KEY WORDS: *Molasses waste water, Phanerochaete chrysosporium, Ligninase, Decolorization*

INTRODUCTION

Molasses as the most important by- product of cane and beet sugar factories contains about 50% sugars which cannot be recovered commercially, This is despite the fact that it has high value mainly due to its use as a source of carbon for various fermentation industries [4,25,26]. The main disadvantage of the use of molasses as a raw material for fermentation is the presence of large amounts of colored substances (residues of the fermentation process that remain in the system even after recovery of the product), [14,16]. Molasses waste water (MWW) from the alcohol fermentation plants contains a large amount of organic matter having dark brown color, which is an indication of presence of melanoidin pigments, MP. These highly colored pigments are synthesized as a consequence of condensation reactions of sugars and amino compounds by the Maillard reaction during heating

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process [13,18]. Variety of organic matters including brown pigments in MWW have harmful environmental impacts with high chemical oxygen demand [4,6,24]. Some of the physical and chemical techniques that can be used to treat colored effluents include: coagulation and sedimentation, bleaching by chlorine, adsorption, ion exchange on appropriate synthetic adsorbents, etc..., all of which have high operating costs and have had limited applications [13,14]. Moreover, the dark color of MWW can be hardly removed by the usual biological treatments such as activated sludge process [13,23]. In face the color may even increase during this type of treatments because of occurrance of variety of repolymerization reactions [13,16]. Furthermore, tighter constraints on effluent discharges have forced industries or waste creators to seek new ways for waste disposal [4,12,14,16]. Indeed finding an effective and inexpensive biological treatment system could have a significant Positive impact on the intented industries.

Many investigations have been done or are under way in order to identify and evalute the ability of microbial systems to decolorize the colored industrial effluents. Filamentous fungi having the ability to decolorize molasses pigments have been screened [26]. Some Basidiomycetes strains mainly white rot fungi have been known to be among the most active microorganisms in decolorizing MWW [3,12]. The selected strains of these fungi are shown to degrade a wide variety of environmentally organic pollutants including a number of dark colored pigments that exist in industrial effluents [3,19]. *Phanerochaete chrysosporium* is a white rot fungus and capable of producing extracellular peroxidase enzymes which are involved in catalytic degradation of lignin [3]. The irregular and recalcitrant nature of lignin as an aromatic polymer and the structural similarity which exists between lignin and many environmental pollutant molecules have led researchers to postulate that ligninolytic system produced by Phanerochaete chrysosporium acts rather nonspecifically and are capable of oxidatively degrading many aromatic pollutants [1,3,8,10]. In this paper we report the use of the white rot fungus Phanerochaete chrysosporium to decolorize MWW. Ligninase activity was detected in the decolorized MWW media. Ligninase is used as a generic name for a group of isozymes which catalyze the oxidative depolymerization of lignin and the oxidation of methoxybenzene - containing lignin - like substrates [24,27]. Lignin peroxidase (LiP) is the major isozyme of ligninase [7,27]. The effect of some additional carbon source on the decolorizing ability of the fungus, along with the influence of veratryl alcohol (VA 3,4 - dimethylbenzyl alcohol) on the decolorization reaction were also evaluted. Veratryl alcohol as the natural secondary metabolite of the fungus has a special role in the catalytic cycle of the lignin degradative system (LDS) [3,5,9,20,22].

MATERIALS AND METHODS

Microorganism. cultivation, and preparation of the inculum

Phanerochaete chrysosporium (1557) was purchased from the DSM culture collection deutshe sammlung von microorganismen and Zelkulturen (DSMZ) and was maintained at low temprature 4°C, on 2% malt extract agar slants. Subcultures were routinely made every two months. Inoculum consisted of conidial suspensions and diluted in a way that its absorbancy at 600 - 650 nm equaled 0.5 in a 1cm path-length cuvette. The number of spores was determined using a thoma counting - cell under the optical microscope at cell × 1000 magnification (2.4×10^6 per ml). For preparation of conidial suspension, spores were taken from three week-old slants (incubation temperature 25°C) [9].

Decolorization of MWW

Molasses waste water was obtained from an alcohol fermentation factory which uses cane molasses as the substrate. The MWW was filtered through a Whatman #42 filter, diluted with distilled water and sterilized in autoclave at 8-9 psi for 30 minutes. Basal mineral salt solution (BS) contained the following agents per liter of distilled water [10,23,24]: basal medium: KH_2PO_4, 0.2 g; $CaCl_2$, 0.01 g; $MgSO_4.7H_2O$, 0.05 g; thiamine solution (1 ml of the thiamine solution with concentration of 1 mg/ml was used)1 ml; and mineral solution, 1 ml. Mineral solution contained followings (per liter) $MgSO_4$, 3g; $MnSO_4.H_2O$, 0.5g; NaCl, 1 g; $FeSO_4.7H_2O$, 100 mg; $CaCl_2$, 82 mg; $ZnSO_4$, 100 mg; $CuSO_4$, 10 mg ; H_3BO_3, 10 mg. The basal mineral salt solution was sterilized in

autoclave at 15 psi for 5 minutes. In order to prepare the MWW at the desired concentration, the sperately sterilized diluted MWW and the basal mineral salt solution were mixed and our preliminary works showed that the mixture should be diluted with distilled water in a way to provide a predetermined absorbance at 475 mm. For example for MWW at the level of 10%, the absorbance was set to be equal to 0.7. The spore suspension prepared as above was then added to the decolorizing medium (diluted MWW) at the ratio of 1:10. Experiments were conducted in 125 ml Erlenmeyer flasks, each containing 10 ml of the decolorization medium. The final pH of the medium was about pH 5. At the end of the decolorization period, the pH of the medium did not change and was about pH 4.5-5. The flasks where incubated without agitation at 37.5°C for five days. At the appropriate time interval a 1 ml sample was taken for analyses. When needed, veratryl alcohol was added to the decolorizing medium at the final level of 0.4 mM, after 2 days of the incubation. Hydrogen peroxide prepared at the concentration of 50 mM and when needed, it was added to the decolorizing medium at the ratio of 1:10. Either glucose (G) or sucrose (S) was used as the source of carbohydrate. The concentration of the sugar used was set at 1%.

Analytical determination

Mycelium dry weights were measured after filtering using a Whatman #1 filter paper, collecting, washing with distilled water and drying at 80°C overnight and then weighing [4,9]. Decolorizing activity of the fungus for MWW was assayed by measuring the decrease in color density spectrophotometrically (absorbance) at 475 nm against uninoculated MWW as the blank at the same wavelength [18,25,26]. The degree of color removal was then calculated. Total reducing sugars in the form of glucose, were determined by the 3,5. dinitrosalicylic acid (DNS) method as described by Miller [17]. Ligninase activity was measured spectrophotometrically according to the enzyme assay described by Tien and Kirk (λ=310nm) [27]. Ligninase catalyzes the oxidation of VA by H_2O_2 to veratralehyde. The reaction mixtures contain 2 mM VA, 0.4 mM H_2O_2, 50 mM tartaric acid, and enough ligninase to give an absorbance change of 0.2 / min [27]. In order to determine that whether the decolorization is due to the adsorption of the dark colored pigments of the MWW or not, the mycelium produced during the experiment was washed with distilled water and it was cultivated under the same conditions as that of the decolorization but with tap water and without any nutrients [16]. The adsorbed substances were desorbed into the water and the absorbance was measured.

RESULTS AND DISCUSSION

At first when *P. chrysosporium* was incubated with MWW as the sole source of carbon and energy for growth of the fungus, there was almost no growth on the medium and no decolorizing activity (DA)therefore, was expressed. For undiluted MWW this could be due to presence of high molecular aromatics, some with growth inhibitory action on the microbes such as polyphenolics and also nitrogen containing compounds especially ammonium nitrogen [13,16]. By diluting MWW solution with (distilled) water to the level of 10%, growth of *P. chrysosporium* became evident although the expression of DA was at very low level (color reduction at < 2%) (Fig. 1). Then effects of adding either glucose or sucrose to the diluted MWW solution, each one as a possible suitable carbon and energy source were investigatel. In many studies relating to the expression of the microbial DA toward dark colored pigments present in the waste waters, effects of variety of sugars and in the different forms have been tested [12,16,18,19,25]. Concentration of the sugar examined was also important for example using *Aspergillus niger* and when sucrose was added at the level of 10 g/l to molasses spent wash, a negative effect on the color reduction was obtain although by adding glucose at the concentration up to 2.5%, the color removal reported was higher than 50% [16]. The concentration of the sugar tested in our study, was set at the level of 1%. Improvement of the DA of *P. chrysosporium* was observed by the addition of either glucose or sucrose to the diluted MWW (Fig.1). It has been shown that for *P. chrysosporium* in order to mineralize the lignin fraction of wood and convert it to CO2, a growth substrate such as glucose or cellulose is required [9,10]. Moreover, as it was said before the filamentous white rot Basidiomycetes including *P. chrysosporium* synthesize and release ligninase enzymes into the culture medium. Lignin peroxidase

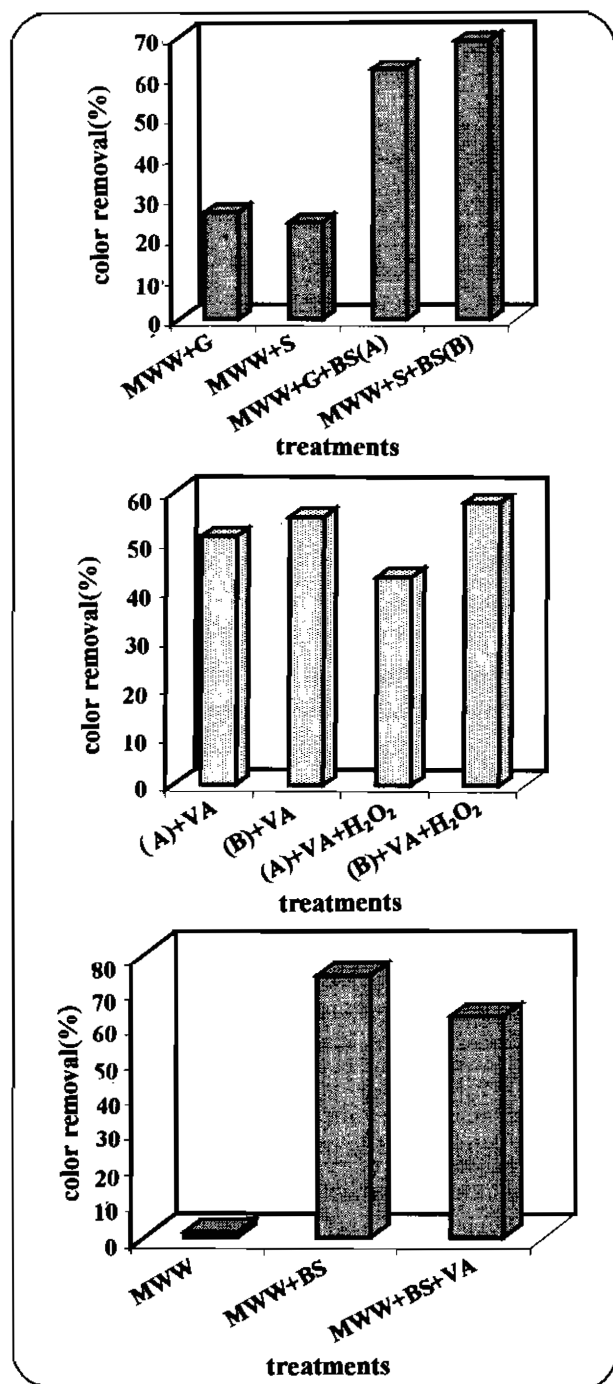

***Fig.1:** Effect of various treatments on the decolorization of MWW by Phanerochaete Chrysosporium.*

Abbreviations used in Fig.1 are: MWW: molasses waste water; G: glucose; S: sucrose; BS: basal salt solution; treatment A: MWW+G+BS; treatment B:MWW+S+BS; VA:veratryl alcohol; H_2O_2:Hydrogen peroxide.

and manganese dependent lignin peroxidase (MnP), are both part of lignin degradative system of the fungus [3,9,22]. Synthesis of the LDS occurs irrespective of the presence of lignin and that lignin alone does not serve as a growth substrate for these fungi [9]. The LDS of *P. chrysosporium* is shown also to be effective in degrading several persistent environmental pollutants such as pyrene, synthetic azo dyes [3,10,12,19]. Contributions of LDS to the decolorization of olive mill waste waters (OMW) by *P. chrysosporium* has been also demonstrated [25,26]. The common factor between these biodegradable compounds and lignin is their recalcirant nature. In our study when the basal salt solution was added to the MWW a significant color reduction by *P. chrysosporium* was obtained (77%) (Fig.1). It appears that by completion of the essential requirements for the fungus growth, the biodegradative behavior toward the colored pigments in the MWW was started. Addition of BS to the MWW in combination to either glucose or sucrose, showed 34-37 percent increase in color reduction over the medium which had only, added glucose or sucrose. While the extent of the color reduction was10-15% less than that obtained when only BS has been added to the MWW without any extra sugar source. It has been demonstrated that the relationship between carbohydrate and nitrogen content of the culture medium could be an important factor in expressing ligninolytic activity: i.e.mediums with the limited amount of carbohydrate and high nitrogen content result in increased levels of LiP production although higher levels of MnP is obtained in mediums with high carbohydrate level and low amount of nitrogen [3,25]. In our study and with the diluted MWW having only BS solution was added, it is very probable that a proper ratio for sugar and nitrogen content exists which could have been led to high level of expression of LDS in the form of DA. Fahy *et al.* reported for the first time that a microorganism (*P. chrysosporium*) had the ability to decolorize molasses spent wash without presence of any additional carbon source [4]. Our finding is in the good agreement with the results of Fahy *et al.* Although the level of the color reduction in our study is even higher (28%) than that reported by Fahy *et al* (77% versus 49%) [4]. Moreover, in. the decolorization by Trametes versicolor of MWW from an alcoholic fermen-

tation factory, good results obtained (82% color reduction). Although, adding surcose and phosphate as the nutrients, to the decolorizing medium were necessary [1].Besides the nutrient requirements for *Trametes versicolor* in the course of the decolorization, extensive attention toward *P.chrysosporium* is expressed because of its powerful enzyme system for degrading lignin [3].

In order to know more about the possible contribution of LDS of *P. chrysosporium* in decolorization of MWW, we also examined the influence of adding veratryl alcohol to the decolorizing medium. In several papers, authors have shown that synthesis of the LDS in *P. chrysosporium* cultures increased rather significatly when VA was supplied to the medium - VA which is produced by the fungus acts as a substrate for LiP [9,20]. It has been also demonstrated that VA addition to the culture medium enhanced production of LiP by protecting the enzyme in completing its role in the catalytic cycle against inactivation by hydrogen peroxide which is produced during the primary phase of the growth of the fungus, [3,20]. We also examined the effect of adding H_2O_2 to the MWW for possible role of H_2o_2 in enhancing the DA of *P.chyrsosporium*. Addition of VA to the decolorizing medium containing either glucose or sucrose along with the BS solution resulted to a rather comparable levels of the color reduction with that in the media having no VA added (see Fig. 1). Even when VA was added to the decolorizing medium with highest degree of color reduction, no improvement was observed and a decreasing trend was obtained (see Fig. 1). Therefore, the results obtained showed that VA had no inducible effect on the DA expression of the fungus and may interfere with the cellular production of VA. Although for some synthetic azo dyes as well as for dark colored pigments in OMW, addition of VA to the culture of *P. chrysosporium* increased degradation level of these compounds which led to the higher the percentage of color removal [20,23,24]. We also examined addition of H_2O_2 along with VA to the decolorizing media. The results showed no improvement in color reduction over that obtained with the media containing only added VA (see Fig. 1). As it has been reported the relationship between the concentration of VA produced by various strains of *P. chrysosporium*, and their DA on lignin fraction of wood is not clear [3,14,20]. For possible improvement in DA of the fungus, levels of the added H_2O_2 along with the levels of VA should be established both at two positions, intra - and extra - cellular level. The oxidation and biodegradation of the synthetic azo dyes was shown to be stimulated at high levels of VA along with low levels of H_2O_2 added to the medium [20].

The medium which gave best result in DA of the fungus on MWW, was chosen for further studies. Increasing the concentration of the MWW by 5%, step by step, resulted in a significant decrease in DA of this microorganism (Table 1). Incubation of P. chrysosporium with MWW at the level of 30% resulted to an Mincrease in intensity of the color of the medium (26% color increase) while *P. chrysosporium* showed to have the growth in a limited manner. This may be due, to not only presence of higher concentration of the microbial growth inhibitory substances in the MWW but also due to occurrence of a kind of enhancement process for condensation reaction between amino- and carbonyl - containing substances and formàtion of more melanoidin type pigments- these dark colored pigments is shown to have antimicrobial properties [13]. In fact in many studies when a readily available source of sugar (sucrose, for example) added to the natural substrate and its effects, on the color removal ability of the microorganisms tested, results showed that a typical pattern exists (increasing level of color reduction with rising the concentration of sugar added reaching to a maximum value then there would be decreasing trend with further increase in the sugar concentration). Although by using natural substrate alone, no such fluctuation in the color removal is seen in this trend [16].

The color reduction level for the medium which gave best result, was monitored as a function of time (Fig. 2). The results showed that color removal increased steadily

Table 1: Effect of the concentration of MWW on the decolorizing activity of P. chrysosporium

MWW [%]	Color removal [%]
10	77
15	59
20	64
25	3.5
30	-26

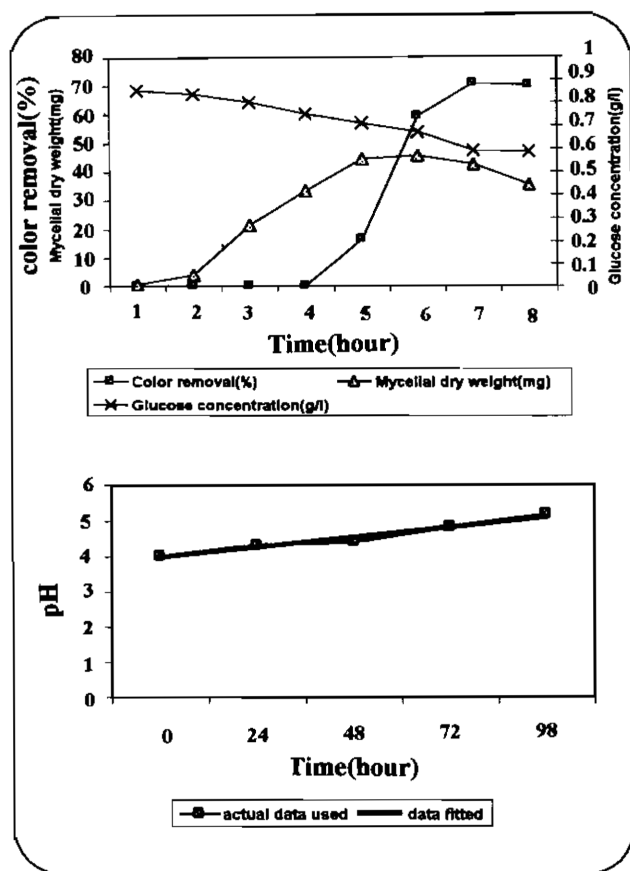

Fig.2: Decolorization of MWW by P. Chrysosporium and the growth pattern of the fungus. Trend of changes of the reducing sugar (glucose) concentration (a) and pH (b) are also, shown.

over time and reached to its highest amount after six days of the incubation at 37.5°C. Formation of the mycelial mass over the surface of the decolorizing medium was occurred in an uniform and smooth manner. There was no fluctuation in pattern of the growth and the trend of the decolorization. Therefore DA of *P. chrysosporium* appears to occur upon the growth on the MWW as the source of carbon and energy. A typical course of growth of thc fungus under best conditions of the decolorization of the MWW, is shown in Fig.2. The decolorization by the fungus was started after 56 hours of the incubation as the fungus entered into the stationary phase of the growth and the maximal color removal was reached at 4 days of the incubation (98 hours). After this point, the growth of mycelia starts to decline. In the present study, the preliminary tests showed that disturbing the mycelial math i.e. transferring the pregrown mycelial mat from the first state of the growth to the decolorizing medium led to significant loss of the DA. In fact variations in the results of the decolorization were reduced significantly when the mycelial pellet size was controled and by keeping its uniformity, therefore from the formation of the clumping mass should be prevented. With regards to the production of ligninase by *P. chrysosporium*, similar observations have been reported elsewhere [13].

The trend of changes in the total reducing sugar in the form of glucose, is also shown in Fig.2. Initial concentration of the reducing sugar of the undiluted MWW was 7.9 g/l and after proper dilution of the MWW (see table 1), the sugar level has changed to about 0.55 g/l during the decolorization process (see Fig. 2). The diluted MWW can be considered as a kind of the carbohydrate- limited medium for the expression of DA of the fungus as for well as ligninase production [7,27].

The results obtained from the determination of the mycelium color adsorption for the treatment with highest level of the color removal, showed that the color mainly has been eliminated by the biological action of the fungus. The adsorption of the color to the mycelium had little contribution in the total color removal (<2%). The adsorption pattern of melanoidin to the mycelia of Aspergillus oryzae has been discussed in the work of Ohmomo *et al* [18]. The adsorption of the color of MWW on the mycelium of Aspergillus niger was also, determined and its contribution toward the total color removal was about 17% [16].

It is well known that the fungal decolorization of the synthetic dyes correlates with the onset of secondary metabolism and ligninolytic activity [11,23]. Moreover, in the course of study on the production of ligninase by *P. chrysosporium* it has been shown that the most effective decolorizer strain (DA was expressed on the bleaching the specific type of effluent from the paper manufacturing plant) was also more better strain in production of higher levels of ligninase [11].

In the present study ligninase activity was measured in the MWW medium which gave best result for the color removal. The activity at level of 224 UL^{-1} was obtained which is rather similar to the level(s) of ligninase activi-

ty obtained through the works reported in several other papers [11,21,27].The microbial culture conditions used in those papers are very much restricted and were defined to provide good media for effective production of ligninase(s) [11]. Stability of ligninolytic enzyme is also of great concern regarding to its production - the enzyme activity loss during the production stage of the growth cycle of the fungus has been addressed clearly [2].

Expression of ligninase activity in order to catalyze the degradation of lignin as well as to degrade many environmental pollutants is best obtained at a constant pH (see Fig.2), with a starting value of pH 5 [5,14]. In almost all papers describing ligninase production, use of a suitable buffer system has been reported [10,11,23,25]. Although, there are cases where the MWW was shown to have good buffer capacity, specifically in the alkaline range of pHs but no microbe's growth was observed under the alkaline conditions [16]. The comprehensive research on the lignin biodegradation shows that the process is mainly oxidative and after decay of lignin, by both white - and brown - rot fungi, content of carbonyl and carboxyl groups increases - the degraded lignins contained a greater number of total (aromatic plus aliphatic) carboxyl groups compared to that of the control treatment [3]. Vanillic acid, isovanillic acid, veratric acid, and dehydrodivanilic acid are among most common products of the lignin degradation [3]. Moreover, it is shown that some ligninolytic enzymes of *P. chrysosporium* are also capable of catalyzing the decarboxylation of vanillic acid [15]. Therefore, for the ligninolytic culture in order to keep its pH at the constant level, variety of the reactions, both in acidic- or alkaline - direction, would occur. More works are needed to identify the occurrence of these types of reacions in those cultures which give good results in the decolorization of MWW.

It is concluded that the fungus (*P. chrysosporium*) used in this study was able to decolorize waste waters from a commerical alcoholic fermentation unit which, uses molasses as the substrate. High rate of the decolorization of the MWW was obtained without using any additional sugar source. In almost all works reported in this area use of an additional carbon source is considered to be the major shortcoming in making the process more economical. However, the results of this study along with relatively moderate level of the MWW used in the decolorization reaction could be considered as a realistic approach in treating this type of waste waters. Further work of course, is necessary to optimize conditions under which the decolorization along Production of enzyme (ligninase) occur. More importantly we report the production of ligninase in the MWW culture medium which showed to be the one with the highest level of the color removal. At present time LDS appears to have key role[s] in the process of decolorization of MWW more works in this area are needed to define these specific Type of actions of the enzymes.

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