ISSN Online: 2349-7785

Decolorization Capability of an Isolated Fungal Strain *Fusarium monoliformes* in Relation to Ligninolytic Enzyme Activity in Digested Distillery Waste Water

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Received 24 October 2014; accepted in revised form 19 November 2014

Abstract: In this study the production of three ligninolytic enzymes viz, laccase, Manganese Peroxidase (MnP) and Lignin Peroxidase (LiP) by *Fusarium moniliforme* and their subsequent use for decolorization of digested distillery waste water is reported. At optimum performance parameters the decolorization of digested distillery waste water by the isolated strain the high degree of decolorization were studied in relation to the excretion of ligninolytic enzymes i.e. Lignin Peroxidase (LiP), Manganese Peroxidase (MnP) and Laccase. Therefore, in the present study a positive co-relation was observed between LiP production and decolorization.. However, a negative co-relation exists between MnP production and decolorization of digested distillery waste water.

Key words: *Fusarium moniliforme.*

Introduction

In India, there are a number of large-scale distilleries integrated with sugar mills. The waste products from sugar mill comprise of bagasse, press mud and molasses. The biochemical oxygen demand (BOD) and chemical oxygen demand (COD) of molasses spent wash range between 35,000-50,000 and 100,000–150,000 mg L^{-1}, respectively [1]. The digested distillery wastewater (DDW) is a potential water pollutant, block out sunlight from entering the bottom layers of rivers and streams, thus reducing oxygenation of the water by blocking photosynthesis and becomes detrimental to aquatic life. Secondly, it has a high pollution load which leads to the eutrophication of water courses. Melanoidin is one of the biopolymers that is hardly decomposed by microorganisms and is widely distributed in nature. Melanoidins have antioxidant properties, which render them toxic to aquatic micro and macroorganisms. In recent years, several basidiomycetes and ascomycetes type fungi have been used in the decolourization of natural and synthetic melanoidins in connection with colour reduction of wastewaters from distilleries [2].

Aspergillus fumigatus, Aspergillus niger, Aspergillus niveus are some fungus which shows color and COD reduction of diluted distillery effluent at different concentrations [3,4,5]. Ascomycetes group of fungi also play an important role in the treatment of distillery waste water such as *Penicillium decumbens, Penicillium lignorum* showed reduction in color and COD and phenol reduction [6].

Pant and Adholeya [7] isolated fungal cultures which produced lignolytic enzymes and at the

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same time showed the decolorization capability of distillery waste water and further remove nitrogen by hydroponic treatment. White rot fungus is another group of fungus which also shows the decolorization of distillery waste water. They also produce some lignolytic enzymes such as laccase, manganese peroxidase and lignin peroxidase which help in the decolorization. Lignin is also responsible for color in distillery waste water, in addition to melanoidin pigments [8]. Therefore, the present work involves the role of ligninolytic enzymes in relation to effluent decolorization by the isolated fungal strain *Fusarium monoliformes* and expression of lignolytic enzyme activity in relation to fungal decolorization was also explored in the presence of carbon and nitrogen concentration.

Materials and method

Soil samples were collected from the various sites of the local distillery (Doon Valley Distillers: a cane molasses based distillery) of Dehradun. Sampling was done three times in a year during different seasons in order to have maximum diversity of microorganisms. Temperature of the sample was determined at the spot with the help of a thermometer. Spread plate technique and Streak plate technique on potato dextrose agar media supplemented with 10 % (v/v) digested waste water used for isolation of microbial culture[9]. The petriplates were incubated at 30°C for 5 days for the growth of the culture. Plates showing decolorization potential were subcultured repeatedly. The pure cultures obtained were maintained on potato dextrose agar (PDA) slants and sub cultured every fortnight. The fungal isolate showing the maximum capability was identified morphologically as *Fusarium monoliformes* and was chosen for further studies.

Measurement of decolorization potential

Decolorization activity of fungus for digested distillery wastewater assayed spectrophotometrically as a decrease in optical density of supernatant of treated medium at 475 nm against uninoculated wastewater medium maintained aseptically as blank and expressed as the percentage decrease in absorption. Experiments were performed in triplicate and samples (2 ml) were withdrawn at regular intervals for determination of decolorization. Decolorization was calculated according to the formula given by Itoh [10];

Decolorization (%) = [(initial absorbance-observed absorbance)/ initial absorbance] x dilution factor x 100.

Ligninolytic enzymes

Lignin peroxidase (LiP, EC 1.11.1.14)

Lignin peroxidase (LiP) activity was determined as per the method of Tien[11] by monitoring the oxidation of veratryl alcohol to veratrylaldehyde at 37°C as indicated by an increase in absorbance $A_{310.}$ One unit of enzyme activity is defined as the amount of enzyme oxidizing 1µmol of substrate per minute.

Manganese peroxidase (MnP, EC 1.11.1.13)

Manganese peroxidase (MnP) activity was measured with phenol red as the substrate at A_{610} by the method of Kuwahara [12]. Activity is expressed as increase in A_{610} per minute per milliliter. One unit of enzyme activity is defined as the amount of enzyme oxidizing 1µmol of substrate per minute.

Laccase (EC 1.10.3.2)

Laccase activity is determined by the oxidation of 2, 2'-azino-bis (3- ethyl thiazoline-6-sulfonate), i.e., ABTS at 37°C as per the method of Buswell and Odier [13]. Oxidation was followed via the increase in absorbance at 420nm. One unit of enzyme activity is defined as the amount of enzyme oxidizing 1mmol of ABTS per minute.

To further determine the role of the ligninolytic enzymes in decolorization capability of isolated strain. The optimum performance parameters were determined for the decolorization potential of fungal strain for the development of bioprocess for the decolorization of digested distillery wastewater (DDW). The optimum concentration of glucose and nitrogen supplementation of (0.8 %, w/v) and (0.03 %, w/v) was observed, respectively. Therefore, the effect of optimum concentration of glucose as carbon source (0.8 %, w/v concentration) and at 1.0 % (w/v) concentration as carbon source on decolorization was tested. The effect of NH_4NO_3 as nitrogen source

at optimal concentration (0.03 %, w/v) as at higher concentration of on decolorization and level of enzyme activity was tested [14].

Results and discussion

A number of fungi were isolated from both the digested distillery waste water as well as soil contaminated with distillery waste water. Isolated strains were tested for their decolorization ability. Based on the morphological characters and decolorization capability the microbial strain selected for the present study was identified as *Fusarium moniliforme* NG4 [14].

In the present decolorization studies by fungal isolate *Fusarium moniliforme* showed about 80 % color removal under optimal performance parameter (supplementation of glucose 0.8 % (w/v), NH_4NO_3 0.03 % (w/v), pH 5.0 and temp 30°C) of fungal decolorization by 6th day. This high degree of decolorization achieved by the isolated fungal strain *Fusarium moniliforme* NG4 were studied in relation to the excretion of ligninolytic enzymes i.e. Lignin Peroxidase (LiP), Manganese Peroxidase (MnP) and Laccase.

The results showed that fungal mycelial growth also reached its peak value within 4 days of culture incubation (Fig 1). The level of enzyme activities increases to maximum level on 6th day with maximum activity of lignin peroxidase at 125U/L and that of Mn-peroxidase at 65U/L. After 6th day the lignin peroxidase (LiP) activity started to decline and reached a low level of 45U/L by the 10th day of culture incubation while activity of manganese peroxidase (MnP) did not show any trend of decline. The activity of manganese peroxidase was in accordance and trend of decolorization, which did not change or show any further increase after the 6th day of culture incubation. It is observed that the activity of manganese peroxidase remain constant at 65 U/L. However, a little activity of laccase was observed after 3rd day of culture incubation (15U/L). The activity of laccase attained its peak value on day 6th (18U/L) and showed decline in activity to a very low value of 7-8U/L till the end of the decolorization experiment.

To further determine the role of the ligninolytic enzymes in decolorization capability of isolated strain. The effect of optimum concentration of glucose (0.8 %, w/v concentration) and at 1.0 % (w/v) concentration as carbon source on decolorization was tested. These concentrations were used in the present study, as the maximum decolorization potential was observed for the fungal isolate at these concentrations during the determination of optimum performance parameters for the bioprocess development for the decolorization of digested distillery waste

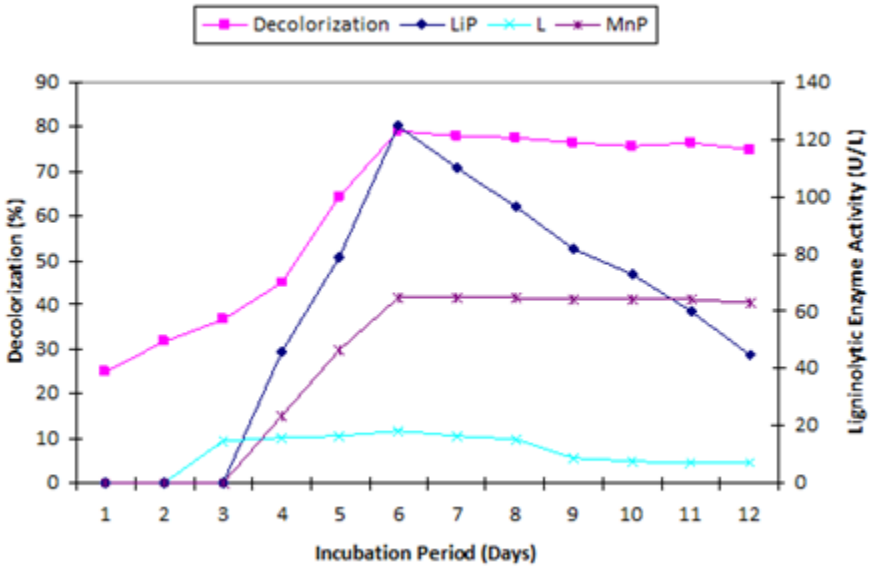

Figure 1. Ligninolytic enzyme activities of fungal strain *Fusarium moniliforme* NG_4 (LiP= Lignin Peroxidase, MnP= Manganese Peroxidase, L= Laccase)
Effect of glucose on decolorization inrelation to ligninolytic enzymes

water. From the results for the process development, it was observed that at (0.8 %, w/v) concentration of glucose supplementation the maximum decolorization capability (80 %) was observed. Further, at 1.0 % (w/v) glucose concentration the decolorization capability of the isolated fungal strain was a little bit lower than the (0.8 %, w/v) of glucose (Fig 2). Therefore, the concentration levels of the lignolytic enzyme activities were determined only at these two concentrations of glucose supplementation to the DDW.

As evident from the results, the enzyme lignin peroxidase (LiP) and manganese peroxidase (MnP) activities were observed after the third day of incubation (Fig 3 & 4). The maximal activity of lignin peroxidase was attained on the 6th day of incubation. A considerable change in degree of manganese peroxidase activity was observed similarly. On day 6th of incubation, the peak value of decolorization was observed. After 6th day, there was no substantial increase in decolorization potential of the fungal strain. Similarily, the enzyme activity of lignin peroxidase at 0.8 % (w/v) concentration of glucose shows the peak value of 125 U/L on 6th day which showed the continuous dip later. However, at the (1.0 %, w/v) concentration

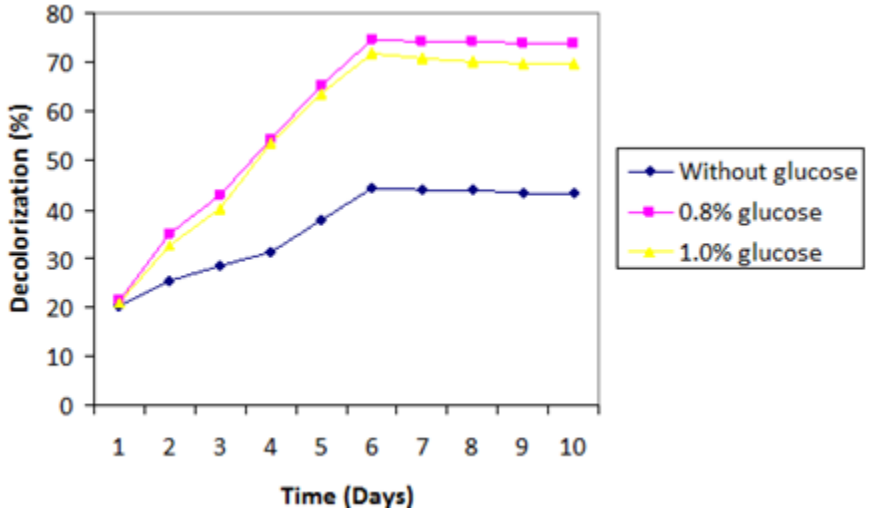

Figure 2. Decolorization ability of isolated fungal strain at different concentration of carbon source (glucose) with reference to period of incubation

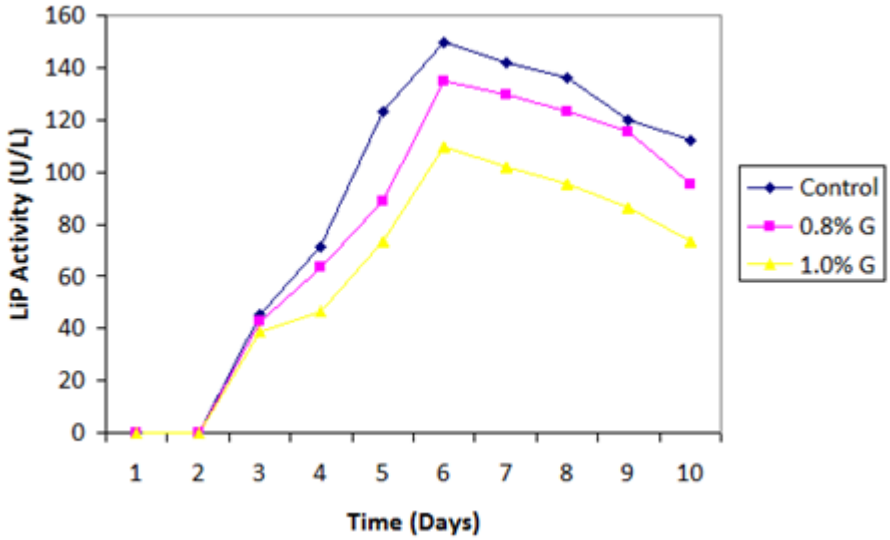

Figure 3. Effect of different concentration of carbon source (glucose) on lignin peroxidase enzyme activity by isolated fungal strain

of glucose supplementation the LiP activity shows low activity of 110 U/L which may be correlated to the decrease in decolorization capability of the strain (Fig 3). At the same time, the activity of manganese peroxidase appeared on 4th day of the incubation and showed an upward trend and it rose from 23.5 to 65 U/L on 6th day of incubation. The activity of MnP remained constant during the entire period of incubation. At higher concentration of glucose (1.0 % w/v) the MnP activity further enhance to almost 20% and attained the peak value of 75-78 U/L (Fig 4). The activity of enzyme shows a similar trend as shown at 0.8 % (w/v) of glucose during the entire period of incubation. The activity of laccase at both the concentration of glucose was negligible during the entire period of incubation. Therefore, correlation of laccase activity was not taken into the consideration for the decolorization potential of the strain.

Effect of nitrogen on decolorization in relation to ligninolytic enzymes

The effect of NH_4NO_3 as nitrogen source at optimal concentration 0.03 % (w/v) was used as discussed previously. Above this optimal concentration the effect on decolorization as well as level of enzyme activity was tested (Fig 5). Above this optimal nitrogen concentration, no

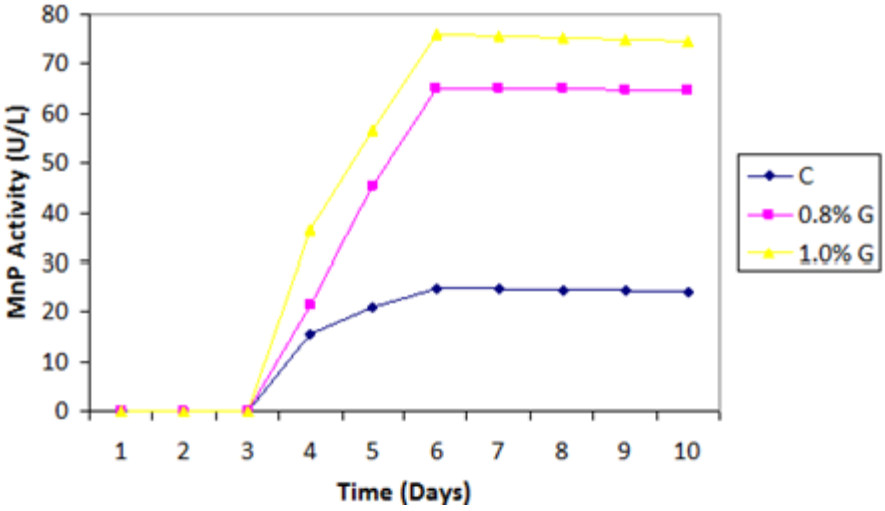

Figure 4. Effect of different concentration of carbon source (glucose) on manganese peroxidase enzyme activity by isolated fungal strain

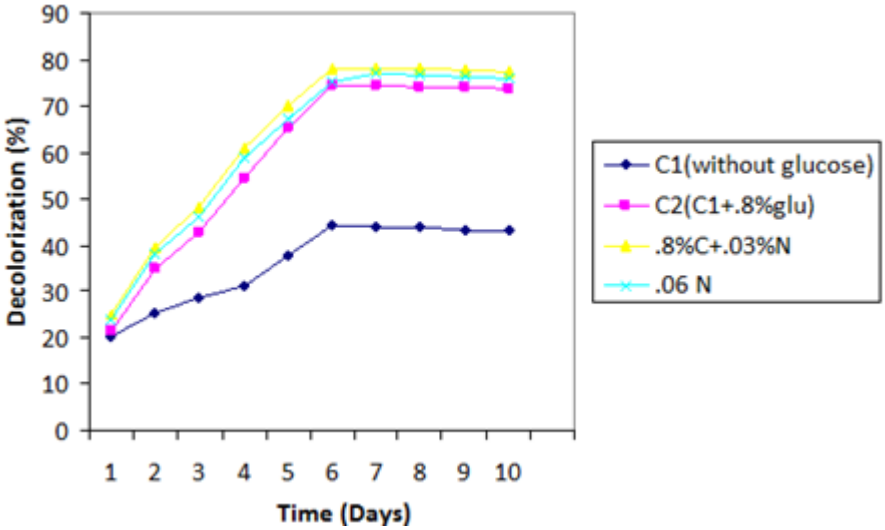

Figure 5. Decolorization ability of isolated fungal strain at different concentration of nitrogen source (Ammonium nitrate)

further increase in activity could be detected for LiP (Fig 6) and MnP (Fig 7). Lignin peroxidase and manganese peroxidase activities were maximally expressed at an optimal concentration of NH_4NO_3 at 0.03 % (w/v) and attained their peak value. Above this concentration, the activity of both the enzymes dropped remarkably co-relating the decrease in decolorization capability of fungal isolate above optimal NH_4NO_3 concentration (0.03 % w/v). Further the lack of additional nitrogen source NH_4NO_3 did not dramatically inhibit the growth of fungal strain but showed a decrease of 5-8 % color removal capability of the isolate.

Therefore, it may be presumed, that the expression of enzyme system in fungal isolate *Fusarium moniliforme* NG4 in the present study was synthesized under low carbohydrate and nitrogen condition as the supplementation of extra glucose to the minimal salt media was required for the growth of the fungal strain as there is nearly absence of readily available sugar in digested distillery waste water. The addition of the carbon and nitrogen help the fungal strain to grow and may further drift to the refractile carbon source of the digested distillery waste water. Further, the

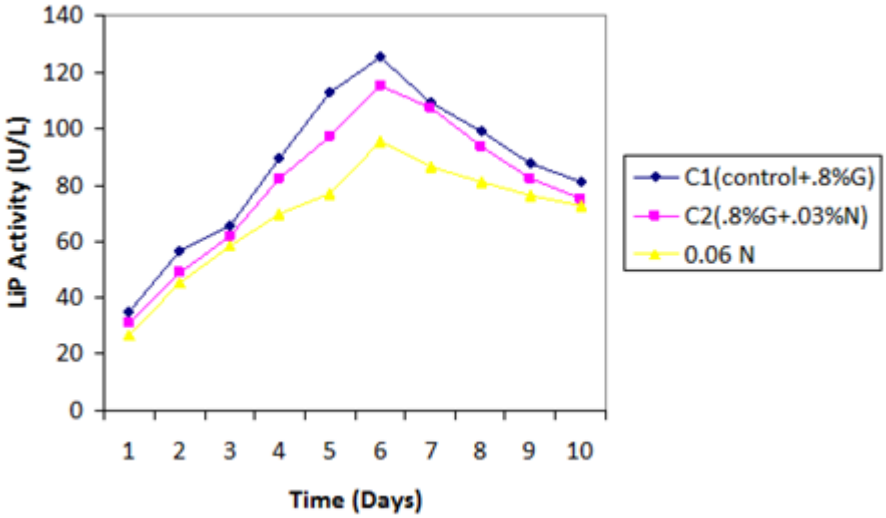

Figure 6. Effect of different concentration of nitrogen source (Ammonium nitrate) on lignin peroxidase enzyme activity by isolated fungal strain

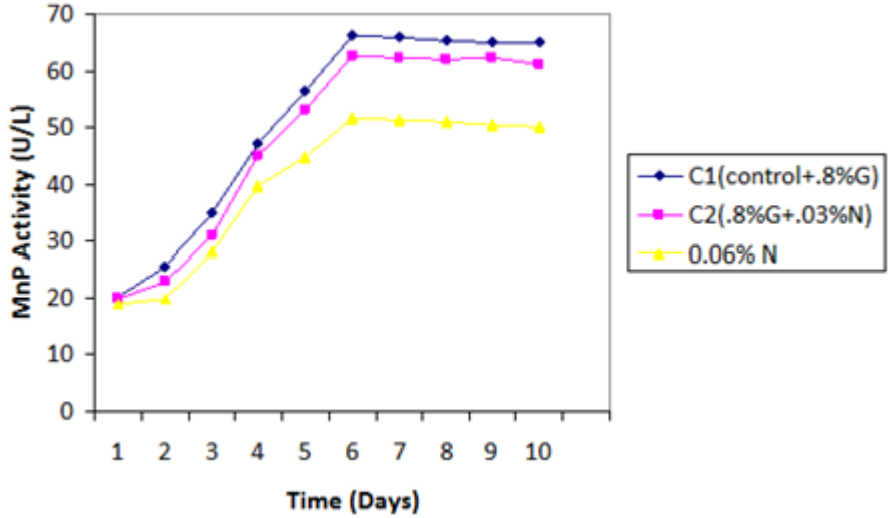

Figure 7. Effect of different concentration of nitrogen source (Ammonium nitrate) on manganese peroxidase enzyme activity by isolated fungal strain

extent and the rate of melanoidin degradation and decolorization were lower in the medium having high nitrogen concentration than that in the low nitrogen medium as already shown in previous experiments.

Traditionally it has been mainly the white rot fungi which are used for the decolorization of distillery waste water [15-18]. The correlation between decolorizing and ligninolytic abilities of white rot fungi has been commented upon by several workers [19,20,21]. Essentially the main color causing compound in distillery waste water is melanoidin, which has a chemical structure quite similar to humic acid, another recalcitrant compound found in soils and lignin [22]. The ligninolytic activity of white rots is thought to be responsible for degradation of melanoidins [23,24]. However other fungi, representatives of different taxonomic and ecophysiological groups, are able to degrade lignocellulosic substrates and produce lignolytic enzymes. The production of MnP activity was reported in *Alternaria alternate* with a possible role in humic acid degradation by Rezacova [25].

In the present study a positive co-relation was obtained between LiP and MnP production and decolorization of digested distillery waste water by an isolated fugal strain of *Fusarium moniliformes*. Similar positive correlation between LiP production and decolorization of waste water from a cane molasses based distillery was reported by the previous worker [23,24]. However a negative correlation was observed between MnP production and molasses waste water decolorization by an obligate marine basidiomycetes fungus, *Flavadon flavus* as reported by Raghukumar and Rivonkar [1]. Recently LiP production by *Penicillium decumbens* was reported byYang [26]. Although enzymatic system related with decolorization of melanoidins is yet to be completely understood, it seems greatly connected with fungal ligninolytic mechanisms. However D'souza [27] reported 100 % decolorization of 10 % (v/v) spent wash by a marine fungal isolate in which laccase production increased several folds in the presence of phenolic acid and non phenolic inducers.

In the present study, color removal ability of fungal isolate *Fusarium moniliforme* at different concentration of carbon and nitrogen and in relation to ligninolytic enzymes was also assessed. From the results obtained, it may be presumed, that the expression of enzyme system in fungal isolate was synthesized under low carbohydrate and nitrogen condition as the supplementation of extra glucose to the minimal salt media was required for the growth of the fungal strain as there is nearly absence of readily available sugar in digested distillery waste water, which may further drift to the refractile carbon source to the digested distillery waste water. Further, the extent and the rate of melanoidin degradation and decolorization were lower in the medium having high nitrogen concentration than that in the low nitrogen medium as already shown in previous experiments. This can be further co-related that in both the condition of low nitrogen and low carbohydrate, high level of manganese peroxidase activity, without lignin peroxidase action was not enough to give a high level of decolorization ability of isolate. Therefore, it seems that lignin peroxidase activity plays a prominent role in decolorization of digested distillery waste water at optimal performance parameter.

Conclusion

In this study the production of three ligninolytic enzymes viz, laccase, MnP and LiP by *Fusarium moniliforme* and their subsequent use for decolorization of digested distillery waste water is reported. This high degree of decolorization achieved using an isolated fungus were studied in relation to the excretion of ligninolytic enzymes i.e. Lignin Peroxidase (LiP), Manganese Peroxidase (MnP) and Laccase. Therefore, in the present study a positive co-relation was obtained between LiP production and decolorization. However, a negative co-relation exists between MnP production and decolorization of digested distillery waste water by an isolated fugal strain of *Fusarium moniliforme.* Of course more work is needed in direction of optimization of other process variable to make the process more effective at industrial scale.

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