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Optimization of different Parameters on Synthetic Dye Decolorization by Free and Immobilized *Mucor hiemalis* MV04 (KR078215)

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Abstract

Decolorization of different dyes by an indigenous strain of fungus was isolated from Eucalyptus tree .Thus based on 18S rRNA gene and ITS region homology, with the references strains, the isolates were identified as Mucor hiemalis MV04 (KR078215). From number of different synthetic dyes (RBBR, Malachite Green, and Congo red) Mucor hiemalis decolorize RBBR dye most efficiently. Maximum decolorization of 50 ppm RBBR dye was achieved temperature at 30°C, pH 5.0 and 130rpm shaking speed for both free and immobilized biomass. Complete decolorization was achieved in 8 days. It was also found from this study that immobilized biomass have higher decolorization capacity as compare to free fungal biomass.

Keywords: Dyes, *Mucor hiemalis* MV04 (KR078215), immobilization, decolorization.

Introduction

Environmental pollution has been recognized as one of the major problems in the modern world. The increasing demand for water and the dwindling supply has made the treatment and reuse of industrial effluents an attractive option. Textile industries consume a major share of dyes in India[1]. Rapid industrialization has necessitated the manufacture and use of different chemicals in day to day life. Synthetic dyes are widely used in the textile, paper, cosmetics, leather, dyeing, color photography, pharmaceutical and food industries because of the simplicity and cost-effectiveness of their synthesis, high stability to light, temperature, detergent and microbial attack[2]. Synthetic dyes with complex structures deriving from the use of different chromophoric groups can be extremely recalcitrant [3]. Synthetic dyes pose a threat to the environment. A significant proportion of these dyes enter to the environment via wastewater. Approximately 10,000 different dyes and pigments are used industrially and over 0.7 million tons of synthetic dyes are produced annually, worldwide[4].

However, the variation in the structure is enormous and many thousand different dyes are produced for commercial use. In general, dyes can be classified according to their chemical structure, particularly chromophore, and the method of application. Several of these dyes are very stable to light, temperature and microbial attack, making them recalcitrant compounds, many of them are also toxic. The chemical classes of dyes employed more frequently on industrial scale are the azo, anthraquinone, sulfur, indigoid, triphenylmethyl (trityl), and phthalocyanine derivatives. However, it has to be emphasized that the overwhelming majority of synthetic dyes currently used in the industry are azo derivatives[5]. Azo bonds present in these compounds are resistant to breakdown, with the potential for the persistence and accumulation in the environment[6]. Currently, a number of different technologies and methods such as biological, physical and chemical are widely used for the removal of dyes from wastewater. Synthetic dyes cannot be efficiently decolorized by traditional biological processes.

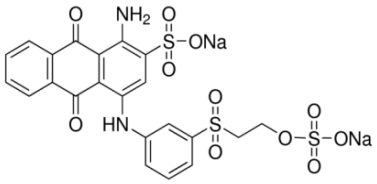
Thus, a number of biological and physicochemical methods have been developed for the efficient removal of dyes[7]. Methods like filtration, use of activated carbon and chemical flocculation are effective but quite expensive and show operational problems such as development of toxic intermediates and low removal efficiency[8].

In recent years a number of studies have focused on some microorganisms capable of degrading and absorbing dyes from wastewater. A wide variety of microorganisms are reported to be capable of decolorization of dyes. It has been reported that microorganisms are capable of degrading dyes and could be used in effluent treatment plants for removal of these dyes[9,10]. Microbial decolorization has been proposed as a less expensive and less environmentally intrusive alternative. Immobilization enhances stability and allows reuse of the enzymes including laccase[11,12]. Selection of immobilization conditions and immobilization matrix is essential to design a system appropriate to each particular purpose[13].

Therefore, the main objective of the present work is to compare different dye (RBBR, Malachite Green and Congo red) decolorization abilities of isolated fungal species *Mucor hiemalis* MV04 (KR078215) by free and immobilized biomass, and to investigate the effects of pH, temperature, agitation speed, carbon sources and nitrogen sources in synthetic dyes.

Material and Methods

Dyes and chemicals: Remazol Brillent Blue-R (RBBR), Melachite Green, Congo Red and (figure-1a, b, c) were obtained from Hi-media and Sigma Aldrich (India). All the chemicals and media such as Potato Dextrose Agar (PDA), Potato Dextrose broth (PDB) and Nutrient agar (NA) were procured from Himedia, Mumbai, India.



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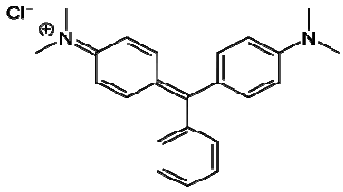
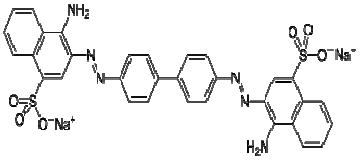


(b)



(c)

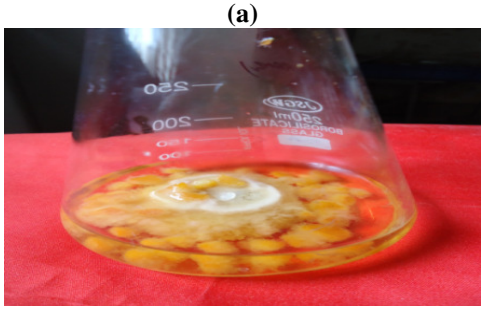
Figure-1
Chemical structures of Dyes (a) Remazol Brilliant Blue (RBBR) (b) Malachite Green (c) Congo Red

Dyes preparation: A synthetic wastewater was prepared by adding dyes (RBBR, Malachite Green and Congo red) (50 mg/L) and starch (2 g/L) two common components in real textile wastewater. The other components of the synthetic wastewater were as follows: 0.1 g/L urea, 2 g/L KH_2PO_4, 0.099 g/L $CaCl_2$, 1.025 g/L $MgSO_4.7H_2O$, 0.001 g/L thiamine and 1 mL/L trace elements. Stock trace elements solution was prepared by dissolving 0.125 g $CuSO_4.5H_2O$, 0.061 g $MnSO_4.5H_2O$, 0.043 g $ZnSO_4.7H_2O$, 0.082 g $Fe_2(SO_4)_3.14H_2O$ in 1 L of distilled water. The pH of this synthetic wastewater solution was 5.0[14].

Isolation and identification of fungal isolate: Fungi MV04 obtained from Eucalyptus tree trunk**.** The fruiting body was cut into small pieces. These chips were washed ten times with sterilize distilled water, dried with sterilize filter paper and isolation of fungal strains was done using potato dextrose agar (PDA) in petriplates incubated at 30±2°C for 48 h (figure-2a). The fungal growth which occurred on the plate was subculture and maintained in PDA slants. This medium was kept at 4°C for two days in an incubator and this was further utilized as inoculants in biodegradation studies.

Preparation of immobilized biomass: Decolourization was also studied with the immobilized fungus in a repeated batch process. Fungus was immobilized on orange peeling (figure-2b). The orange peeling was cut into 1×1 cm^2 pieces and washed with distilled water and sterilized at 60°C for 2 h. Immobilization of matrix was carried out in 250 ml Erlenmeyer flasks containing 100ml of culture media. Flask containing culture medium and dyes (50 mg/L) were sterilized. After sterilization, the flasks were inoculated with fungal mycelia disc and then incubated on a rotary shaker set at 130 rpm at 30^0C for 48 h. Immobilized cells were removed from the flasks and used for dye decolorization study. Immobilization occurred in situ by natural adsorption through the direct contact with the biomaterials. The orange peeling immobilized cells were removed from the flasks and mycelium was used repeatedly for decolourization study.

(a)



(b)

Figure-2
(a) Free and (b) Orange peeling immobilized *Mucor hiemalis* MV04 (KR078215)

Molecular characterization of fungal isolate: The fungi were identified based on molecular characterization and morphological structures as color, texture, mycelium and spore formation and attachment into the filaments[15]. Molecular characterization and identification of fungal isolate MV04 was done ITS region and 18S rRNA sequencing respectively. The 18S rRNA partial sequencing analysis was carried out in order to identify the fungal isolates and deposited in the GeneBank database. The sequenced data thus obtained were subjected to blast analysis using public database GeneBank at www,ncbi.nlm.nih.gov/BLAST suggesting the identity of isolates. The sequences were aligned using the CLUSTAL W program and analyzed with the MEGA 4.0 software[16]. The phylogenetic tree were constructed using the neighbor-joining (NJ) algorithm, with bootstrap values calculated from 1000

replicate runs, using the routines included in the MEGA software[17]. Thus based on 18S rRNA gene and ITS region homology (≥97%), with the references strains, the isolates were identified as *Mucor hiemalis* MV04 (KR078215) (figure-3). For microscopic characterization, the isolates (4 days old culture) were subjected to lactophenol cotton blue staining and studied for their shape, size of the mycelium and spores attachment to the mycelia under microscope at 40X[18].

Test of decolorization potential of *Mucor hiemalis* MV04 for different dyes: The decolourization studies were done in a 250 ml conical flask with 100 ml of potato dextrose broth (PDB) was prepared by following the MSM composition described by Khalid et al.[19]. All the dyes were added at different concentration of 50 to 200 mg/L and pH of the broth was adjusted to 5.0. The culture mediums containing the respective dyes were autoclaved at 121°C for 30 minutes. The 100 mL of broth in 250 mL conical flask were inoculated with the respective isolated strain. The media incubated at room temperature on an incubator shaker at 130rpm, the experiment was carried out in duplicates. Every 2 days absorbance was measured at 603 nm using a UV-Vis spectrophotometer to determine the extent of dye degradation. Optimum decolorization was observed after 8 day of incubation. After testing different dyes RBBR dye shows maximum decolorization by *Mucor hiemalis* MV04 (KR078215) as compare to Melachite Green and Congo red.

Decolorizing experiment: The efficacy of free and immobilized cell on the decolourization of the dyes was examined in culture medium containing actively grown fungal biomass (free and immobilized). The OD of each of the sample was taken at the respective absorption maxima of each of the dye at different time intervals[20]. The percentage of decolourization was calculated as per the following equation:

$$\text{Decolorization (\%)} = \frac{\text{(Initial Absorbance – Final Absorbance)}}{\text{Initial Absorbance}} \times 100$$

Parameter optimization: The decolorization process was optimized by studying the effect of different physical and nutritional factors on decolorization of RBBR, Melachite Green and Congo Red by *Mucor hiemalis* KR078215. Decolorization of dye (50 mgl^{-1}) in potato dextrose broth by *M. hiemalis* KR078215 isolate was optimized with respect to the effect of 1% carbon sources (dextrose, sucrose, maltose and fructose), 1%, nitrogen sources (Ammonium Nitrate, Urea, Sodium Nitrate, Yeast extract), pH (3-9) and temperature (20-50°C). All experiments were carried out with 1% (v/v) inoculums and PDB without culture was served as control. All the flasks were incubated at 30°C under shaking conditions (130 rpm) for 8days.

Results and Discussion

Screening of dye for decolorization by fungal strain: All of the 3 dyes were tested for decolorization by MV04 fungal isolate in enrichment culture medium PDB. Fungal isolate showed variable potential to decolorize RBBR, Melachite Green and Congo Red in PDB medium. All selected dyes were degraded by MV04 fungal strains but percent decolorization was different for all dyes. The growth of the fungi may be effected by the presence of dyes at toxic concentrations.

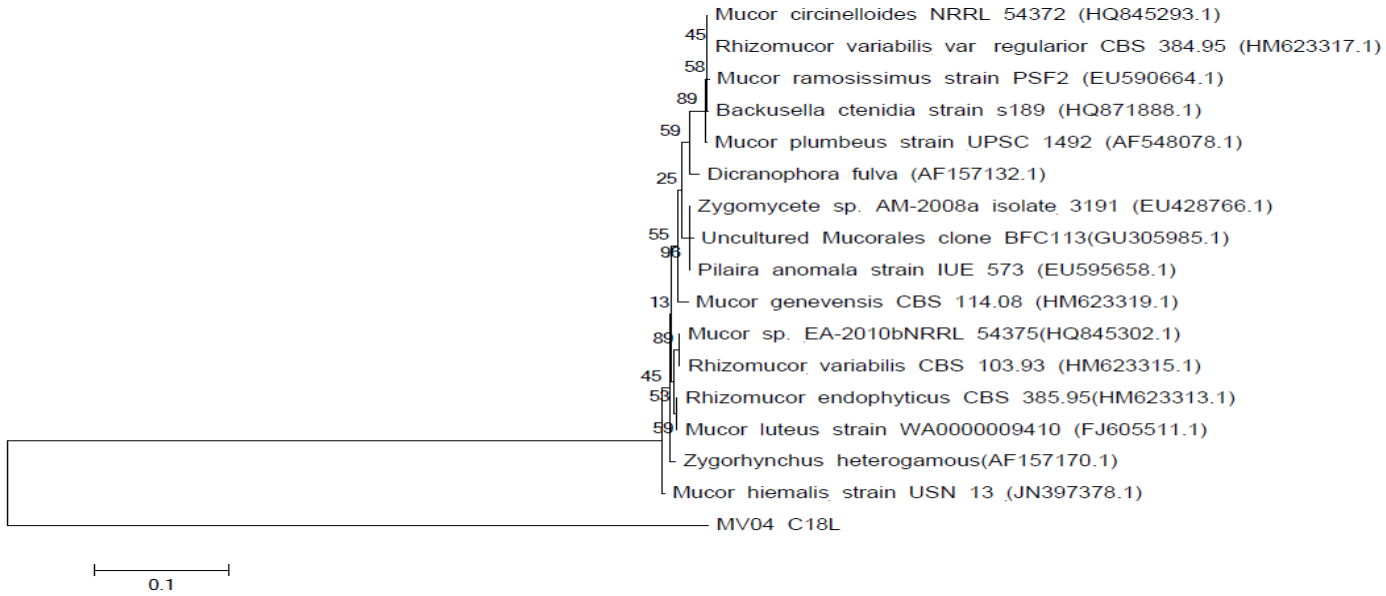


Figure-3

Neighbour-joining phylogenetic analysis resulting from the aligment of 18S rRNA partial gene sequences of *Mucor hiemalis* MV04 (KR078215) fungal strain. The tree was conducted by using MEGA4 software. Bootstrap values (%) indicated as the nodes. The phylogenetic distance is shown on scale bar

This also has an effect on dye decolorization efficiency of the fungal species[21]. It is clear from (table-1) the decolorization activity decreases with increasing initial dye concentration. The most effective decolorization for all dyes was observed at 50ppm concentration in 8 days incubation period at 130 rpm shaking speed. Immobilized biomass showed better result as compare to free biomass of fungal isolate. It is reported that higher dye concentration strongly inhibits decolorization, which may be due to desorption or toxic effects. The ability of enzyme for recognizing the substrate efficiently at very low concentrations may be present in some waste water[22]. Increased dye concentrations have an adverse effect on dye decolourizing efficiency of the fungi. Also, the class of the dye which defines its structure also influences the dye degradation[23].

RBBR dye has best decolorization potential with MV04 fungal isolate (figure-4). Next effective dyes in descending order were Malachite green and Congo red. Congo red was very slowly degrading dye with MV04 fungal strain. Based on these results, most efficiently degraded dye (RBBR) was selected for further experiments. For industrial applications, it is important to know whether the microorganisms that decolorize dyes can bear high concentrations of the compound since the dye concentration in a typical industrial effluent can vary between 10 and 50mg l^{-1}[1,24]. Toxic effect was probably due to inhibition of cellular metabolic activities and cell growth.

Determination of optimum pH, temperature and agitation speed for decolorization of RBBR

Fungus has a strong capability to grow on wide range of pH. *M. hiemalis* MV04 (KR078215) could effectively decolorize (51.82% for free and 63.04% for immobilized biomass) RBBR at relatively broad range of pH (3-9), it showed maximum decolorization of RBBR at pH 5 as shown from (figure-5) It is an established fact that by controlling the $[H^+]$ concentration, the pH of the medium influences degradation of a pollutant[25].

To study the effect of varying temperature on dye decolorization efficiency of the fungus, the media were adjusted to initial optimum pH 5 and incubated at 20, 30, 40 and °C 50 for 8 days. It was found that with an increase in temperature from 20 to 30°C the decolorization rate increased (maximum observed was at 30°C) and then a further increase in temperature to 50°C drastically affected decolorization activity of MV04 fungal isolate. The maximum decolorization (50.14% for free and 66.88% for immobilized fungal biomass) was observed at 30°C (figure-6). Suppressed decolourizing activity at 50°C, this might be due to the loss of cell viability or deactivation of the enzymes responsible for decolourization at higher temperature[26].

Table-1
Effect of Different dyes Concentration on % Decolorization by Free and Immobilized *Mucor hiemalis* MV04 (KR078215) Fungal Isolate

Fungal Isolate MV04	**Concentration (mg l^{-1})**	**% Dyes decolorization**		
		RBBR	Malachite Green	Congo Red
Free biomass	50	39.39	30.70	32.88
	100	37.48	28.38	31.49
	150	34.40	26.17	26.64
	200	32.70	24.27	25.59
Immobilized biomass	50	62.93	50.01	55.34
	100	56.38	46.14	48.51
	150	42.87	34.22	36.72
	200	40.17	28.11	31.43

Figure-4
Change in dye color before and after treatment with *Mucor hiemalis* MV04 (KR078215)

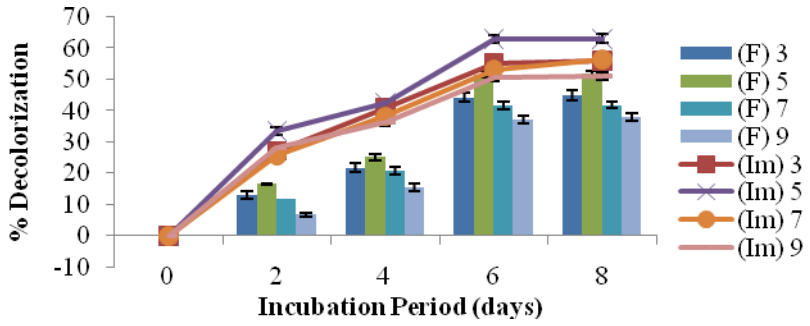


Figure-5

Effect of different pH on % RBBR dye decolorization with Ammonium nitrate (0.1 ml^{-1}), dextrose (0.1ml^{-1}), temperature(30°C), agitation (130rpm)and duration 2-8 days by MV04 fungal isolate. Error bars represent the standard deviation which was within 5 % of the mean. (F) Represents for free fungal biomass and (Im) immobilized fungal biomass.

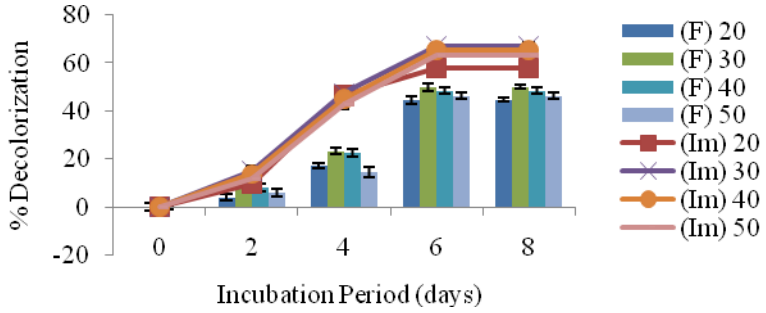


Figure-6

Effect of different temperature on % RBBR dye decolorization with Ammonium nitrate (0.1 ml^{-1}) dextrose (0.1 ml^{-1}), pH 6.0,agitation (130rpm)and duration 2-8 days, MV04. Error bars represent the standard deviation which was within 5 % of the mean. (F) Represents for free fungal biomass and (Im) immobilized fungal biomass.

Different shaking levels (110,120,130,140 and 150rpm) were tested to find out the percent dye decolorization by MV04 fungal isolate. Upto 130rpm as increasing shaking speed decolorization also increases. After that decolorization efficiency decreases, it is due to less attachment of fungal cell. As shown from (figure-7) at 130rpm dye decolorization is maximum for both free and immobilized fungal isolate (43.27% and 59.11%). Agitation increased mass and oxygen transfer between cells and the medium. In addition, enzyme activity also could have depended on the presence of oxygen[36]. According to Chakraborty et al.[27] at agitation speed of 150 and 120 rpm, the fungus was able to decolorize 99.99% of 600 mg/L CR.

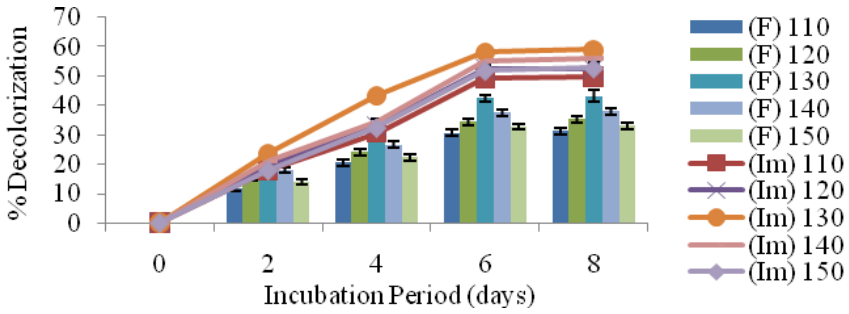


Figure-7

Effect of different agitation speed on % RBBR dye decolorization with Ammonium nitrate (0.1 ml^{-1}), dextrose (0.1 ml^{-1}), temperature(30°C), pH 6.0, and duration 2-8 days, MV04. Error bars represent the standard deviation which was within 5 % of the mean. (F) Represents for free fungal biomass and (Im) immobilized fungal biomass

Effect of supplementation of carbon sources on RBBR Dye Decolorization: The fungi needs readily usable carbon source to grow and then produce secondary metabolites and extracellular enzymes for biodegradation. To assess the effects of different carbon sources on dye decolorization by fungal strain, four carbon sources, namely dextrose, sucrose, maltose, and fructose were used at the rate of 1 g l^{-1} in 250 ml flask and agitated using orbital shaker at 130 rpm. From the study dextrose was found to be the most effective for dye decolorization (38.17% free and 64.5% immobilized biomass) and among all carbon sources used. Decolorization ranged between 80 to 100% in 8 days (figure-8). The primary mechanism of decolorization may be due to dye adsorption/degradation by mycelium of fungi as well as the reduction of dye intensity in solution because of changes caused by them[28]. Furthermore, the rate of dyes removal can be linked with the available co-substrates and with the exponentialgrowth phase[29]. However at higher concentrations the same carbon sources on metabolism produced organic acids, which in turn decreased the pH of media.

Effect of different Nitrogen source on RBBR Dye Decolorization: Nitrogen source supplementation in medium plays a critical role in dye decolorization activity. In present study media was supplemented with nitrogen source (Ammonium Nitrate, Urea, Sodium Nitrate, Yeast extract). As shown in (figure-9) among all different nitrogen sources, Ammonium Nitrate (1g l^{-1}) was the best with 35.73% for free and 65.53% for immobilized biomass decolorization within 8d. The amount of nitrogen present in the media effects dye decolorization by altering the enzyme production by fungi. For several fungal species the ligninolytic enzyme activity is suppressed rather than stimulated by high nitrogen concentrations (20-100mM). Singh et al. found that the range of decolorization activity of Brown GR with ammonium chloride, ammonium sulphate, ammonium nitrate, yeast extract and peptone were 72%, 64%, 68%, 68 % and 76% respectively with *Aspergillus* sp[30].

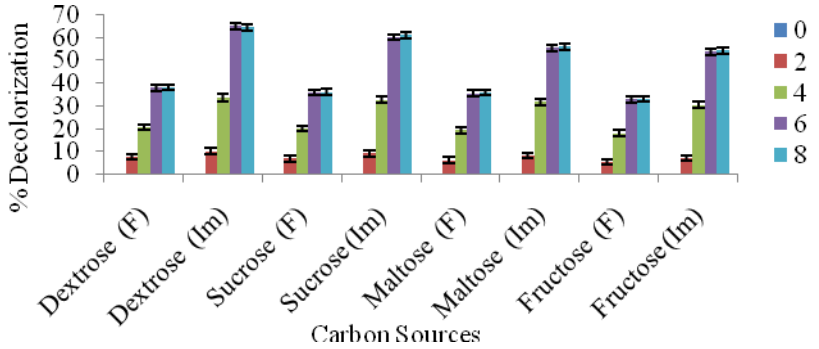


Figure-8

Effect of different carbon concentration on % RBBR dye decolorization with Ammonium nitrate (0.1ml^{-1}) temperature(30°C), pH 6.0,agitation (130rpm)and duration 2-8 days, MV04. Error bars represent the standard deviation which was within 5 % of the mean. (F) Represents for free fungal biomass and (Im) immobilized fungal biomass

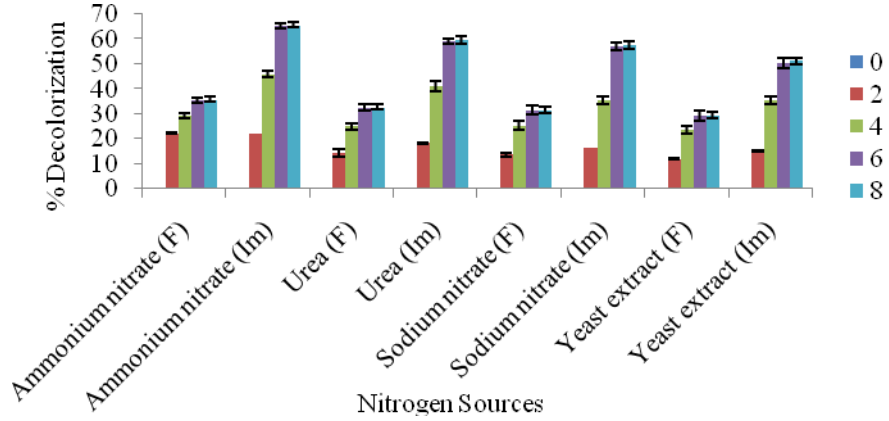


Figure-9

Effect of different nitrogen concentration on % RBBR dye decolorization with dextrose (0.1mgl^{-1}) temperature (30°C), pH 6.0, agitation (130rpm) and duration 2-8 days, MV04. Error bars represent the standard deviation which was within 5 % of the mean. (F) Represents for free fungal biomass and (Im) immobilized fungal biomass

Conclusion

Our results show that the fungus *Mucor hiemalis* MV04 (KR078215) isolated from tree trunk have capability to decolorize a number of synthetic dyes, even at relatively high concentrations. Temperature, pH and agitation were found to be an important parameter, while dextrose and ammonium nitrate were the best carbon and nitrogen sources. The result clearly shows that both free and immobilized have different impact on utilization of dye. In this study we have observed that immobilized biomass have higher decolorization capacity as compare to free fungal biomass. Removal of dye may be due to biodegradation of chromophore in dye molecule because of extracelluar enzyme production by fungi.

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