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Degradation of Rhodamine B, A Xanthene Dye by *Aspergillus niger* MSA2

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ABSTRACT

Dyes and dyestuff play a major role in creating water pollution. Rhodamine B (RhB) is a xanthene class of dyes that have fluorescence and hazardous property. The higher solubility of RhB in water leads to higher application in various industries thus selected for the study. Screening of RhB degrading fungal culture was carried out from industrial wastewater and identified as *Aspergillus niger* MSA2 by using 18S rRNA sequencing. The maximum decolorization of 90.25 % was obtained in 5 ppm dye concentration, starch (0.15 %) and NH_4NO_3 (0.05 %) as carbon and nitrogen source, inoculum (10 % v/v), pH 6 and temperature 30 °C in 100 rpm. The Laccase, MnP, and LiP enzyme activity were checked after decolorization. The induction in enzyme activity in presence of RhB is responsible for degradation. The analytical technique, UV-Visible scanning, and FTIR were studied for confirmation of RhB degradation. In conclusion, the fungal culture *Aspergillus niger* MSA2 has great potency for the degradation of RhB.

Keywords *Aspergillus niger* MSA2, Degradation, Enzymes, Optimization, Rhodamine B.

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INTRODUCTION

Dye and dyestuff are a major source of water pollution as widely utilized in various industries to impart color. Around 10,000 different dyes and pigments are produced per year. These dyes have a broad-spectrum, different colors, structures, and applicability. The 10-15 % dyes are found as wastage in various industrial processes. The colorant water is directly discharged without treatment into the environment as it has many other contaminates and causes several issues (Nanghini *et al.* 2012). Traditional treatments are physical and chemical methods utilized, but these treatments create secondary issues as chemicals were in higher concentration (Balaji *et al.* 2012). Biological treatment implementation is necessary for the treatment of wastewater. Many researchers had done successfully treated colored wastewater through application of biological treatments. Bioremediation has great potential for the removal of hazardous compounds from contaminated sites (Mathur *et al.* 2018).

Fungi have the potency to remediate hazardous compounds and treatment of wastewater. Fungi have filamentous structures and have a high surface

area. Insoluble compounds can contact with fungi; the fungal enzyme converts insoluble into a soluble form which is useful for remediation (Hefnawy *et al.* 2017). Many fungal species; *Penicillium, Pleurotus, Aspergillus, Candida, Rhizopus* have efficiency to degrade the dyes form wastewater. Fungi have extracellular enzymatic system such as laccase, manganese peroxidase (MnP) and lignin peroxidase (LiP) which help to remediate dyes efficiently (Ameen *et al.* 2021). Fungal enzymes have potency to modified in higher contaminated area and secrete the enzyme which help to remediate contaminated site and wastewater treatment (Pan *et al.* 2017).

Aspergillus genus has versatility for remediation of azo dyes. Many researcher has use the *Aspergillus* for degradation of dyes in their study. *Aspergillus* capable to degrade various dyes such as, acid blue 29, disperse red and congo red (Ameen *et al*. 2021), diazinon (Hamad and Soliman 2020), thiazole yellow (Bankole *et al.* 2019), remazol blue and red (Mohamed *et al.* 2019), acid blue 161 (Almeida and Corso, 2018), malachite green, nigrosin and basic fuchsin (Ranjitha *et al.* 2018) and many more. The process parameters play role in degradtion of dyes. The different parameters; nutrient sources, pH, temperture, inoculum, concentration, oxygen transfer are imporatant factors for removal of dyes from wastewater (Asses *et al.* 2018).

This present study was based on RhB decolorization and degradation by using *Aspergillus niger*. RhB is highly utilized in cosmetic induatries, painting and printing industries. RhB have xanthene group in their structure and have high resisatant toward degradtion (Saigl 2021). The lower concentration of RhB in environment cause various issues in living organisms (Joshiba *et al.* 2021). So, removal of RhB by using microorganisms have the novelty than the study found in literarture. Since the methods were developed for degradation of RhB; based on physical and chemical processes. The higher concentrtaion of RhB causing the effect on growth of microorganisms. So, biological method for removal of RhB was effective for lower concentration. This aim of study to found out culture capable to degrade the RhB potencially. The aim of study was fulfilled by using *Aspergillus niger* MSA2 as its have the higher potency to degrade RhB with other textile dyes.

MATERIALS AND METHODS

Dyes, media and chemicals

RhB (CI No. 45170) is widely used in different industries. The lower concentration of RhB shows toxicity to humans, animals, and plants. Thus selected as a model dye for study. The dye was procured from the dye industry. All other media, chemicals and reagents were analytical grade.

Screening, isolation, enrichment and identification of RhB degrading fungi

Screening of RhB degrading fungi was carried out from chemical industry wastewater. The contaminated wastewater sample was serially diluted and spread on 1 ppm RhB-containing potato dextrose agar (PDA) plates. The plates were incubated at 30 °C temperature for 10 days. The fungal culture was screened based on the zone of decolorization. The isolated fungal culture spores were added into potato dextrose media embedded with 1 ppm RhB. The spore suspension was prepared as per described by (Mishra *et al*. 2011). The fungal cultures were screened based on decolorization ability. The potent culture was enriched from 1 to 5 ppm with an increase of 1 ppm increment in each transfer. Identification of fungal culture was carried out with morphologically and 18 S rRNA sequencing. DNA quality was checked with agarose gel electrophoresis. Sequences were amplified with PCR and purified by column purification. DNA sequencing of PCR amplicon was carried out with ITS1and ITS4 primers using BDT v3.1 Cycle Sequencing Kit on ABI 3500xl Genetic Analyzer. The 18S rRNA sequence was used to carry out BLAST with the database of NCBI GenBank. Based on the maximum identity score first fifteen sequences were selected and aligned using multiple sequence alignment software programs.

Decolorization study

Aspergillus niger MSA2 was grown in PDB containing RhB, the decolorization study were performed. The sample were withdrawn in 2 mL eppendorf tube and centrifuge at 10000 × g for 10 min. The supernatant were checked for OD taken at 554 nm (Garg *et*

al. 2020). Percentage and rate of decolorization was checked with below formula.

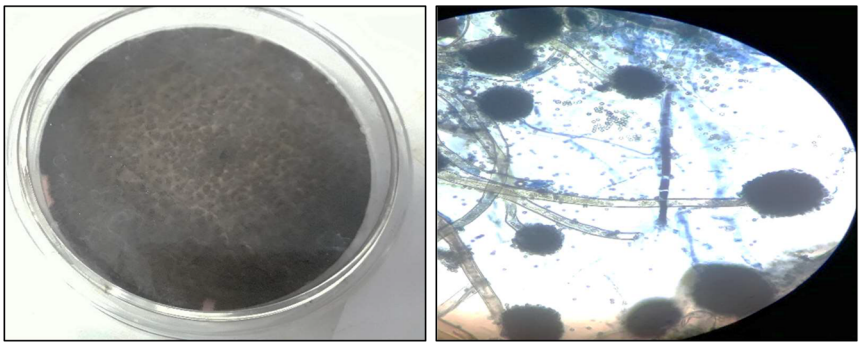
$$\text{Decolorization}\,(\%) = \frac{\text{Initial absorbance—Final absorbance}}{\text{Initial absorbance}} \times 100$$

Optimization of growth conditions of RhB degrading fungi

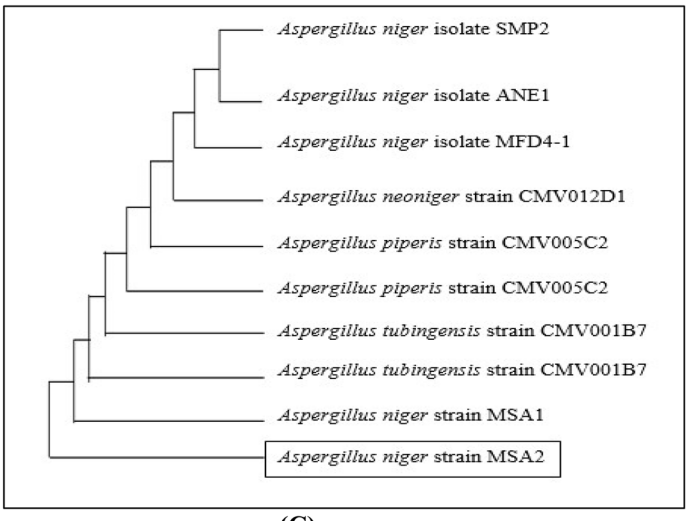
Optimization of growth conditions study to obtain maximum decolorization. The nutrient parameters carbon and nitrogen sources and its concentration were checked for efficient decolorization. The environmental parameters such as static and shaking condition; pH, temperature, inoculum size and dye concentration were selected for 4 days incubation time.

Enzyme study

Laccase, manganese peroxidase (MnP) and lignin peroxidase (LiP) were studied for degradation of RhB. The grown fungal culture were centrifuge and supernatant were taken for extracellular enzyme assay. Laccase and its enzyme activity performed based on the oxidation of ABTS substrate (Aslam *et al.* 2012). Manganese peroxidase and its enzyme activity performed based on the oxidation of guaiacol substrate (Patrick *et al.* 2011). Lignin peroxidase and



(A) **(B)**



(C)

Fig. 1(A). Growth of *Aspergillus niger* on PDA, **Fig. 1(B).** Microscopic observation of *Aspergillus niger,* **Fig. 1(C).** Phylogenetic tree of *Aspergillus niger.*.

its enzyme activity performed based on the oxidation of veratryl alcohol substrate (Yadav and Yadav 2006).

Degradation study

Degradation of RhB were confirmed by UV-Visible spectrophotometer and Fourier transformed infrared spectroscopy (FTIR). The UV-Visible scanning of RhB (Control) and treated effluent were carried out in 190 to 700 nm. FTIR were performed for identification of functional groups generated in treated effluents. Sample was scanned in KBr pellets in 400 -4000 cm^{-1}.

RESULTS AND DISCUSSION

Screening, Isolation and Identification of fungal culture

Screening of 6 fungal culture was carried out based on decolorization ability. The morphological characteristics confirms *Aspergillus genus* shown in Fig. 1(A)-(B). As per 18S rRNA sequencing and ITS region, potent fungal culture was identified as *Aspergillus niger* MSA2 (NCBI Accession No. OL604498). First 15 sequences was aligned through BLAST. The evolutionary history and analysis were performed with MEGA7 as per (Saitou and Nei 1987) and (Kumar et al. 2016b). Phylogenetic tree analysis were carried out as per (Nei and Kumar 2000). Phylogenetic tree is given in Fig. 1(C).

Optimization of growth conditions of RhB degrading fungi

Effect of static and shaking condition on RhB decolorization

Fungal culture were able to decolorize the RhB efficiently in shaking conditions than static conditions. The uniform pellets size were cultivated in 100 rpm speed. The shaking condition gives higher oxygen which also helps to oxidation of dyes (Taskin and Erdal 2010). Present study also in accordance with these statement. The fungal culture potentially remove 45.81 % RhB in shaking condition (Fig. 2). In further study, shaking conditions were preferred throughout study.

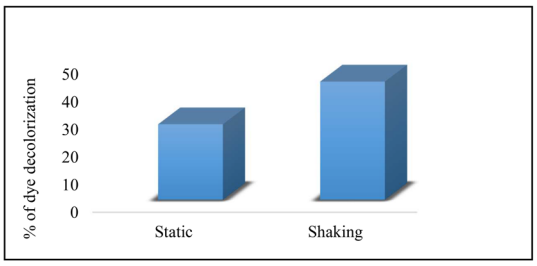
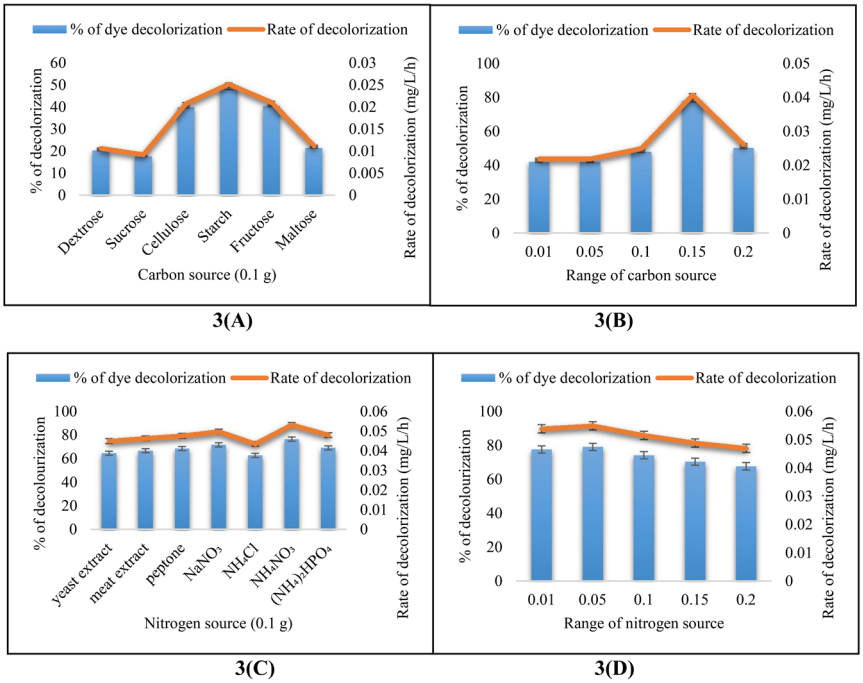


Fig. 2. RhB decolorization in static and shaking conditions.

Effect of nutrients on RhB decolorization

Carbon and nitrogen are utilized as energy source and utilized as substrate by many microorganisms which enhance the enzyme system and helps to increase decolorization (Martorell *et al.* 2018). Fungal culture was tested for RhB dye decolorization by utilizing different carbon sources. The different carbon sources; dextrose, maltose, fructose, sucrose, starch and cellulose were utilized to obtain maximum decolorization. The maximum result 48.39 % decolorization was observed in presence of starch as carbon source in 96 h incubation time with rate of decolorization 0.025 mg/L/h (Fig. 3A). The similar study for decolorization of true blue was obtained in presence of maltose as a carbon source (Ponraj *et al*. 2011). Optimization was carried out by changing different concentration of starch (0.01 to 0.2 %). The highest decolorization by fungal culture was observed in 0.15 % starch concentration. Maximum decolorization up to 78.03 % and rate of decolorization was 0.025 mg/L/h (Graph 3(B)). Effect of nitrogen sources; Yeast extract, Meat extract, Peptone, $NaNO_3$ (sodium nitrate), NH_4Cl (ammonium chloride), NH_4NO_3 (ammonium nitrate), and $(NH_4)_2HPO_4$ (ammonium hydrogen phosphate) were checked with its concentration range (0.01 to 0.2 %). The highest decolorization were observed in NH_4NO_3, 76.54 % decolorization at rate of 0.053 mg/L/h (Fig. 3(C)). The variation in concentration were studied and maximum decolorization were observed in 0.05 % NH_4NO_3 79.03 % decolorization at rate of 0.053 mg/L/h was observed in 72 h incubation time (Fig. 3D). Presence of nitrogen source was responsible for enhance dye decolorization with reduction in time. The maximum decolorization were obtained in presence of NH_4NO_3, for direct blue dye decolorization (Hefnawy *et al.* 2017).



Fig, 3. Effect of nutrients on RhB decolorization.

Effect of environmental factors on RhB decolorization

Environmental factors play important role in dye decolorization. The density of organisms play important role for decolorization of dye. The density of spores responsible for pellets formation and optimized pellets size was gave maximum decolorization (Zhou *et al.* 2022). The 2 to 14 % v/v range was selected for RhB decolorization. The results were observed maximum RhB decolorization at 10 % v/v inoculum at arte of 83.99 % at rate of 0.058 mg/L/h (Fig. 4A) (Ewida *et al.* 2019). Dye concentration have role in decolorization process. Toxicity of dye increase in higher concentration of dyes (Bankole *et al.* 2019). The dye concentration 1 to 9 ppm was considered for study. The RhB is highly toxic in nature, so the concentration of RhB taken in study was low. The maximum 83.52 % decolorization was obtained at 5 ppm dye at a rate of 0.058 mg/L/h (Fig. 4B). pH and temperature are influencing factor for decolorization of dyes. The growth of microorganism differ with pH. Fungal culture can able to grown in slight acidic conditions. The temperature is important for enzyme activity, higher and lower temperature responsible for deactivation of enzymes. The present study showed maximum decolorization 85.65 % at rate of 0.06 mg/L/h at 6.0 pH (Fig. 4C). The maximum decolorization 90.25 % was observed at a rate of 0.062 mg/L/h (Fig. 4D).

Enzyme activity

Dye molecules have different integrity and diversity in structure point of view, degradation of dyes can possible with few of the enzymes which share common mechanistic features for catalysis *Aspergillus* have majorly three enzymes for dye degradation; Laccase, MnP and LiP (Singh 2017). The present study showed this three enzyme activity after decolorization

Table 1. Enzyme activity after decolorization of RhB.

Enzyme	Enzyme activity (U/mL)	Specific activity (U/mg/min)
Laccase	1.01 ± 0.13	0.55 ± 0.075
MnP	10.90 ± 1.19	5.92 ± 0.64
LiP	7.93 ± 0.60	4.31 ± 0.32

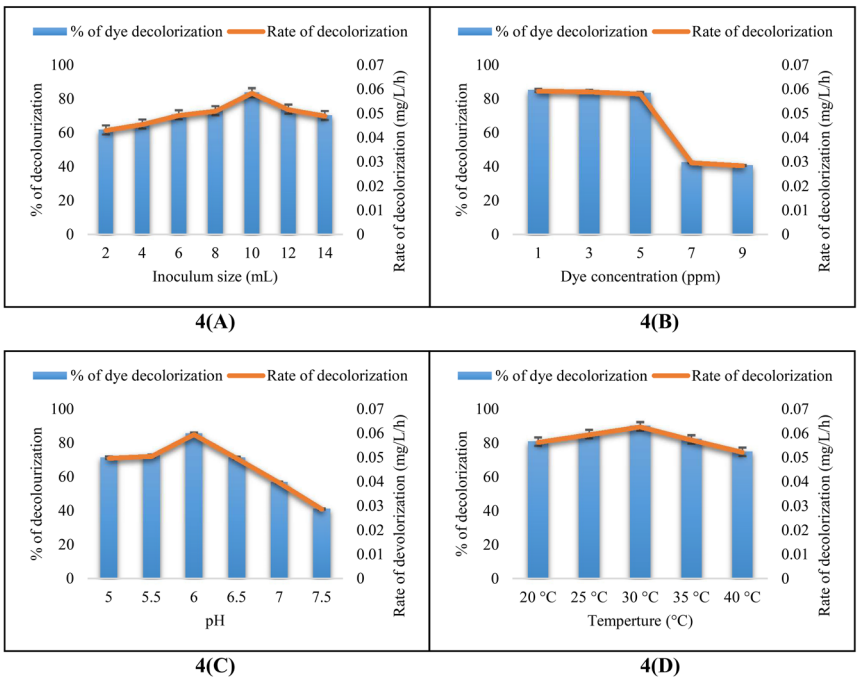


Fig. 4. Effect of environmental parameters on RhB decolorization.

of RhB. The results were given in Table 1. Specific activity per unit of mg of protein measured. Control without dye was run to check original enzyme activity. The control shows lesser activity than decolorized effluent. The induction in enzyme activity due to presence of RhB responsible for dye degradation. The MnP activity was higher than the Laccase and LiP; the results with in accordance with (Pan *et al*. 2017)

Degradation study

UV-Visible spectrophotometer

The decolorization of RhB was carried out with UV-Visible spectrophotometer. The peak of RhB at 554 nm was reduced with time. The peak at around 300 nm in lower intensity also reduced with time.

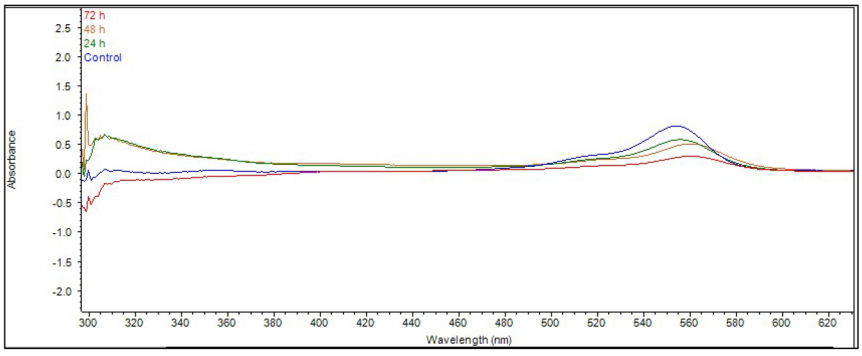


Fig. 5. UV-Visible scanning of RhB and its metabolites.

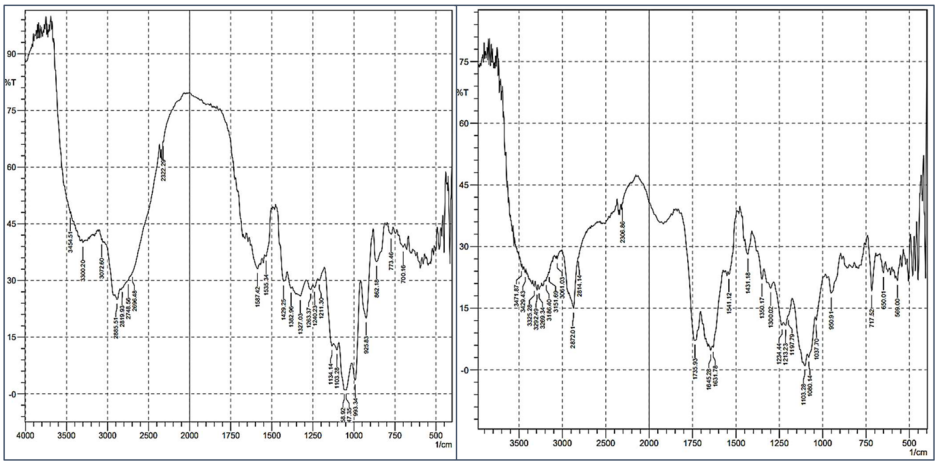


Fig. 6. The FTIR spectra of (A) RhB control, (B) Treated effluent.

The reduction in RhB intensity was confirming decolorization. The Fig. 5 was showing the UV-visible scanning of RhB and their metabolites.

Fourier transformed infrared resonance spectroscopy (FTIR)

The FTIR analysis of RhB (control) with its metabolites were performed for identification of functional groups present in treated metabolites. The RhB control shows (Fig.6A), C-O stretching (1103), C-C stretching (1211), C-N stretching (3300), C=C stretching (1535) stretching (3072), aromatic groups (700-900), C-H stretching (3600-3000) in graph confirms the structure of RhB. The decolorized effluent shows (Fig. 6B) disappearance of peaks and formation of new peak due to metabolites generations, confirms RhB degradation. In decolorized effluent shows, generation of mono substituted compound (717) generation, alkyl ketone (1300), changes in C=C stretching intensity (1500-1800); C=N stretching (1645), formation of different peaks in C-H stretching (2700-3500) region. The changes in intensity due to degradation of RhB and formation of new metabolites present in treated effluent. The structural vibrations shows the changes in intensity in decolorized effluent. The absence and presence of different peaks in treated effluent confirms the degradation of RhB by *Aspergillus niger* MSA2.

CONCLUSION

Aspergillus niger MSA2 has prominent source for removal of hazardous compounds due to highly adapted enzyme system. RhB is highly carcinogenic and mutagenic dye and cause these effects in lower concentration. 90.25 % decolorization was observed by using *Aspergillus niger* MSA2 in 5 ppm dye concentration, starch (0.15 %) and NH_4NO_3 (0.05 %) as carbon and nitrogen source, inoculum (10 % v/v), pH 6 and temperature 30 °C in 100 rpm. The conditions were optimized with OFAT. The presence of activity of Laccase, MnP and LiP confirms the role of enzyme in degradation. The degradation was confirmed by UV-Visible scanning and FTIR analysis. The changes in functional groups of control and decolorized effluent; confirms the degradation of RhB efficiently.

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