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Degradation Potential and Enzyme Characterization of *Aspergillus Flavus* SKF8 Isolated from a Textile Industrial Effluent against Reactive Yellow 145

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Abstract Globally, azo dyes contamination is becoming of the major environmental issues which require an urgent consideration for their effective treatment and disposal. During this decade, microbial remediation have found to be a promising solution, however, many research strategies have yet to be fixed to achieve an effective treatment of azo dyes containing textile effluents. In this context the present study was undertaken to isolate a potential azo dye degrading microbe and as a result a promising Reactive Yellow 145 (RY 145) dye degrading fungus was isolated from textile effluent sediment samples from a textile industry located at Salem district, Tamil Nadu, India and the strain was identified as *Aspergillus flavus* SKF8 based on molecular methods (rDNA sequencing). This strain demonstrated a growth dependent biodegradation activity of the Reactive Yellow 145 in which the highest rate of dye degradation was recorded during its stationary growth phase. Further, this strain decolorized the chosen dye to a maximum of 88.4%. Results revealed that the strain could synthesize five dye degrading enzymes viz., laccase, veratryl alcohol oxidase, lignin peroxidase, polyphenol oxidase and NADH-DCIP reductase and biodegrade the azo dyes effectively. The overall results proved that this novel isolate, *Aspergillus flavus* SKF8 have the potential for real-time applications in the decolorization and biodegradation activities of azo dyes containing textile effluent.

Keywords: *Reactive Yellow 145, Aspergillus flavus, Biodegradation, Textile effluent, azo dyes*

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1. Introduction

In this decade, increased importance has been given by environmentalists to the textile effluent discharges, containing azo dyes. Worldwide, 280,000 tons of textile dyes are discharged annually into various water bodies [1]. The discharge of these effluents containing synthetic dyes into the environment is undesirable because of the serious environmental issues linked with the dyes and their by-products [2]. Among the total synthetic dyes, azo dyes represents up to 70% of the total textile dyestuffs used across the globe [3]. Azo dyes have applications in wide industrial sectors like textile, leather, paper, etc. Azo dyes belong to the class of aromatic and heterocyclic compounds with one or more azo bonds (-N=N-) which are recalcitrant and can cause cancer in humans and animals [4].

The accumulated forms of azo dyes in water bodies can cause adverse effects in terms of dissolved oxygen, biological oxygen demand, chemical oxygen demand, photosynthesis of the aquatics organisms, etc., [5]. Hence, their removal from the industrial effluents is very important before they enter into the water ecosystem [6]. In the recent times, extensive research and development activities have been focussed on biological methods for the eco-friendly remediation of these dyes [7]. Most studies have focused on azo dye decolorization or biodegradation using bacteria due to their high efficiency, diversity and adaptability, however, degradation of some products like aromatic amines can strongly inhibit the bacterial growth [8]. By contrast, fungi can degrade complex organic as well as inorganic compounds through the synthesis of various ligninolytic enzymes viz., laccase, manganese peroxidase, lignin peroxidase, etc. [9]. Many fungal strains viz., *Pichia* sp., *Penicillium* sp., *Candida tropicalis* and *Pleurotus ostreatus* have been reported to

decolorize azo dyes effectively using adsorption and/or by degradation process; further, some fungal strains can completely mineralize azo dyes [10]. Moreover, fungi have been reported for their strong adaptability and high efficiency in the removal of aromatic azo dyes [11], however, very limited scientific information are available in these regards, therefore the present study is carried out. In this study a novel fungal strain was isolated which proved its potential in the effective degradation of the chosen azo dye. It was isolated from the textile effluent sediment samples from a textile industry located at Salem district, Tamil Nadu, India. A thorough investigation was carried out to understand the mechanism behind the biodegradation of the dye by the novel isolate.

2. Materials and Methods

2.1. Sample Collection and Isolation of Dye Degrading Fungi

Textile effluent sediment samples were collected aseptically from a textile industry situated at Salem district, Tamil Nadu, India. Three samples were collected with a 100 m distance from each sampling spot using individual pre-sterilized spatulas and all aseptic procedures were followed to avoid contaminations. These collected samples were transported immediately to the laboratory and were stored at 4°C until further process. Each sample was individually processed for the isolation of dye degrading fungi in which the samples were serially diluted using saline (0.9% NaCl in distilled water) and the each dilution was spread plated on Sabouraud dextrose agar plates. After 96 hrs incubation at 30°C, fungal colonies with unique morphologies were repeatedly pure cultured using freshly prepared Sabouraud dextrose agar plates. All the individual pure cultured strains were preserved under refrigeration in slant tubes for further studies.

2.2. Screening of Potential RY 145 Degrading Fungi

All the isolated fungal strains were individually screened for their potential RY 145 degradation activities in sabouraud dextrose broth with the dye concentration of 100 ppm/L (100 mg / L of RY 145). The experiment was carried out for 144 hrs at 30°C. After incubation, the broth cultures were centrifuged at 10,000 rpm for 10 min and the absorbance of the cell free supernatants were evaluated at λ max (590 nm) of the RY 145 using UV-Vis spectrophotometer. The uninoculated Sabouraud dextrose broth containing the RB 194 dye was used as blank. The percentage of dye degradation was calculated using the following formula as given below [12]:

$$Dye\ degradation\ (\%) = \frac{Absorbance\ of\ blank - Absorbance\ of\ treated\ sample}{Absorbance\ of\ blank} \times 100$$

2.3. Molecular Identification of Potential Reactive Yellow 145 Degrading Fungus

DNA extraction was performed by using 5 mm fungal pure cultured colony collected at the mid-exponential growth phase with help of Roche Kit (Germany) according to manufactures instructions.

Among all the isolates the fungal strain which demonstrated the maximum RY 145 dye decolourizing ability was subjected to molecular identification involving Internal transcribed spacer (ITS) and 5.8S rRNA region [13] using the primers as follows, ITS1 (5' - TCCG TAGG TGAA CCTG CGG - 3') and ITS4 (5' - TCCT CCGC TTAT TGAT ATGC - 3'). The sequence analysis was carried out using an ABI Prism 377 automatic sequencer (Applied Biosystems, CA, USA) and gene sequence was compared with the available sequence database of the NCBI with the 2.5.0 BLAST version. The evolutionary distance was computed using the neighbour joining method [14] and MEGA 7 was used for evaluating the evolutionary relationships of this fungus [15].

2.4. Time Course on Dye Degradation

The identified potential fungal strain SKF8 was studied for the time course on dye degradation with reference to its cell biomass concentration. The experiment was performed in a 250 ml conical flask with 100 ml working volume and the analysis were carried out at every 12 hrs regular time interval from lag to decline growth phases of the chosen isolate SKF8. The log phase culture of the chosen strain was used as inoculum in Sabouraud dextrose broth as the basal growth medium supplemented with 100 mg/L of RY 145 along with the other cultural conditions of pH 6.0, 30°C temperature, 100 rpm agitation was maintained. The inoculum preparation was carried out with spores of five days old fungal culture and physiological saline was used as diluent. All the required evaluations were made using a portion of cultured broth (cell free supernatant) obtained by centrifuge separation of cell pellet at 10,000 rpm for 10 min. Cell biomass density was estimated using the dry weight of cell biomass concentration resulted from centrifuged cell pellets which was hot air oven dried at 50 °C for 30 min and the study of dye degradation was evaluated in cell free supernatant as per the screening procedure mentioned above in the section 2.2.

2.5. Degradative Enzymes Study

Seven various degradative enzyme activities viz., azoreductase, laccase, veratryl alcohol oxidase, lignin peroxidase, manganese peroxidase, polyphenol oxidase and NADH-DCIP reductase were evaluated in this study for their role during the biodegradation of RY 145 and this experimental study was carried out at 12 hrs regular time intervals from lag to decline growth phase of the chosen strain. A portion of cultured broth was taken for sonication at 4°C under 20 KHz for 45 sec. using Ultra-sonicator (Hielscher, USA) followed by centrifugation at 3000 rpm for 15 min and the supernatant was analysed for the seven enzyme activities using the below described assay methods.

2.5.1. Azoreductase Assay

Azoredutase activity was estimated at 30°C in 1 ml reaction volume containing 50 mM sodium phosphate buffer at pH 7.0, 50 µM FMN, 150 µM NADH, 25 µM Methyl Red and 200 µl of crude enzyme [16]. The addition of NADH prepared in 50 mM sodium phosphate buffer (pH 7.0) initiated the reaction and enzyme activity was measured using UV-Vis spectrophotometer with the decrease of absorbance at 430 nm per min time. The Methyl Red at 430 nm has the extinction coefficient of 23,360 M^{-1} cm^{-1}. A unit of enzyme activity was expressed as the decrease in the concentration of azo dye (µM) per min.

2.5.2. Laccase Assay

The laccase activity was measured using 2,2'-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid (ABTS) in which the reaction volume has the mixture of 2.450 ml citrate phosphate buffer at pH 4.0, 500 µL of 1.5 mM ABTS and 50 µl crude enzyme which was incubated for 5 min [17]. The oxidation of ABTS was estimated by the increase of absorbance at 420 nm (ε420 = 36mM^{-1} cm^{-1}) and enzyme activity was expressed as Unit/ml/min.

2.5.3. Veratryl Alcohol Oxidase Assay

The veratryl alcohol oxidase activity was examined using the reaction mixture which has 1 ml veratryl alcohol (10 mmol/L), 0.4 ml crude enzyme supernatant and 1.5 mL sodium tartrate buffer (100 mmol/L) at pH 3.0 and the reaction was initiated by the addition of 0.1 ml H_2O_2 (10 mmol/L). The enzyme activity was quantified by the oxidation of veratryl alcohol to veratraldehyde with the changes in absorbance at 310 nm at 30°C using UV-Vis spectrophotometer [18] and the enzyme activity was represented in international units (U) that is one unit was defined as 1 µmol of veratryl alcohol oxidized per 1 min.

2.5.4. Lignin Peroxidase Assay

The lignin peroxidase activity was evaluated based on the oxidation of azure B dye [19]. The reaction mixture has 1 ml sodium tartarte buffer (125 mM) at pH 3.0, 500 µl azure B (0.160 mM), 500 µl crude enzyme supernatant and 500 µl hydrogen peroxide (2 mM). The reaction was initiated by addition of 10 mmol/L hydrogen peroxide and one unit of enzyme activity was represented by a decrease in 0.1 OD per minute per ml of the crude enzyme supernatant.

2.5.5. Manganese Peroxidase Assay

The manganese peroxidase activity was estimated by the oxidation of DMP 2,6-dimethoxyphenol results into the formation of coerulignone [20] which can be measured by the rise in absorbance at 469 nm using a spectrophotometer. Reaction mixture has 200 µL sodium tartrate (50 mM) at pH 7.0 containing 5 µg/mL manganese peroxidase extract from crude enzyme supernatant, 0.5 mM DMP, 1mM $MnSO_4$ and 0.1mM H_2O_2 and the reaction was carried out at 30°C. One unit of enzyme activity was estimated by the quantity of enzyme required to oxidize 1 mmol of 2,6-DMP per min.

2.5.6. Polyphenol Oxidase Assay

The polyphenol oxidase activity was evaluated using the reaction mixture consisted of 20 µL crude enzyme supernatant and 180 µL substrate solution which has 0.2 ml tyrosine in 0.05 M phosphate buffer at pH 6.8 [21]. The reaction was carried out at 25°C, and the change in absorbance was recorded at 410 nm using a spectrophotometer. One unit of enzyme activity was expressed as an increase in absorbance by 0.001 units per minute per ml.

2.5.7. NADH-DCIP Reductase Assay

The NADH-dependent 2,6-dichlorophenolindophenol (NADH-DCIP) reductase activity was evaluated by the reduction in DCIP concentration which was estimated under its absorption maximum at 590 nm using a spectrophotometer. The reaction mixture has 3 ml which contains 50 M DCIP, 50 mM potassium phosphate buffer at pH 7.4 and 50 M NADH [22]. One unit of enzyme activity was expressed as one microgram of DCIP reduced/min/ml.

3. Result and Discussion

3.1. Isolation and Screening of a Potential Dye Degrading Fungus

Textile industrial effluents are the main causes of water pollution which has now becoming an alarming concern worldwide. In the recent years, biological treatments have received increasing attraction in treating and detoxifying textile effluents than other treatment processes identified till date [23]. The present study concentrated in the isolation of indigenous fungi from textile effluent sediment samples, since fungal species were less concentrated than bacteria strains till date for the treatment of textile dyes containing wastewater. All the collected effluent sediment samples of this study were individually studied for the isolation of potential RY 145 (model textile industry azo dye) degrading fungi. The samples were serially diluted, spread plated, visualized for the fungal colonies of unique morphologies and the selected colonies were pure cultured. 14 different fungal strains were isolated in this study and were named as SKF1 to SKF14. All the strains were individually screened for the biodegradation of an azo dye, RY 145 and among the total strains; SKF8 showed promising activity of 61.3% degradation followed by strain SKF2 which revealed 56.7% degradation activity in the preliminary screening, whereas the rest of the strains showed poor biodegradation activities. Based on the preliminary screening, the strain SKF8 was chosen for molecular identification and detailed study of biodegradation. To the best of our knowledge, till date, RY 145 degrading fungi were poorly studied and there are no reports on indigenous strains with promising potential on the chosen azo dye.

3.2. Molecular Identification of the Potential Dye Degrading Fungus

The molecular identification of the promising dye degrading fungus SKF8 was done by amplifying the ITS

and 5.8S rRNA regions of the genome. The total length of the amplified sequence was 616 base pairs and based on the BLASTn homology search against the NCBI Gen Bank sequence database, this strain SKF8 was identified as *Aspergillus flavus*. The amplified sequence revealed 100% similarity with the available sequences of the NCBI database. Further, the sequence was deposited in the NCBI GenBank sequence database and provided with the accession number, **MT378333**. The genus *Aspergillus* belongs to the family Trichocomaceae and the phylum Ascomycota.

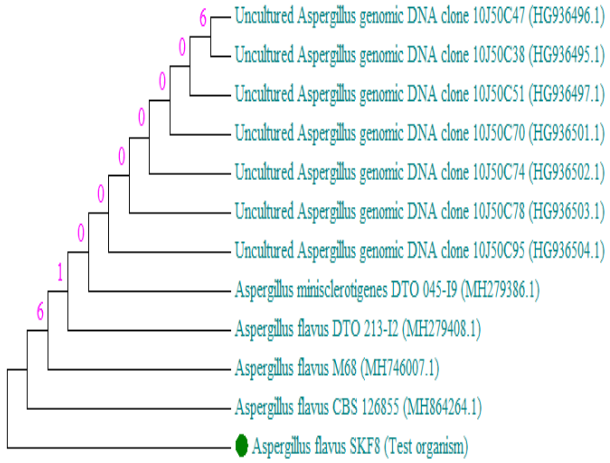

Figure 1. Evolutionary relationships of *A. flavus* SKF8 against the highest similar nucleotide sequence of the NCBI GenBank database inferred using Neighbor-Joining method

The phylogenetic tree of *Aspergillus flavus* SKF8 was plotted against the twelve maximum similarity sequences obtained from the NCBI nucleotide collection (Figure 1).

3.3. Time Course on Dye Degradation

The time course on the biodegradation of the azo dye, RY 145 was investigated using the potential fungus, *A. flavus* SKF8 with reference to its cell biomass concentration. The biodegradation of RY 145 got initiated from the first hours of the fungus growth and observed till the decline of growth; a gradual increase in the dye degradation was observed during the lag phase. However, the maximum rate of biodegradation was witnessed during the stationary phase of growth by the fungus from 108 to 144 hrs in which 8.22 ± 0.41 g/L fungal cell biomass was observed (Figure 2). Finally a gradual decrease in the degradation was observed in the decline phase of growth. The strain demonstrated a maximum decolourization of 88.4%. (Figure 3). The pattern of biodegradation which is observed here, during the entire growth phase of the fungus reveal that the metabolites are involved in the process are growth dependent metabolites. In a recent study, on the degradation of RY 145 using the pure cultures of *Pseudomonas aeruginosa* and *Thiosphaera pantotropha by* Garg et al. [24] revealed less than 50% degradation with dye concentration of 100 mg/L. However in the present study, *A. flavus* SKF8 demonstrated 88.4% of decolorization at 100 mg/L dye concentration revealing its efficiency.

3.4. Degradative Enzymes Study of the Potential Fungus

Microbial degradation of dyes is mainly associated with the synthesis of enzymes which mediate the conversion of dyes into degraded products. A panel of seven different dye degradative enzymes were evaluated in this study during the time course on the biodegradation of RY 145 using the potential fungus, *A. flavus* SKF8 and is illustrated in the Figure 4. This fungal strain revealed a potential biosynthesis of laccase enzyme in which the activity was observed during the entire growth phase; however the maximum activity was recorded during the 144 hrs with 1.83 U/ml/min. It is evident from the results that Laccase production was growth dependent which has a gradual increase and decrease during the lag and decline growth phase of the fungal strain.

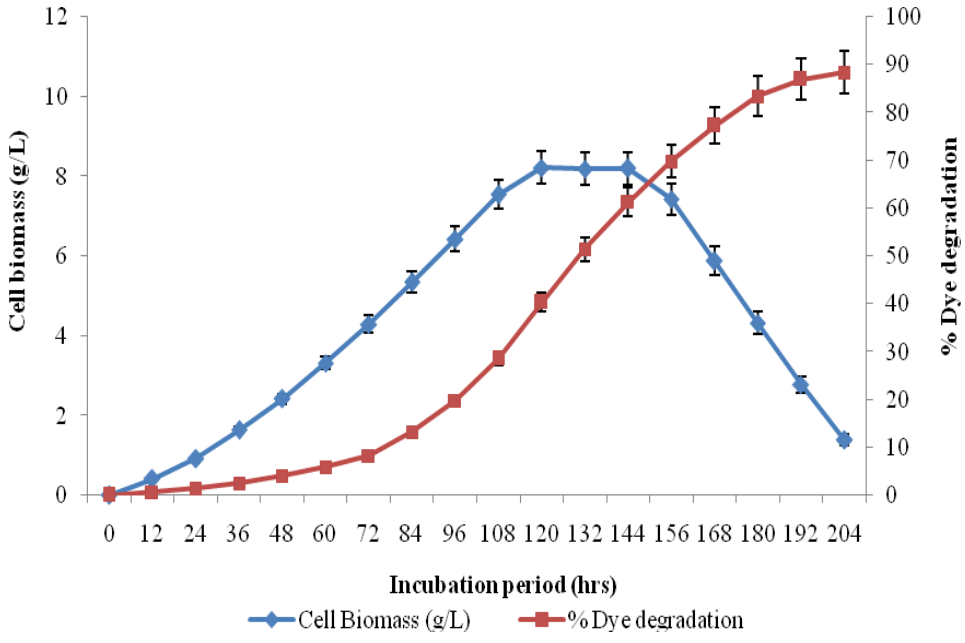

Figure 2. Time course on RY 145 biodegradation with reference to the cell biomass concentration of the potential fungus, *A. flavus* SKF8

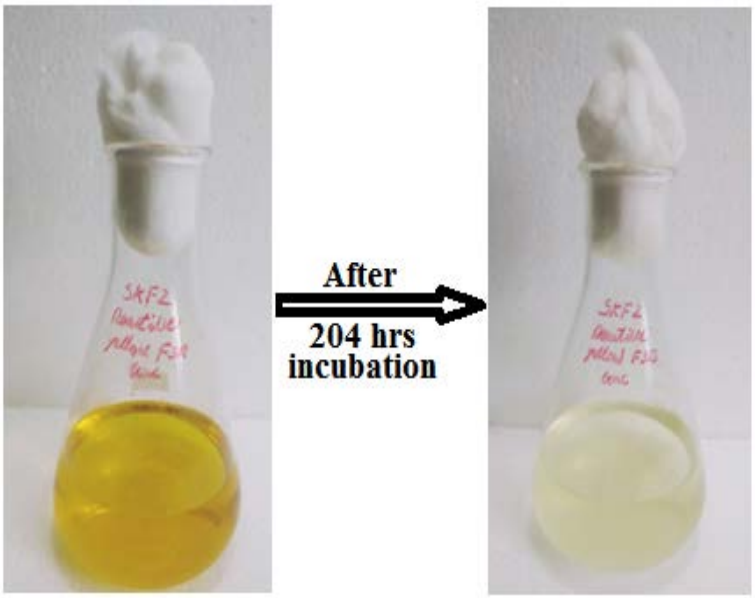

Figure 3. Biodegradation of RY 145 using the potential fungus, A. *flavus* SKF8

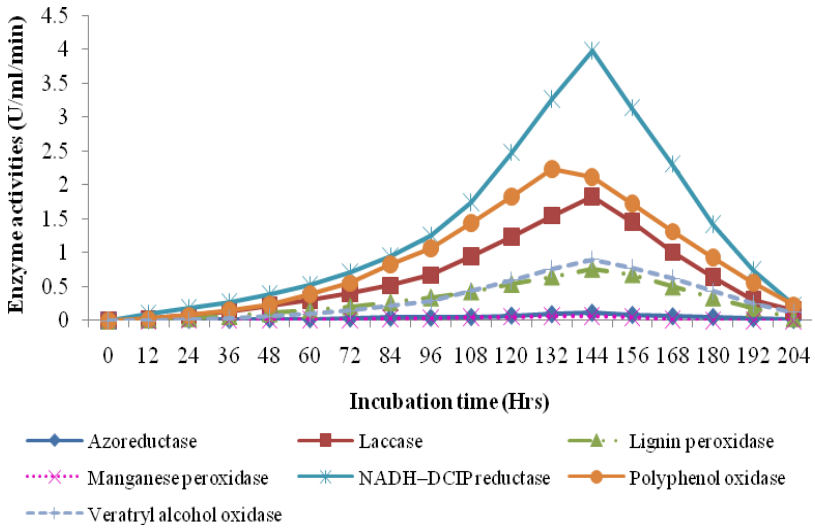

Figure 4. Quantitative estimations of seven different degradation enzymes during the biodegradation treatment of Reactive Yellow 145 using A. *flavus* SKF8

Similarly, this fungus showed an appreciable production of veratryl alcohol oxidase and the synthesis was initiated during the lag growth phase and achieved its peak production during the end of stationary growth phase (144 hrs) with 0.89 U/ml/min. Similar to the laccase enzyme, veratryl alcohol oxidase also revealed to be a growth dependent enzyme. Likewise, this fungus evidenced a similar growth dependent pattern of lignin peroxidase production with its maximum production at 144 hrs with 0.75 U/ml/min, respectively. However, this strain showed poor biosynthesis of manganese peroxidase. Interestingly, this strain revealed a growth dependent and potential production of polyphenol oxidase and NADH-DCIP reductase enzyme activities during the end of stationary growth phase (144 hrs) with 2.92 U/ml/min and 3.98 U/ml/min activities, respectively. However, this strain demonstrated a negligible production of azoreductase enzyme.

Similar to this study, a previous study observed the synthesis of laccase and azoreductase during the biodegradation treatment of Drimaren Red CL-5B achieved using *Aeromonas hydrophila* MTCC 1739 and *Lysinibacillus sphaericus* MTCC 9523, respectively and these enzymes synthesis enhanced the biodegradation activity of this azo dye [25]. Likewise**,** Thanavel et al. [26] observed a significant increase in the concentration of dye degrading enzymes such as laccase, lignin peroxidase, azoreductase, tyrosinase, and riboflavin reductase using biodegradation treatment of azo dyes viz., Reactive Red 180, Reactive Black 5 and Remazol Red using the bacterial strain, *A. hydrophila* SK16.

4. Conclusion

This study isolated a potential RY 145 degrading fungus, *A. flavus* SKF8 from a textile effluent sediment sample. This strain demonstrated its dye degradation ability from the initial hrs of its growth cycle. Further, the analysis of the degradation mechanism revealed that the strain produce five dye degrading enzymes viz., laccase, veratryl alcohol oxidase, lignin peroxidase, polyphenol oxidase and NADH-DCIP. From the overall observations,

this research suggests that the isolated fungus can be employed in real time applications for the effective biodegradation activities of azo dye containing textile effluents.

Conflict of Interest

The authors declare that they have no conflict of interest on publication of this article.

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