Asian J Biol Life Sci., 2026; 15(1):252-257.
https://ajbls.com

Original Article

Selective Isolation and Molecular Characterization of *Streptomyces* sp from Dye Discharged Site and its Enzyme Screening in Prospective to Azo Dye Degradation

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ABSTRACT

Background: Reactive azo dyes discharged from the textile industry have the great impact to the environment. The present study of using microorganism particularly *Streptomyces* sp from dye discharged site showed its effectiveness in the process of azo dye degradation. **Materials and Methods:** A laccase-producing *Streptomyces* sp. was isolated and initial Screening was performed followed by Morphological Characterization and Molecular Identification. Phylogenetic analysis and genetic analysis was also performed. Dye Decolorization Performance with respect to different carbon source was tested. Enzyme activity analysis includes reductase, manganese peroxidase and laccase was performed. **Results:** A reddish-brown colony on agar plates among the developed colonies (4×10^4 CFU) showed that laccase was active. Morphological characterization showed that the colonies were white and powdery, with well-developed aerial and substrate mycelia and a spiral spore chain, which is typical of *Streptomyces* sp. Using 16S rRNA gene sequencing to identify the isolate at the molecular level confirmed that it was *Streptomyces* sp. Phylogenetic analysis showed that it was closely related to *Streptomyces somaliensis, S. albu*,*S. sampsonii* and *S. hydrogenans*. Genetic distance analysis revealed the greatest similarity with *S. somaliensis* (0.006974), succeeded by *S. albous* (0.00835), suggesting negligible evolutionary divergence. The distance matrix strongly supports the results of the phylogenetic tree, which show that isolate RP belongs to the genus *Streptomyces* and is most closely related to *S. somaliensis*. The isolate effectively decolorized Reactive Red dye, with carbon sources having a major impact. Dextrose (82.53±0.30%) caused the most decolorization, followed by starch (68.8±0.30%), lactose (45.46±0.2%), and sucrose (23.2±0.2%). Enzyme profiling showed that reductase had more activity (72.8±0.2 U) than manganese peroxidase (48.13±0.23 U) and laccase (23.87±0.30 U). **Conclusion:** These results show that several enzymes are involved in breaking down dyes, with reductase being the most important one. The study underscores the potential of laccase-positive *Streptomyces* sp. as an efficient biocatalyst for azo dye remediation.

Keywords: Amines, Azo reductase, Bioremediation, Laccase, Reactive red, *Streptomyces* sp.

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Received: 15-01-2026;
Revised: 09-03-2026;
Accepted: 28-05-2026.

INTRODUCTION

Actinomycetes have a lot of potential for cleaning up the environment, especially when it comes to pollution from artificial colors like azo dyes that are widely used in the food, cloth and medication sectors (Bautista-Pinzón *et al*., 2024). Because of their stable structure the azo dyes are distinguished by their bright hues and nitrogen-nitrogen (N = N) linkages which are persistent pollutants (Benkhaya and El Harfi, 2020). Aquatic toxicity, mutagenicity and carcinogenicity are only a few of the serious health and environmental concerns caused by ineffective disposal to landfills (Singh *et al*., 2024). Thousands of different dyes are produced by the textile and paper industries and these dyes must be stable in the face of various chemicals, light exposure, and microbial deterioration. Globally, more than 10,000 synthetic colors are currently in use. Ninety percent of reactive textile dyes are left in sewage plants and eventually dumped into waterways due to this intrinsic recalcitrance. Many physical, chemical, and biological treatments have historically been applied using a single technique. Biological and advanced oxidation mechanisms are becoming more significant since they cause the contaminants to completely mineralize (de Silva *et al*., 2019). The method is used, which results in low biodegradability, low mineralization, and low dye removal, along with high post-treatment costs and

DOI: 10.5530/ajbls.20260141

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requirements to meet discharge regulations. Recalcitrant dyes can be broken down by a variety of specialized oxic and anoxic treatment methods. A crucial enzyme in the decomposition of azo dyes, azoreductases depends on NADH and catalyzes the oxidative cleavage of azo bonds, which breaks down complex compounds of dye into colorless aromatic amines (Preethi and Pathy., 2020). They can be broadly divided into oxygen-sensitive and oxygen-insensitive azoreductases based on their sensitivity to oxygen, with the latter being more useful for real-world aerobic applications. Azoreductases are known to be produced by many of microorganisms includes bacteria, fungi and actinomycetes. According to reports, certain bacteria, such as *Aeromonas hydrophila* and *Lysinibacillus sphaericus*, produce laccase and azoreductase, which are used in the elimination of reactive red (Srinivasan *et al.*, 2017). As a result of their metabolic diversity and capacity to produce several degradative enzymes, *Streptomyces* species have garnered significant interest across bacteria (Ramírez-Tapias *et al.*, 2018). There is a lot of concern in developing applications that use *Streptomyces* to biotransform resistant materials. Thus, the goal of the current investigation was to separate and filter dye-degrading enzymes from *Streptomyces* species.

MATERIALS AND METHODS

Isolation and Phenotypic Characterization of Actinomycetes (Ganesan *et al.*, 2017)

Textile dye discharged soil sample was collected at Karut region and pre-treated at 50ºC for 30 min to suppress non-spore-forming bacteria. One g of pre-treated soil was serially diluted up to 10^{-6}, and 1 mL of the 10^{-5} dilution was inoculated onto actinomycetes isolation agar by using the pour plate method. The medium composition (g/L): sodium propionate (4.0), sodium caseinate (2.0), dipotassium phosphate (0.5), magnesium sulfate (0.1), ferrous sulfate (0.001), 0.01% guaiacol and agar (15.0), with pH adjusted to 8.1. Plates were incubated at 25ºC for 10 days and pure actinomycetes cultures were obtained by repeated subculturing. Phenotypic identification was performed based on colony morphology and slide culture method was used to differentiate aerial and substrate mycelium followed by staining with Sudan Black.

Molecular characterization

The molecular characterization performed by 16srDNA sequencing as described by Babadi *et al.* (2022). For seven days, the isolates were grown in an Erlenmeyer flask using an ISP4 medium at 180 rpm and 28ºC. The cells were then separated by centrifugation process at 8000 rpm for 10 min using 15 mL culture in a falcon tube. The phenol-chloroform technique was used for isolating genomic DNA. 16S rDNA was amplified using universal primers 27F: AGAGTTTGATCCTGGCTCAG and 1492R: GGTTACCTTGTTACGACTT. A 50 µL reaction mixture containing oligonucleotides of 10 µM, a 5× premix containing Taq polymerase and 2µL (100 ng) genomic DNA was used for the 29 cycles of PCR amplification. First denaturation at 95ºC, annealing at 51.4ºC and at 95ºC extensions comprised the cycle. Following the purification of the resultant PCR products using the Himedia Cleanup kit (Mumbai, India), 16S rDNA sequencing was completed, and the BLAST search was run using the MEGA tool.

Dye degradation on Minimal Medium

Laccase positive isolate was subjected to preliminary dye screening using 100 ppm reactive red. The process was carried out in Erlenmeyer flasks (250mL) containing 100mL basal medium composed of (g/100 mL): K_2HPO_4 (0.5), $MgSO_4$ (0.01), $(NH_4)_2SO_4$ (0.1), sodium citrate (0.05) and 0.01% guaiacol. The sources of carbon were dextrose, lactose, sucrose and starch were amended to the medium at 1% concentration. The medium was sterilized and inoculated with 5-day-old *Streptomyces* sp while uninoculated medium served as the control. After 24 and 48 hrs of incubation, the culture was centrifuged at 10,000 rpm for 5 mins to obtain the cell-free supernatant and the percentage of decolorization by measuring OD at 520nm.

$$\% \text{ of decolorization} = \text{Blank OD - Test OD/ Blank OD } x \text{ 100}$$

Azo reductase Assay

Azoreductase activity was determined by monitoring the decrease in optical density at 430nm. The standard reaction mixture (3 mL) contained 25 mM Tris-HCl buffer (pH 7.4), 25 µM Reactive Red, 100 µM NADH and 250 µL cell free supernatant. The reaction mixture without Reactive Red was pre-incubated for 3 min at 30ºC, after which the reaction was initiated by the addition of dye. Azo dye reduction was measured by recording the initial rate of decrease in absorbance at 430 nm. A control lacking the dye was maintained under identical conditions. One unit of azoreductase activity was defined as the amount of enzyme required to decolorize 1 µmol of dye per minute under the assay conditions.

Laccase Assay (Suganya and Soundhari, 2019)

Activity of Laccase was assayed using the substrate guaiacol. The reaction mixture includes 2mM guaiacol solution-1 mL, 10 mM sodium acetate buffer-3 mL and culture supernatant-1 mL. By replacing the enzyme with 1 mL of distilled water, the blank was prepared. The reaction mixtures were incubated at 30ºC for 15 min. The formation of a reddish brown colour due to guaiacol oxidation was observed, and the absorbance was measured at 450 nm using a UV-visible spectrophotometer. Laccase activity was expressed in International Units (IU), where one unit corresponds to the quantity of enzyme required to oxidize guaiacol of 1 µmol per minute under the assay conditions.

The Laccase activity in U/ml is calculated by this formula

$$\text{E.A} = \text{A x V/t x e x v}$$

Where, E.A = Enzyme activity, A =Absorbance, V =Total mixture volume (mL), v = enzyme volume (mL), t =incubation time, e = extinction coefficient for guaiacol (0.6740 µM/ cm).

Screening of manganese peroxidase

Manganese peroxidase screening can be assessed by the transformation of phenolic compounds to phenoxy radicals by the oxidation of Mn(II) to Mn(III) with the existence of an oxidant, H_2O_2. MnP activity was observed using the substrate phenol red. Newly prepared culture broth of 1 mL was used and treated to centrifugation for 10 min at 4°C and 7000 rpm. The supernatant from each centrifuge tube were collected for MnP assay as it contains manganese peroxidase as it is the extracellular enzyme. The 5 mL reaction mixture contains 1.0 mL sodium acetate buffer (50 mM, pH 4.5), 0.1mL of phenol red (0.1 mM), 0.4 mL of H_2O_2, and 0.4 mL of manganese sulphate (0.1 mM). The mixture was then incubated at 30°C. 40 µl of 5N NaOH was added into 1 mL of the reaction mixture after the reaction to stop the reaction and absorbance was measured at 610 nm. The aforementioned procedure was repeated at 1 min intervals, utilizing a 1 mL aliquot of the reaction mixture, for the duration of up to 4 min. A 0.1 U mL^{-1} min^{-1} rise in absorbance equals 1 U of enzyme activity

RESULTS AND DISSCUSSION

From the sample 4 x10 ^{4} colonies were developed and one colony found to be reddish brown on agar plates confirms laccase positive. The isolates was white powdery produced aerial and substrate mycelium with spiral spore identified as *Streptomyces* sp further confirmed by 16s rDNA analysis. Figure 1 represents the phylogenetic relatedness of laccase positive *Streptomyces* sp. The phylogenetic study based on 16S rRNA gene-sequences indicates that the *Streptomyces* isolate with accession PZ193905 has a nearby relationship with multiple reference species within the genus. The tree shows that isolate RP is related to *S. albus, S. somaliensis, S. sampsonii, and S. hydrogenans*, which means that they all come from the same evolutionary line. The short branch lengths (0.000-0.002) seen in these reference species show that their sequences are very similar, which means that they are closely related and have been preserved in the group. Although isolate RP is in the same cluster, it has a slightly longer branch length (0.012), which means that it is genetically different from its closest relatives. The matrix (Table 1) demonstrates that the isolate has the least genetic variation from *Streptomyces somaliensis* (0.006974), then from *Streptomyces albus* (0.00835) and finally from *Streptomyces sp.* (0.00834). These low scores show that isolate RP is most similar to *S. somaliensis* when compared to the other taxa. On the other hand, *S. sampsonii (0.017308), S. hoynatensis (0.0166338), and S. hydrogenans* (0.017249) had higher long-distance which means they were less similar to these species.

The very small distances between several reference species show that the sequences in the genus are very similar. This is common because the 16S rRNA gene is conserved. Nonetheless, it has been noted that 16S rRNA gene sequences frequently display exceedingly high similarity (>99%) among various *Streptomyces* species, complicating the precise delineation of species boundaries using this marker alone (Komaki, 2023). Kaur *et al*., (2022) have also reported laccase-positive *Streptomycetes*, which correlates with our findings.

Reactive red degradation under different carbon

The present study illustrates that the carbon source significantly influenced the decolorization of Reactive Red by laccase-positive *Streptomyces*, with the highest decolorization rate observed in dextrose (82.53±0.30%), succeeded by starch (68.8±0.30%), lactose (45.46±0.2%), and sucrose (23.2±0.2%). Figure 2 represents the percentage of reactive red decolorized by *Streptomyces* sp. This difference could be due to differences in how well carbon is used, which has a direct effect on how fast microbes grow and how much laccase they make. The Alba Blánquez *et al.* (2019) conducted similar studies, suggesting that carbon factors have significant impacts on reductase enzyme synthesis and removal of dyes by *Streptomyces* species. Microorganisms can easily break down simple sugars like glucose, which makes more enzymes and speeds up the breakdown of dye. Studies on microbial laccase production have shown that easily available carbon sources increase enzyme activity, while sugars that are hard to break down or take a long time to break down lower enzyme yield. The significant decolorization observed in dextrose

Table 1: Distance matrix of isolate and reference strains.

Strains	Isolate_ RP	*S. albus*	*Streptomyces*	*S. sampsonii*	*S. hoynatensis*	*S. somaliensis*	*S. hydrogenans*
Isolate_RP							
S. albus	0.00835						
Streptomyces	0.00834	0.000					
S. sampsonii	0.01730	0.000	0.000				
S. hoynatensis	0.01663	0.000	0.000	0.00134			
S. somaliensis	0.00697	0.000	0.000	0.000	0.000		
S. hydrogenans	0.01724	0.000	0.000	0.00271	0.00203	0.000	

medium in this investigation aligns with evidence that laccase produced by *Streptomyces* species efficiently decolorizes textile dyes, and the enzyme is integral to the oxidative destruction of azo and reactive dyes. Numerous studies indicate that bacterial laccase can degrade synthetic dyes in the presence of appropriate nutrients. The diminished decolorization in sucrose medium may be attributed to the slower absorption of disaccharides, which subsequently reduces enzyme synthesis. Lakshmaiah *et al.* (2023), conversely, identified lactose and the yeast extract as the optimal nitrogen and carbon sources for the bioremediation of reactive blue, accordingly Kameche *et al.* (2022) found that glucose and sucrose were the best sources of carbon for bio-decolorization, and ammonium chloride and yeast extract were the best sources of nitrogen.

Enzyme activity of isolated *Streptomyces* sp

The enzyme analysis of *Streptomyces* showed that different enzymes had different properties. For example, reductase activity (72.8±0.2 U) was higher than that of Mn-peroxidase (48.13±0.23 U) and laccase (23.87±0.30 U). This means that the strain makes more than one enzyme that helps break down dyes. However, the amount made depends on the type of enzyme. Figure 3 represent activity of extracellular enzyme produced by *Streptomyces* sp. The enzyme studies reveal that that reductase activity surpassed that of laccase and manganese peroxidase, indicating that catalytic

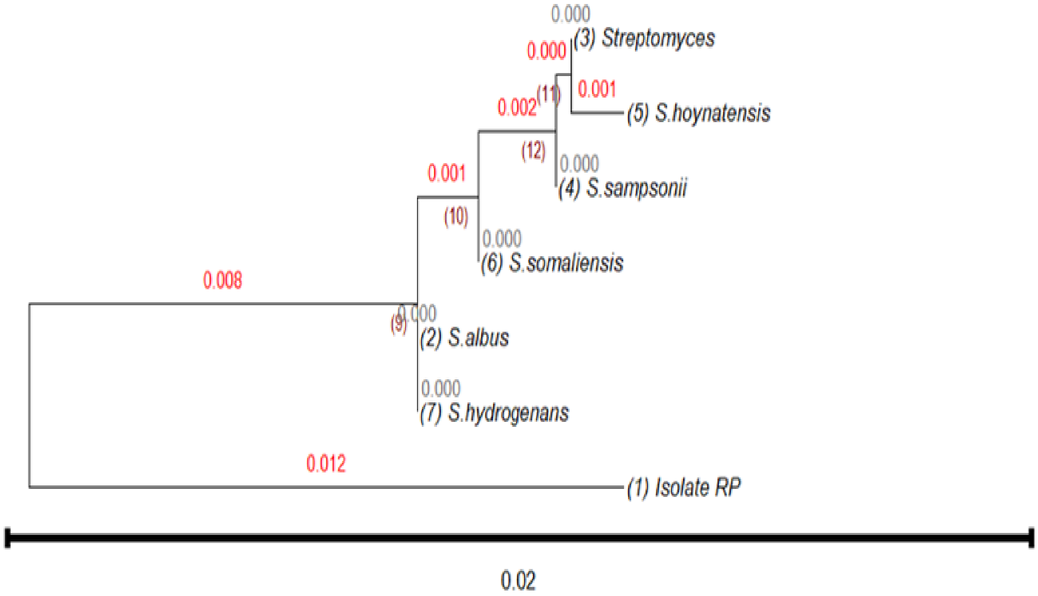

Figure 1: Phylogenetic relatedness of 16s rDNA of *Streptomyces* isolate.

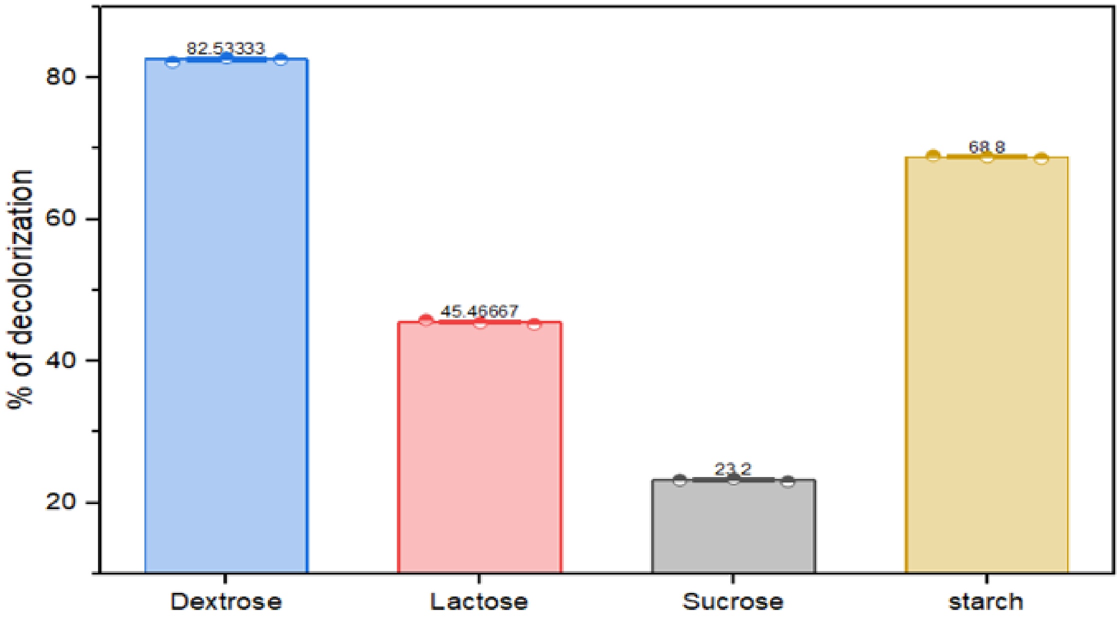

Figure 2: Percentage of Reactive red decolorized by *Streptomyces* sp.

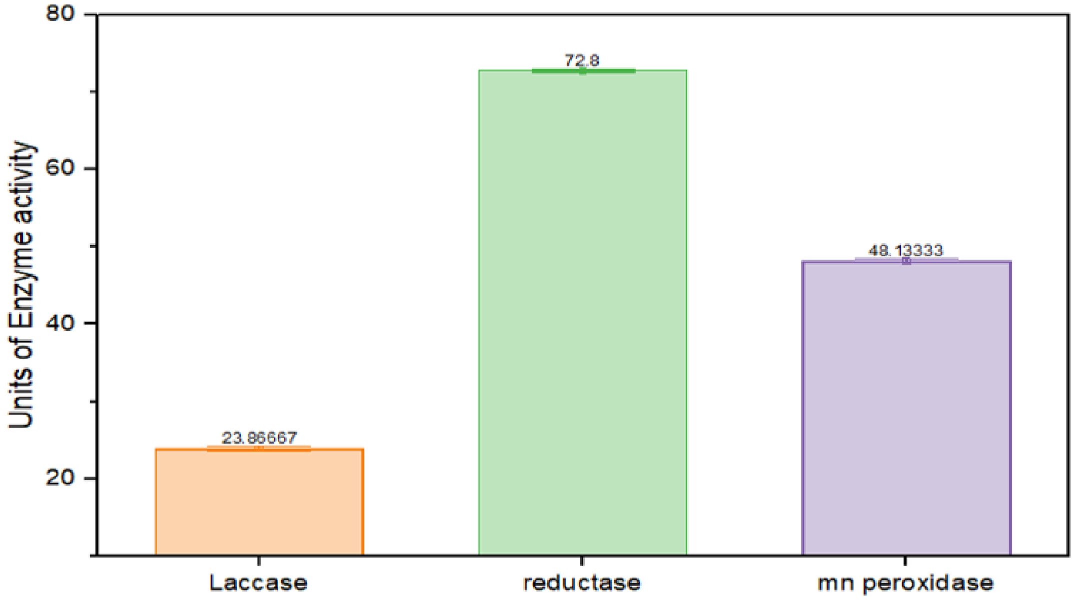

Figure 3: Activity of extracellular enzyme produced by *Streptomyces* sp.

enzymes are paramount for dye degradation by *Streptomyces*. Dong *et al*. (2019) reported analogous findings, demonstrating that azoreductase from *Streptomyces sp*. Lu *et al*. (2013) also found that *Streptomyces* that makes laccase breaks down textile dyes very well during biodegradation. Peroxidases and laccases are known to be involved in oxidative reactions that break down complicated dye structures into simpler ones. Qin *et al*. (2021) said that enzymes from *Streptomyces* that are like peroxidases and laccases work together to break down dyes and are very good at oxidizing synthetic dyes.

CONCLUSION

The current study evaluated the role of laccase-positive *Streptomyces* species with notable dye decolorization potential. Morphological, biochemical, and molecular examinations validated its taxonomic classification and its close phylogenetic affinity with *Streptomyces somaliensis*. The isolate effectively degraded Reactive Red dye, with dextrose recognized as the optimal carbon source for achieving maximum decolorization. Enzymatic analysis demonstrated that dye degradation is facilitated by a consortium of enzymes, with reductase activity surpassing that of laccase and manganese peroxidase. This means that even though the strain has laccase, reductase is very important for the process of biodegradation. In general, the results show how important enzymatic synergy is in breaking down dyes and suggest that this strain of *Streptomyces* could be very useful for eco-friendly bioremediation of textile dye pollutants.

ACKNOWLEDGEMENT

We thank the PG and Research Department of Microbiology, Dhanalakshmi Srinivasan College of Arts and Science for Women (Autonomous), Perambalur for supporting us to carry out this work.

ABBREVIATIONS

BLAST: Basic Local Alignment Search Tool; **CFU:** Colony Forming Units; **IU:** International Units; **ISP4:** International Streptomyces Project medium 4; **MnP:** Manganese Peroxidase; **NADH:** Nicotinamide Adenine Dinucleotide; **OD:** Optical Density; **PCR:** Polymerase Chain Reaction; **rRNA:** Ribosomal Ribonucleic Acid.

ETHICAL STATEMENT

This research work does not contain any studies with human participants or animals performed since it involved with soil samples and microbial isolates.

FINANCIAL SUPPORT AND SPONSORSHIP

No funding was received for this work from any organization.

CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

SUMMARY

The present study reveals the effectiveness of microorganisms particularly *Streptomyces* sp with respect to the degradation of harmful textile dyes when it is discharged into water ecosystem cause serious damage to the environment.

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Cite this article: Pavitra R, Raja A, Uma Devi P. Selective Isolation and Molecular Characterization of *Streptomyces* sp from Dye Discharged Site and its Enzyme Screening in Prospective to Azo Dye Degradation. Asian J Biol Life Sci. 2026;15(1):252-7.