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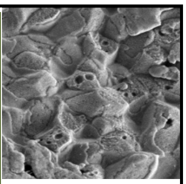

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SEM of Cocoa husk

Isolation and Characterization of a Methyl Red-Degrading *Pseudomonas* sp. from Contaminated Soil

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Abstract

Microbial-based bioremediation is a greener dye removal method than physicochemical methods like coagulation-flocculation, adsorption, membrane filtration, and advanced oxidation. In this study, we isolated and characterized a methyl red-degrading *Pseudomonas* sp. from contaminated soil, examining its growth and degradation under different environmental conditions. The bacterium, tentatively designated as *Pseudomonas* sp. strain Aft-12 demonstrated strong methyl red decolorization capacity. Time-course growth analysis in minimal salt medium supplemented with 200 mg/L methyl red, glucose as a carbon source, and ammonium sulphate as a nitrogen source revealed a minimal lag phase (~12 h), followed by rapid exponential growth and decolorization, reaching completion by 72 h. Strain Aft-12 performed effectively across a range of conditions, showing optimal growth between pH 6.5 and 7.0 and between 30 and 35 °C. Growth was optimal at dye concentrations of between 200 and 250 mg/L. Co-substrate screening revealed that glucose and sucrose significantly enhanced biomass, although Aft-12 maintained activity without supplementation. Azoreductase activity peaked at 1 µmol/min/mg protein after 48 h, supporting enzymatic decolorization via reductive cleavage. These characteristics make this bacterium a promising candidate for sustainable bioremediation of azo dye-laden effluents.

INTRODUCTION

Synthetic dyes are widely used in industries such as textiles, leather, food, cosmetics, and paper, owing to their vibrant colors, durability, and cost-effectiveness. Among them, azo dyes form the largest and most versatile class, characterized by the presence of one or more azo bonds (–N=N–) linking aromatic rings. Their application, however, comes at an environmental cost. Azo dyes are recalcitrant to degradation due to their complex molecular structures and often escape conventional wastewater treatment processes, accumulating in aquatic environments. Their azo bond (–N=N–) and aromatic rings give it strong chromophoric properties and make it very stable chemically. HoweverThese compounds pose health and ecological risks due to their toxicity, mutagenicity, and potential carcinogenicity. Methyl red ($C_{15}H_{15}N_3O_2$) is a synthetic monoazo dye that is commonly used as a pH indicator in analytical chemistry, as well as for dyeing and printing textiles and for biological tests, these same features also make it last a long time in the environment and make it toxic. Methyl red does not break down easily when it gets into ecosystems through industrial waste. It can build up in sediments and water bodies, which can cause long-term pollution problems [1].

Methyl red stays in the environment for a long time and breaks down into toxic products when there is not enough oxygen. When microbes or chemicals break the azo bond, they make aromatic amines like N,N-dimethyl-p-phenylenediamine (DMPD) and o-aminobenzoic acid (OABA). These chemicals are often more dangerous than the original dye because they can cause mutations and cancer. Studies have shown that azo dyes like methyl red can turn into aromatic amines, which are thought to be likely human carcinogens. This raises serious concerns for the environment and public health [2]. Methyl red has been shown to be harmful to fish, invertebrates, and algae in water. Sublethal levels make fish less able to eat, reproduce, and cause histopathological damage to their gills and liver. Also, its bright color makes it harder for light to get through, which slows down photosynthesis and changes the primary productivity of aquatic ecosystems. Studies that tested the effects of methyl red on algae have shown that it can lower the amount of chlorophyll and the efficiency of photosynthesis in green algae, which can throw off the balance of the ecosystem [1].

Methyl red is not used as a food dye, but people who work with textiles, paper, or in labs are exposed to it. Aerosols can irritate the lungs when breathed in, and contact with the skin can cause allergic dermatitis. Taking it in can cause stomach pain and, in severe cases, methemoglobinemia because of the release of aromatic amines. The International Agency for Research on Cancer (IARC) has put several aromatic amines made from azo dyes into Group 1 carcinogens (carcinogenic to humans), which shows how important it is to be safe when handling and getting rid of them [3].

Before being released into the environment, many countries' environmental laws require that effluents containing azo dyes be treated first. Adsorption on activated carbon, coagulation-flocculation, and advanced oxidation processes are all effective ways to get rid of things, but they are also expensive and can create more waste. Using certain bacteria and fungi to break down biological materials is a cheap and eco-friendly option that can turn methyl red into less harmful end products [4].

Microbial bioremediation offers a sustainable, eco-friendly, and cost-effective alternative to traditional dye removal techniques, which include coagulation-flocculation, adsorption, membrane filtration, and advanced oxidation. Several bacterial genera, including Bacillus, Klebsiella, Sphingomonas, and particularly *Pseudomonas*, are known for their metabolic versatility and dye-degrading capabilities. This study aimed to isolate and characterize a methyl red-degrading *Pseudomonas* sp. from contaminated soil, examining its growth and degradation profile under varying environmental conditions.

MATERIALS AND METHODS

Growth medium

Soil samples were collected from a textile effluent site in Pakistan. First, ten grams of each soil sample were suspended in 90 mL of sterile tap water, then serially diluted, and 0.1 mL was plated on nutrient agar supplemented with 100 mg/L of methyl red. A minimal salt medium (MSM) agar was prepared containing KH_2PO_4 (0.136%, w/v), Na_2HPO_4 (0.139%, w/v), $(NH_4)_2SO_4$ (0.77%, w/v) as the nitrogen source, $MgSO_4$ (0.001%, w/v), $CaCl_2$ (0.001%, w/v), and agar (1.5%, w/v) as described by [5]. Methyl red was filter-sterilized and added to a final concentration of 100 mg/L unless otherwise specified.

The cultures were incubated at room temperature in the dark. Colonies producing visible decolorization zones were selected and purified by serial transfer. Phenotypic characterization included Gram staining, motility, oxidase, catalase, nitrate reduction, citrate utilization, and ONPG activity tests. Identification was aided by the ABIS online system. Optimization experiments tested pH (5.5–8.5), temperature (25–45°C), and methyl red concentrations (50–400 mg/L). Glucose and yeast extract were evaluated for their effects on degradation rates. All experiments were performed in triplicate, and statistical significance was determined using one-way ANOVA.

Azoreductase enzyme activity determination

Azoreductase activity was quantified spectrophotometrically by observing the reduction in absorbance of methyl red at 430 nm in the presence of the substrate NADH. Crude enzyme was obtained from freshly cultivated bacterial biomass by resuspending the harvested cells in a sodium phosphate buffer (50 mM, pH 5.5) and disrupting the cells with a sonicator, utilizing multiple short bursts while maintaining the temperature at 4 °C to avert thermal inactivation. The disrupted suspension was centrifuged at 10,000 g for 30 minutes at 4 °C to eliminate cell debris, and the resultant clear supernatant was utilized as the enzyme source.

The standard reaction mixture (total volume of 2.2 mL) comprised 152 µM methyl red, 50 mM sodium phosphate buffer (pH 5.5), 20 mM NADH, and a suitable volume of the crude enzyme extract. The reaction commenced with the addition of the enzyme, and the reduction in absorbance at 430 nm was monitored over time using a UV–visible spectrophotometer. Controls devoid of either NADH or enzyme were incorporated to account for non-enzymatic reduction. One unit of azoreductase activity is defined as the quantity of enzyme necessary to reduce 1 µg of methyl red per minute per mg of protein under the specified assay conditions [6].

RESULTS AND DISCUSSION

Identification and Morphological Characterization

The isolate Aft-12, obtained from dye-contaminated soil near a textile effluent discharge site, displayed high methyl red decolorization activity during preliminary screening. On nutrient agar containing methyl red, the isolate produced a distinct decolorization halo, indicating active dye degradation. Microscopically, the cells were Gram-negative rods, motile, and arranged singly or in pairs. Colonies on nutrient agar were creamy-white, smooth, circular, and slightly raised after 24 h at 30 °C. Biochemical profiling confirmed oxidase and catalase positivity, nitrate reduction, and citrate utilization, consistent with *Pseudomonas* characteristics. The ABIS online system assigned the isolate to *Pseudomonas putida* with 96% probability. The bacterium is tentatively identified as *Pseudomonas* sp. strain Aft-12. These results align with earlier studies where *Pseudomonas* spp. were consistently associated with dye-degrading capacity due to their broad enzymatic repertoire [7].

Time-course of Methyl Red Decolorization

Fig. 1 displays the growth profile of isolate Aft-12 over 108 hours in minimal salt medium (MSM) containing 200 mg/L methyl red at pH 7.0 and 25 °C. The bacterial population, expressed as Ln CFU/mL, showed a distinct lag phase lasting up to approximately 12 hours, followed by a rapid exponential growth phase between 12 and 72 hours. The growth plateaued beyond 72 hours, suggesting entry into the stationary phase. The initial lag phase suggests that Aft-12 either constitutively produces azoreductase or rapidly induces its synthesis upon exposure to methyl red, enabling efficient decolorization as soon as growth is initiated. Like Sugiura et al. [8] *Pseudomonas* sp. PR41-1, which made azoreductase that could turn methyl red into dimethyl-p-phenylenediamine and o-aminobenzoic acid, Aft-12 seems to use reductive cleavage to remove color from dyes. However, unlike PR41-1, which was grown in a nutrient-rich medium, Aft-12 retained its decolorization ability in minimal medium without added organic nitrogen. This shows that it can be used for cheap wastewater treatment.

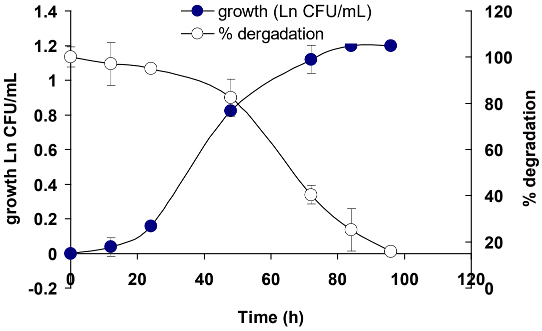

Fig. 1. Time-course growth and decolorization of methyl red (200 mg/L) by *Pseudomonas* sp. Aft-12 under optimal conditions. Error bars represent SD (n=3). Glucose concentration was set at 0.5% (w/v).

Aft-12 worked just as well as *Galactomyces geotrichum* MTCC 1360, which completely removed methyl red (100 mg/L) from deionized water in 1 hour and could handle a wide range of pH (3–12) and temperature (5–50 °C) [9]. However, Aft-12's ability to grow in a minimal salts medium without adding co-substrates is better than co-metabolic systems that need extra glucose or molasses to speed up the process. *G. geotrichum* and Aft-12 can adapt to a wide range of environments, which suggests that they could be useful in a variety of wastewater situations. Also, Aft-12's ability to completely remove methyl red without the need for LB medium with copper, unlike laccase-producing Stenotrophomonas maltophilia AAP56, which needed LB medium with copper for effective dye removal [10], shows that it has even more potential for bioremediation. Being able to work well in effluents with few nutrients can cut costs and lower the risk of secondary pollution by a lot.

Influence of pH

Strain Aft-12 worked best between pH 6.5 and 7.0, as indicated by ANOVA analysis (**Fig. 2**). At pH levels below and above this range, the efficiency of growth on this dye dropped significantly. This neutral-pH optimum is the same as that of *Klebsiella pneumoniae* RS-13, which removed 100% of methyl red at pH 7.0 in 24 hours under aerobic conditions (Wong & Yuen, 1996), and *Sphingomonas paucimobilis*, which showed quick decolorization (99.6% at 750 mg/L in 10 hours) at pH 9.0 but stayed effective over a wide range (pH 3–11) [7]. On the other hand, *Galactomyces geotrichum* MTCC 1360 could grow in a wide range of pH levels (3.0–12.0) and temperatures (5 to 50 °C), but it needed extra glucose or other sugars to grow quickly [9], consistent with this study. Also, *Stenotrophomonas maltophilia* AAP56, which makes laccase, worked best at pH 7.0 when grown in LB medium with copper. This shows that enzymatic oxidation systems can also be set to work well at almost neutral pH [10]. From a practical point of view, Aft-12's preference for neutral pH is good because textile effluents that have been treated once often stay close to neutral, which means that pH adjustment is less necessary.

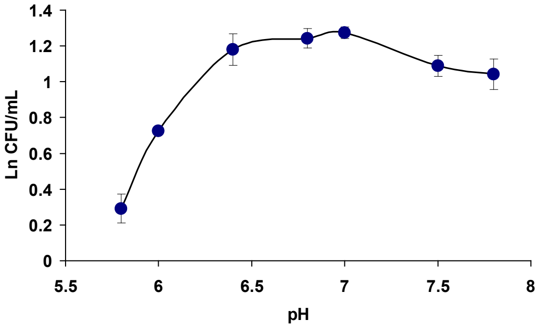

Fig. 2. Effect of pH on methyl red decolorization efficiency by *Pseudomonas* sp. Aft-12. Error bars represent SD (n=3). Glucose concentration was set at 0.5% (w/v).

Influence of Temperature

The effect of temperature on the growth of the bacterium on methyl red (**Fig. 3**) shows that the optimal temperature was between 30 and 35 °C, with good activity maintained between 25 and 35 °C. At 40 °C, efficiency dropped, and further decreased above 45 °C. Comparable optima have been reported for *Pseudomonas luteola*, which achieved >90% methyl red removal at 35 °C under static conditions in nutrient broth [11], and for *Enterobacter* strains isolated from dye-contaminated soils, which maintained high decolorization rates between 30 °C and 37 °C [12].

Similar mesophilic ranges are observed in *Sphingomonas paucimobilis* (optimum 35–40 °C; [7] and *Galactomyces geotrichum* MTCC 1360 (5–50 °C tolerance; [9], though the latter is psychrotolerant and thermotolerant due to its eukaryotic metabolism. This mesophilic profile suggests that Aft-12 would perform well in ambient tropical climates, consistent with the environmental conditions of its isolation site, and would not require external heating or cooling in most field applications.

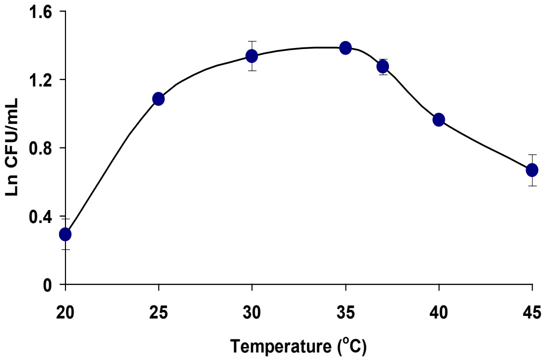

Fig. 3. Effect of temperature on methyl red decolorization efficiency by *Pseudomonas* sp. Aft-12. Error bars represent SD (n=3). Glucose concentration was set at 0.5% (w/v).

Effect of Initial Dye Concentration

The effect of different methyl red concentrations (100–400 mg/L) on the growth of isolate Aft-12 over a 96-hour period measured as natural log (Ln) CFU/mL showed that the bacterial growth exhibited an increase with elevated methyl red concentrations, reaching a peak between 200 and 250 mg/L, at which point the maximum biomass was observed (Ln CFU/mL ~1.4–1.5) (**Fig. 4**). A decline in growth was observed beyond 250 mg/L, with significantly reduced cell density at 350 and 400 mg/L, indicating potential substrate inhibition or stress response at elevated dye concentrations. This trend suggests that Aft-12 can tolerate and thrive in the presence of methyl red at concentrations up to 250 mg/L; however, higher dye concentrations may impede cell proliferation and adversely affect decolorization efficiency. The findings indicate that a methyl red concentration range of 200–250 mg/L is optimal for promoting robust Aft-12 growth and biotransformation activity. Such high tolerance exceeds that of *Klebsiella pneumoniae* RS-13 (max = 100 mg/L) and *Citrobacter* sp. (max = 150 mg/L) but is comparable to the tolerance of *Galactomyces geotrichum* MTCC 1360 (300 mg/L) [9]. The ability to tolerate and degrade high dye loads is critical for practical wastewater treatment, especially for concentrated effluents from dye-bath discharges.

Fig. 4 illustrates the effect of methyl red concentration on decolorization efficiency. Strain Aft-12 completely decolorized 50–100 mg/L methyl red within 48 h. At 200 mg/L, removal efficiency was 95%, dropping to 74.2% at 400 mg/L. This high tolerance exceeds that of *Klebsiella pneumoniae* RS-13, which achieved complete removal only up to 100 mg/L [5], and *Citrobacter* sp., which tolerated up to 150 mg/L before efficiency dropped significantly [13]. It is comparable to *Galactomyces geotrichum* MTCC 1360, which decolorized up to 300 mg/L methyl red but required co-substrate supplementation [9].

In addition, *Sphingomonas paucimobilis* degraded methyl red efficiently at concentrations up to 750 mg/L within 10 h [7], though performance decreased sharply at higher loads. The ability of Aft-12 to tolerate and degrade high dye concentrations without co-metabolism is advantageous for practical wastewater treatment, particularly for concentrated dye-bath discharges where dilution prior to treatment is not feasible.

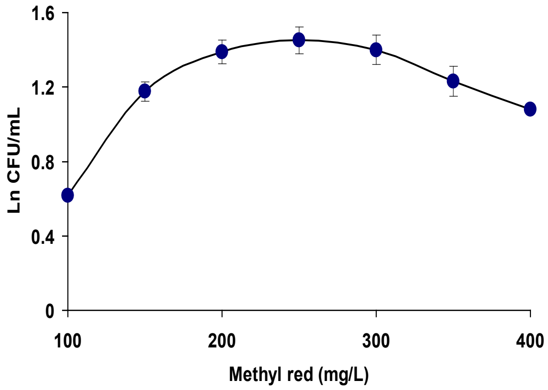

Fig. 4. Effect of initial methyl red concentration on decolorization efficiency by *Pseudomonas* sp. Aft-12. Error bars represent SD (n=3). Glucose concentration was set at 0.5% (w/v).

Effect of Co-substrates

The process ofn glucose supplementation (0.1% w/v) was found to significantly enhanced cell growth, as indicated by the highest biomass (Ln CFU/mL ≈ 1.5), which is followed closely by sucrose (**Fig. 5**). In contrast, other carbon sources such as mannitol, acetate, and citrate displayed no considerable effect on bacterial growth, with cell densities comparable to the no-carbon control with the dye as the sole carbon source. These findings suggest that glucose and sucrose are the most favorable carbon sources for supporting this strain's metabolic activity, which is likely to enhance its decolorization potential in subsequent assays. This suggests a co-metabolic mechanism in Aft-12, where readily metabolizable carbon sources increase intracellular NADH availability, thereby fueling azoreductase activity. Similar glucose stimulation has been reported in *Pseudomonas putida* for azo dye degradation, where additional carbon enhanced reductive cleavage rates [14].

Comparable effects have been observed in *Sphingomonas paucimobilis*, which achieved complete methyl red degradation within 6–8 h in the presence of glucose, and in *Galactomyces geotrichum* MTCC 1360, where co-substrate addition was essential for rapid color removal [7,9]. In contrast, *Stenotrophomonas maltophilia* AAP56 required nutrient-rich LB medium and copper supplementation to maximize laccase-mediated dye oxidation [10]. The ability of Aft-12 to function without co-substrates, yet respond positively to glucose, provides operational flexibility in varying wastewater compositions.

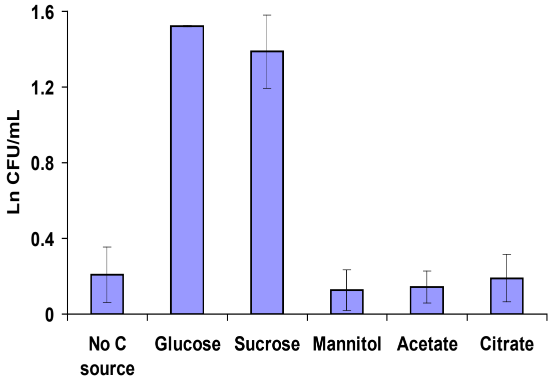

Fig. 5. Effect of different carbon sources on the growth of strain Aft-12 as measured by Ln CFU/mL. Glucose and sucrose significantly enhanced biomass production compared to the no-carbon control and other tested carbon sources (mannitol, acetate, citrate). Error bars represent standard deviation from triplicate experiments.

Azoreductase Activity

Azoreductase activity in crude extracts peaked at 1 µmol/min/mg protein after 48 h (Data not shown), coinciding with the point of maximum methyl red removal by Aft-12. This level of activity is consistent with aerobic azo bond cleavage, where NADH-dependent enzymes catalyze the reductive cleavage of the –N=N– linkage. Comparable activities have been reported for *Pseudomonas luteola* degrading azo dyes (0.88 µmol/min/mg protein [11]) and for *Enterobacter* sp. Aft-3 isolated from dye effluents (0.95 µmol/min/mg protein [12].

Similar enzymatic capacities were also observed in *Sphingomonas paucimobilis*, where azoreductase activity paralleled rapid decolorization within 10 h at 750 mg/L methyl red [7]), and in *Bacillus* sp. OY1-2, which reduced methyl red to dimethyl-p-phenylenediamine and o-aminobenzoic acid under optimal pH 7.5 conditions [8]. These findings reinforce that Aft-12's decolorization is enzyme-driven rather than solely due to adsorption or non-specific degradation.

Table 1. Comparative characteristics of methyl-red decolorizing bacterium.

Strain	Optimal pH	Temp (°C)	Max MR Conc. (mg/L)	Ref.
Pseudomonas sp. Aft-12	6.5 to 7.0	25 to 35	200 to 250	This study
Klebsiella pneumoniae RS-13	7.0	30	100	[5]
Galactomyces geotrichum MTCC 1360	3.0–12.0	30	300	[14]
Sphingomonas paucimobilis	6.5	32	200	[7]
Citrobacter sp.	6.0	30	150	[6]
Enterobacter sp. Aft-3	n.a.	n.a.	100	[12]

Note: n.a. Not available

CONCLUSION

Pseudomonas sp. strain Aft-12 shows good potential as a cost-effective tool for the azo dye methyl red bioremediation. Isolated from dye-contaminated soil, the strain efficiently decolorized methyl red in minimal medium without the need for rich nutrient supplementation. Optimal performance was recorded at pH 6.5–7.0 and 30–35 °C, which are conditions commonly found in secondary-treated textile wastewater. The bacterium exhibits good tolerance and decolorizes methyl red up to 250 mg/L, with azoreductase activity aligning with peak degradation periods. Unlike many strains requiring co-substrates, Aft-12 achieved high efficiency autonomously, although glucose and sucrose improved performance. Compared to previously reported strains, Aft-12 offers operational simplicity, metabolic flexibility, and high dye tolerance. These findings provide preliminary results for the bacterium in eco-friendly future usage in wastewater treatment systems, especially for concentrated dye discharges, without the need for costly additives or extreme pH/temperature adjustments.

REFERENCES

1. Gupte S, Keharia H, Gupte A. Toxicity analysis of azo Red BS and Methyl Red dye solutions on earthworm (*Pheretima phosthuma*), micro-organisms, and plants. Desalination Water Treat. 2013;51(22–24):4556–65.
2. Chung KT, Fulk GE, Andrews AW. Mutagenicity testing of some commonly used dyes. Appl Environ Microbiol. 1981 Oct;42(4):641–8.
3. IARC. Some Aromatic Amines, Organic Dyes, and Related Exposures. In: IARC monographs on the evaluation of carcinogenic risks to humans [Internet]. 2010 [cited 2025 Aug 9]. Available from: http://publications.iarc.who.int/Book-And-Report-Series/Iarc-Monographs-On-The-Identification-Of-Carcinogenic-Hazards-To-Humans/Some-Aromatic-Amines-Organic-Dyes-And-Related-Exposures-2010
4. Adedayo O, Javadpour S, Taylor C, Anderson WA, Moo-Young M. Decolourization and detoxification of Methyl Red by aerobic bacteria from a wastewater treatment plant. World J Microbiol Biotechnol. 2004;20(6):545–50.
5. Wong PK, Yuen PY. Decolorization and biodegradation of methyl red by Klebsiella pneumoniae RS-13. Water Res. 1996;30(7):1736–44.
6. Feng J, Heinze TM, Xu H, Cerniglia CE, Chen H. Evidence for significantly enhancing reduction of Azo dyes in *Escherichia coli* by expressed cytoplasmic azoreductase (AzoA) of Enterococcus faecalis. Protein Pept Lett. 2010;17(5):578–84.
7. Ayed L, Mahdhi A, Cheref A, Bakhrouf A. Decolorization and degradation of azo dye Methyl Red by an isolated *Sphingomonas paucimobilis*: Biotoxicity and metabolites characterization. Desalination. 2011 July 1;274(1):272–7.
8. Sugiura W, Miyashita T, Yokoyama T, Arai M. Isolation of azo-dye-degrading microorganisms and their application to white discharge printing of fabric. J Biosci Bioeng. 1999;88(5):577–81.
9. Jadhav SU, Kalme SD, Govindwar SP. Biodegradation of Methyl red by *Galactomyces geotrichum* MTCC 1360. Int Biodeterior Biodegrad. 2008 Sept 1;62(2):135–42.
10. Galai S, Limam F, Marzouki MN. A new *Stenotrophomonas maltophilia* strain producing laccase. use in decolorization of synthetics dyes. Appl Biochem Biotechnol. 2009;158(2):416–31.
11. Kalyani DC, Telke AA, Dhanve RS, Jadhav JP. Ecofriendly biodegradation and detoxification of Reactive Red 2 textile dye by newly isolated *Pseudomonas* sp. SUK1. J Hazard Mater. 2009 Apr 30;163(2):735–42.
12. Abd Shukor MS, Aftab K, Norazlina M, Effendi Halmi M, Sheikh A, Shukor M. Isolation of a Novel Molybdenum-reducing and Azo Dye Decolorizing *Enterobacter* sp. Strain Aft-3 from Pakistan. Chiang Mai Univ J Nat Sci. 2016 Jan 1;15:95–114.
13. An SY, Min SK, Cha IH, Choi YL, Cho YS, Kim CH, et al. Decolorization of triphenylmethane and azo dyes by Citrobacter sp. Biotechnol Lett. 2002 June 1;24(12):1037–40.
14. Jadhav JP, Parshetti GK, Kalme SD, Govindwar SP. Decolourization of azo dye methyl red by Saccharomyces cerevisiae MTCC 463. Chemosphere. 2007;68(2):394–400.