Received: 8 January 2018 | Revised: 6 April 2018 | Accepted: 16 April 2018

DOI: 10.1111/lre.12216

ORIGINAL ARTICLE

WILEY Lakes & Reservoirs Science, Policy and Management for Sustainable Use

# Biotreatment of paper mill effluent using alkaliphilic *Rhizobium* sp. NCIM 5590 isolated from meteoric alkaline Lonar Lake, Buldhana District, Maharashtra, India

**Avinash A. Raut**[1] | **Swapnil S. Phugare**[2] | **Dayanand C. Kalyani**[3] | **Shyam S. Bajekal**[4]

[1]Department of Biotechnology, Smt. Kasturbai Walchand College, Sangli, India

[2]Department of Biotechnology, Yashwantrao Chavan College of Science, Karad, India

[3]Department of Chemical and Biochemical Engineering, Technical University of Denmark, Lyngby, Denmark

[4]Department of Microbiology, Yashwantrao Chavan College of Science, Karad, India

**Correspondence**
Avinash A. Raut, Department of Biotechnology, Smt. Kasturbai Walchand College, Sangli, India.
Email: rautavinash@gmail.com

## Abstract

Bacterial strain *Rhizobium* sp. was isolated from littoral soil of meteoric alkaline Lonar Lake and used to treat paper mill waste water. The bacterium was found to fix 125.7 nmol of $C_2H_4$ formed $ml^{-1}$ culture media in 72 hr by ARA (acetylene reduction assay). The optimum pH for its growth was 12, whereas the optimum temperature was 40°C. The bacterium could tolerate salt concentrations up to 4%. The collected paper mill effluent used two dyes in combination, namely Acid Orange 7 (AO) and Acid Yellow 17 (AY) to produce brown paper, eventually producing coloured effluent containing unbound AO and AY. *Rhizobium* sp. was found to be efficient for decolorization of both AO and AY individually, as well as in a mixture of AO and AY, suggesting the ability of this strain for treating paper mill effluent. The strain was found to be efficient in reducing up to 85% of the colour from undiluted paper mill effluent within 18 hr. In addition, biotreatment also was effective in reducing COD, metals and toxicity from the waste water. Induction in the enzymes azo reductase and DCIP reductase indicated their possible involvement in biotreatment of paper mill effluent. The effluent toxicity before and after treatment was studied using *Allium cepa* root cells, evaluating various biochemical parameters to assess the toxicity, including lipid peroxidation, protein oxidation, antioxidant enzyme status (catalase, superoxide dismutase) and genotoxicity assays using single cell gel electrophoresis (SCGE).

**KEYWORDS**

*Allium cepa*, diazotrophic, protein oxidation, toxicity

## 1 | INTRODUCTION

There has been a significant increase in the release of organochemicals from industrial, agricultural and domestic activities into environment over the past few decades. Waste waters from the industrial sector have become a prime concern in this regard, with paper and pulp mills being one of the largest wastewater-producing industries. It is estimated that for every ton of paper produced, these mills generate about 60,000–300,000 L of highly coloured and toxic waste water (Thompson, Swain, Kay, & Forster, 2001). The wastewater colour is attributable primarily to lignin and its derivatives, which are discharged in effluents from the pulping, bleaching and chemical recovery stages of the plant processes. In addition to lignin and its derivatives, dyes are also responsible for furnishing colour to paper industry effluents. Dyes are commonly used to produce coloured paper, with unbound dyes eventually released into waste waters. In addition to the colour concerns, paper industry effluents also contain high chemical oxygen demand (COD) and biological oxygen demand (BOD) concentrations. Physical and chemical processes for

  

treating paper and pulp mill effluents are less desirable than biological treatment because of cost-ineffectiveness and residual effects. Increased environmental concerns, however, are pressuring the paper and pulp industry to look for cost-effective and environmental-friendly alternatives for wastewater treatment. Biological processes are attractive in this regard, as they can reduce colour, as well as BOD and COD in the effluents (Bajpai, Mehna, & Bajpai, 1993). To this end, attempts have been made to treat paper and pulp mill waste water with biological methods, using various microorganisms. These microbial sources include white-rot fungi, which possess efficient lignin-degrading potential, thereby being most widely used for biological treatments of lignin- and phenol-containing waste waters. One of the white-rot fungi (*Trametes versicolor*) was previously reported useful for colour removal from baggasse-based paper mill effluent (Modi, Chandra, & Garg, 1998). In view of these problems, recent research has focused on biotechnological approaches with white-rot fungi (WRF) because of their powerful lignin-degrading enzyme system (Haddadin, Al-Natour, Al-Qsous, & Robinson, 2002). Among them, *Phanerochaete chrysosporium* (Yin, Joyce, & Chang, 1989; Zouari, Labat, & Sayadi, 2002), *Lentinus edodes* (Esposito, Canhos, & Nelson, 1991; Wu, Xiao, & Yu, 2005) and *Trametes* (*Coriolus*) *versicolor* (Martin & Manzanares, 1994; Modi et al., 1998) have been reported to be effective in reducing the various pollution parameters of the Kraft bleach plant effluents. Further, although extensive work has been published on bioremediation of the paper industry effluents, work has focused in most cases on the removal of lignin and lignin-related derivatives, with little attention given to the dyes used in colouring the paper. Further, most of the effluent processes have focused on removal of colour, with little attention paid to its detoxification. These effluents, however, can cause considerable damage to receiving waters as they contain high levels of BOD, COD, chlorinated compounds (measured as adsorbable organic halides), AOX, suspended solids mainly fibres, fatty acids, tannins, resin acids, lignin and its derivatives, sulphur and sulphur compounds, etc. (Ali & Sreekrishnan, 2001). Thus, toxicity analysis of effluents after treatment is also an important factor requiring attention.

This study focuses on the removal of colour and the reduction in toxicity from paper mill waste water. Bioremediation has been coupled with toxicological and nitrogen fixation studies. The diazotrophic nature of *Rhizobium* sp. is an added advantage as, along with bioremediation, it can be useful as a nitrogen fixer, and possibly eventually playing an important role in phytoremediation. It is well known that *Rhizobium* sp. are symbiotic nitrogen fixers, with few exceptions (Sullivan, Eardly, Berkum, & Ronson, 1996). The isolate used in this study, however, was found to fix nitrogen non-symbiotically. Toxicological parameters before and after treatment of the effluent (antioxidant enzymes; protein oxidation; lipid peroxidation; genotoxicity), using an *Allium Cepa* test, were studied. Biodegradation of raw effluent liberated through paper mill by *Rhizobium* sp. was also evaluated with the aid of various analytical studies.

## 2 | MATERIALS AND METHODS

### 2.1 | Microorganism and culture conditions

The bacterial isolate was obtained from littoral soil at the south-eastern region of Lonar Lake in the Buldhana District, Maharashtra, India, at coordinates 19°58′23″.2627N and 76°30′46″.42289E, as detected by GPS. The soil at this location was found to be muddy, sticky and grey in colour, with a pH of 9.5. Enrichment of the bacteria was achieved by inoculating 1 g soil samples in sterile modified nitrogen-free mannitol (NFM) broth, with the following composition: 2% mannitol; 0.02% $K_2HPO_4$; 0.02% $MgSO_4{\cdot}7H_2O$; 0.02% NaCl; 0.01% $K_2SO_4$; and 0.5% $CaCO_3$, at a pH of 9.5. Isolation from the enriched culture was achieved by streak plate technique on the above-noted agar medium. Morphologically distinct colonies were selected and screened for their nitrogen-fixing ability, with the most potent isolate identified by 16S rRNA gene sequence analysis. The pure isolate culture was submitted to the National Collection of Industrial Microorganisms (NCIM), Pune, India.

### 2.2 | Phylogenetic analysis

A partial sequence of the 16S rDNA of the isolate was obtained from Microbial Culture Collection (MCC), Pune, India. The 16S rRNA sequence was initially analysed at the National Center for Biotechnology Information in the United States, (http://www.ncbi.nlm.nih.gov), using the BLAST tool, with corresponding sequences of homologous species obtained online from NCBI and used for phylogenetic analysis. The sequence was submitted to GenBank/EMBL/DDBJ under the accession number LC089016. The phylogenetic tree was constructed using 1000 base pair aligned sequences by the neighbour-joining method (p-distance model) using MEGA 4.0 (Tamura, Dudley, Nei, & Kumar, 2007).

### 2.3 | Acetylene reduction assay (ARA)

The nitrogen-fixing ability of the bacterium was analysed by acetylene reduction assay (ARA), as described by Barua, Tripathi, Chakraborty, Ghosh, and Chakrabarti (2011). Sterile glass vials poured with modified ANFM semi-solid medium were inoculated with a pure culture of the isolate (~$10^8$ cells/ml) and incubated at 35°C for 72 hr. Vials were then sealed with sterile rubber stoppers, with air from the headspace replaced with 10% v/v analytical grade acetylene gas with a sterile gas tight syringe (Hamilton make). The vials were incubated at 35°C for 72 hr. After incubation, the headspace gas was analysed for the presence of ethylene ($C_2H_4$) gas, using gas chromatography equipped with a Porapak N column (80/100 mesh), with nitrogen as the carrier gas and a hydrogen flame ionization detector (FID). Peaks were analysed and

the ethylene produced expressed in terms of nmol of $C_2H_4$ formed per ml culture media over 72 hr.

### 2.4 | Effects of environmental factors on growth and nitrogen

Environmental factors include pH, temperature and NaCl concentration effects on nitrogen fixation of the isolate. Cultivation of the organism was done in modified NFM broth. Total nitrogen was analysed with the micro-Kjeldahl method. Inoculated broth was withdrawn aseptically after 24-hr intervals and their nitrogen content (%) estimated with the above-noted method (Ma & Zuazaga, 1942).

### 2.5 | Chemicals and effluent

Nitro blue tetrazolium (NBT) was purchased from Hi-Media (Mumbai, India) and NADH from the Sigma Chemical Company (USA). Lower melting point agarose (LMPA) and normal melting point agarose (NMPA) were purchased from Sigma-Aldrich, USA. Water testing kits were obtained from Hi-media Laboratories (Mumbai, India). Paper mill waste water was obtained from local paper manufacturers in Ichalkaranji, India.

### 2.6 | Effluent analysis and biotreatment studies

Characterization of the effluent involved analysing various parameters, including COD, BOD, total solids (TS), total dissolved solids (TDS), total suspended solids (TSS), pH, nitrates, fluorides, chlorides and total hardness. The microbial culture was inoculated in 50-ml culture tubes containing 25 ml of yeast extract broth, and incubated for an initial 24 hr. and then inoculated to 250-ml Erlenmeyer flasks containing 100 ml undiluted paper mill effluent. The flasks were further incubated and observed for decolorization. Aliquots of 3 ml were withdrawn at different time intervals and centrifuged at 7,000 *g* for 15 min to separate the bacterial cell mass. Decolorization of the effluent was analysed using UV–Vis spectrophotometer (Systronics model 119). All analyses were performed in triplicate, with the activity expressed in terms of percentage decolorization.

### 2.7 | Enzymatic analysis

The bacterial cells, considered as control, were grown in yeast extract medium at 30°C for 24 hr and centrifuged at 8,000 *g* for 20 min. The cells (100 mg/ml) were suspended in potassium phosphate buffer (50 mM; pH 7.4) and sonicated (Sonics-vibracell ultrasonic processor), with the sonifier output set at 50 amp, with seven strokes each of 30 s at 3-minute intervals at 4°C. This homogenate was centrifuged at 10,000 *g* for 20 min and the supernatant used as a source of crude enzyme. A similar procedure was used to quantify enzyme activities after biotreatment of paper mill effluents. Azoreductase and NADH-DCIP reductase activity were assayed spectrophotometrically, using previously quoted methods (Jadhav et al. 2010; Phugare et al. 2011). DCIP reduction was calculated using an extinction coefficient of 19 mM/cm.

### 2.8 | Analytical studies

The metabolites produced after degradation of the paper mill effluent were extracted with an equal volume of ethyl acetate. The extracts were dried by evaporation, with the remaining residue redissolved in HPLC-grade methanol and used for FTIR, HPLC and GC-MS analysis. FTIR analysis was conducted using a Shimadzu 8400S spectrophotometer in the midinfrared region of 400–4,000 $cm^{-1}$, with a 16-scan speed. HPLC analysis was performed in an isocratic Waters 2690 system equipped with a dual absorbance detector, using $C_{18}$ column (4.6 mm × 250 mm) and HPLC-grade methanol as the mobile phase with flow rate of 1 ml/min. Identification of metabolites formed after degradation of paper mill effluent was carried out with a QP2010 gas chromatography, coupled with mass spectroscopy (Shimadzu). Catalase (CAT EC 1.11.1.6) activity was determined by measuring the $H_2O_2$ decomposition at 240 nm, using previously reported methods (Phugare et al. 2011). Superoxide dismutase (SOD EC 1.15.1.1) was assayed by the method of Achary, Jena, Panda, and Panda (2008).

### 2.9 | Biotreatment analysis

Lipid peroxidation and protein oxidation were measured using previously reported protocol (Achary et al., 2008; Phugare et al. 2011). The tissue for comet assay was taken by removing the carapace and excising the gills. Tissue homogenates were prepared in ice-cold 0.05-M Tris–HCl (pH 7.4) buffer and used for the comet assay. The comet assay was performed using earlier-reported protocol (Achary et al., 2008; Phugare et al. 2011).

## 3 | RESULTS

### 3.1 | Microorganism and phylogenetic analysis

The bacterial isolate was deposited at NCIM having number NCIM 5590. The neighbour-joining tree (p-distance model) shows the isolate SE-I identical with *Rhizobium* sp. (Figure 1).

### 3.2 | Acetylene reduction assay

Based on the acetylene reduction assay, the bacterium was capable of fixing atmospheric nitrogen, obtaining a value of 125.7 nmol of $C_2H_4$ formed per ml culture media in 72 hr. Barua et al. (2011) previously analysed the nitrogen-fixing potential of isolates by ARA, obtaining a value of 164.52 nmol of $C_2H_4$ formed per ml culture media in 72 hr.

### 3.3 | Effects of environmental factors on growth and nitrogen fixation

The optimum pH was 12 for both N-fixation and growth, at a temperature of 40°C. The bacterium could fix nitrogen

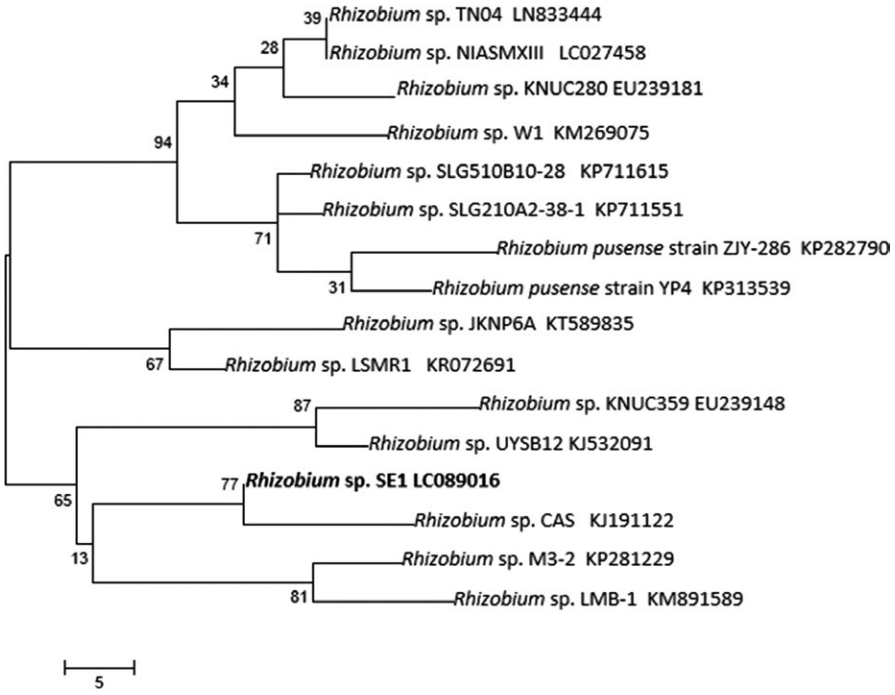


**FIGURE 1** Phylogenetic analysis of 16S rRNA sequence of *Rhizobium* sp. (per cent numbers at nodes indicate levels of bootstrap support, based on interior branch test of 1,000 replicates; scale bar (5) indicates interior branch distance)

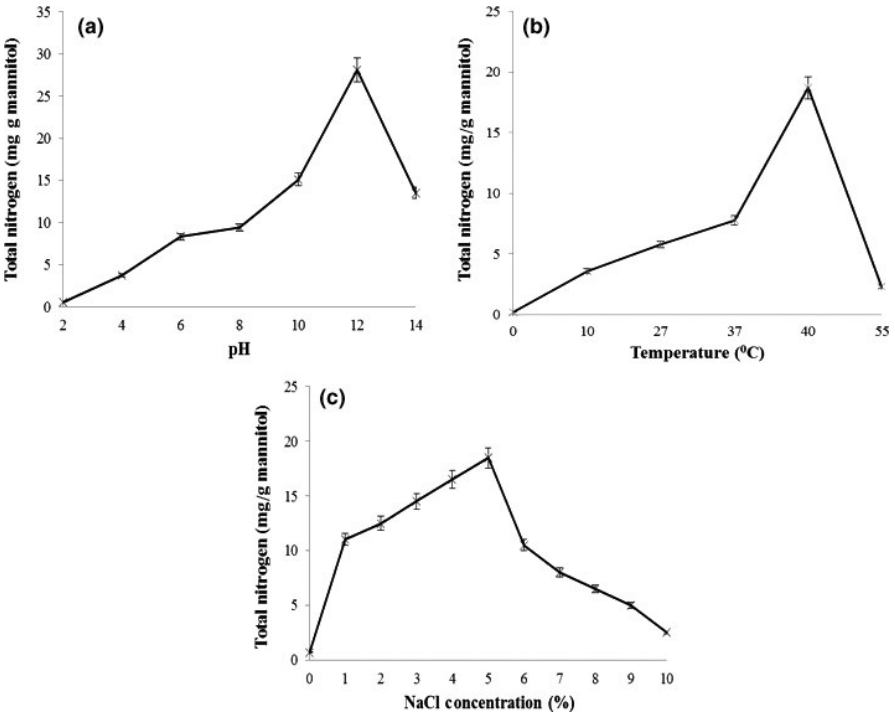


**FIGURE 2** (a) Effect of pH on growth and nitrogen fixation by *Rhizobium* sp. cultivated in modified ANFM broth; (b) effect of temperature on growth and nitrogen fixation by *Rhizobium* sp. cultivated in modified ANFM broth; (c) effect of NaCl concentration on growth and nitrogen fixation by *Rhizobium* sp. cultivated in modified ANFM broth

significantly up to 5% of salt concentration. In contrast, there is a steep decrease in its nitrogen-fixing ability as the salt concentration increases above 5% (Figure 2). These observations corroborate the hypothesis that extreme environmental conditions exert selective pressures causing organisms to differ in characteristics from those in conventional environments, as evidenced by the culture being alkaliphilic and moderately halotolerant in nature.

### 3.4 | Effluent analysis and biotreatment studies

This study revealed higher pollution loads in the paper mill effluents, compared to the allowable limits set by the National Environmental Quality Standards for sewage treatment facilities (Rehman, Bhatti, & Athar, 2009; Table 1). The effluent was characterized by a pH of 8.7, BOD and COD concentrations of 310 and 1,154 mg/L, respectively, TDS and TSS concentrations of 250

**TABLE 1** Summary of physicochemical characterization of paper mill effluent before and after treatment

| Parameter | NEQS values | Untreated effluent | Treated effluent |
|---|---|---|---|
| pH | 6–10 | 8.7 | 8.5 |
| $\lambda_{max}$ (nm) | – | 530 | 240 |
| COD (mg/L) | 400 | 1154 | 547 |
| BOD (mg/L) | 250 | 310 | 267 |
| TDS (mg/L) | 3,500 | 250 | 186 |
| TSS (mg/L) | 400 | 564 | – |
| Total solids (mg/L) | – | 814 | – |
| Cu (mg/L) | 1 | 0.026 | 0.020 |
| Fe (mg/L) | 2 | 0.865 | 0.246 |
| Zn (mg/L) | 5 | 0.043 | 0.037 |
| Pb (mg/L) | 0.5 | 0.012 | 0.012 |
| Ni (mg/L) | 1 | 0.015 | 0.009 |
| Cr (mg/L) | 1 | 0.017 | 0.0124 |
| Cd (mg/L) | 0.005 | 0.0078 | 0.0045 |

TDS, total dissolved solids; TSS, total suspended solids; NEQS, National Environmental Quality Standards.

and 564 mg/L, respectively, and a TS concentration of 814 mg/L. However, these values were significantly reduced after treatment with *Rhizobium* sp. (Table 1). Overall, *Rhizobium* sp. was found to be a potential candidate for biodegradation and detoxification of the paper mill effluents. A literature survey revealed that both bacteria and yeast species were effective candidates for biotreatment of industrial effluents, respectively (Patil, Phugare, Kalyani, Surwase, & Jadhav, 2012, Phugare et al. 2011). The bacterium reported herein is not only a potential candidate for biodegradation, but also appears capable of significant nitrogen fixation. Compared to earlier reports, this bacterium exhibits dual properties that would be beneficial for future bioremediation and phytoremediation strategies.

## 3.5 | Enzymatic analysis

A significant increase was noted for azo reductase and DCIP reductase activity during the biodegradation experiments. An induction in enzyme activity indicates possible involvement of *Rhizobium* sp. in the biodegradation of paper mill effluent. Table 2 provides an overview of the behaviour of the enzyme system before and after degradation. The paper mill effluent examined in this study mainly contained Acid Orange and Acid Yellow (Dyes with azo bond), supporting the azo reductase induction. Many researchers previously reported azo reductase induction during dye decolorization (Dhanve, Kalyani, Phugare, & Jadhav, 2009; Patil, Phugare, Jadhav, & Jadhav, 2010).

## 3.6 | Biotreatment analysis

Biodegradation of the paper mill effluent was confirmed using various analytical techniques, including HPLC, FTIR and GC-MS. The HPLC elution profile of untreated effluent sample produced

**TABLE 2** Enzyme activities (values are means of three experiments and *SEM* (±) is significantly different from the control cells at $^*p < 0.001$ by one-way analysis of variance (ANOVA) test with Tukey–Kramer multiple comparison tests)

| Enzyme | Control | After treatment |
|---|---|---|
| Azo reductase ($min^{-1}$ $mg^{-1}$ protein) | 0.125 ± 0.023 | 0.321* ± 0.017 |
| DCIP reductase (μg of DCIP reduced $min^{-1}$ $mg^{-1}$ cells) | 8.91 ± 1.457 | 10.36* ± 1.146 |

six peaks at retention times of 2.6, 2.8, 3.0, 3.4, 3.6 and 4.1 min (Figure 3a). The treated effluent obtained exhibited three peaks at retention times of 2.0, 2.6 and 3.9 (Figure 3b). Comparative analysis of the peak pattern before and after microbial treatment indicated the number of peaks and absorbance were reduced, suggesting both effluent degradation and decolorization. FTIR spectrum of the effluent (Figure 4a) indicated peaks at 3,437.28 $cm^{-1}$, suggesting the possible presence of N-H stretching of primary amides, 2,926 $cm^{-1}$ for C–H stretching of asymmetric alkanes, 1,620 $cm^{-1}$ for five-membered ring structure, 1,444 $cm^{-1}$ indicative of alkanes and 1,043 $cm^{-1}$ for trisubstituted benzene. The presence of the various peaks is indicative of complex substances in the effluent. FTIR spectrum of the treated effluent (Figure 4b) indicates significant variation in the fingerprint region of the spectrum, concluding that microbial action leads to the transformation of complex paper mill effluent. FTIR spectrum exhibits peaks at 1,749 $cm^{-1}$ for secondary amines, 1,246 $cm^{-1}$ confirming the presence of sulphur group and 1,021 $cm^{-1}$ for primary alcohol with C–OH stretching.

GC-MS analysis indicated the appearance of distinct mass peaks. On the basis of the fragmentation pattern of these mass peaks, a degradative pathway for the effluent (containing mainly Acid Orange 7 and Acid Yellow 17 molecules) was proposed

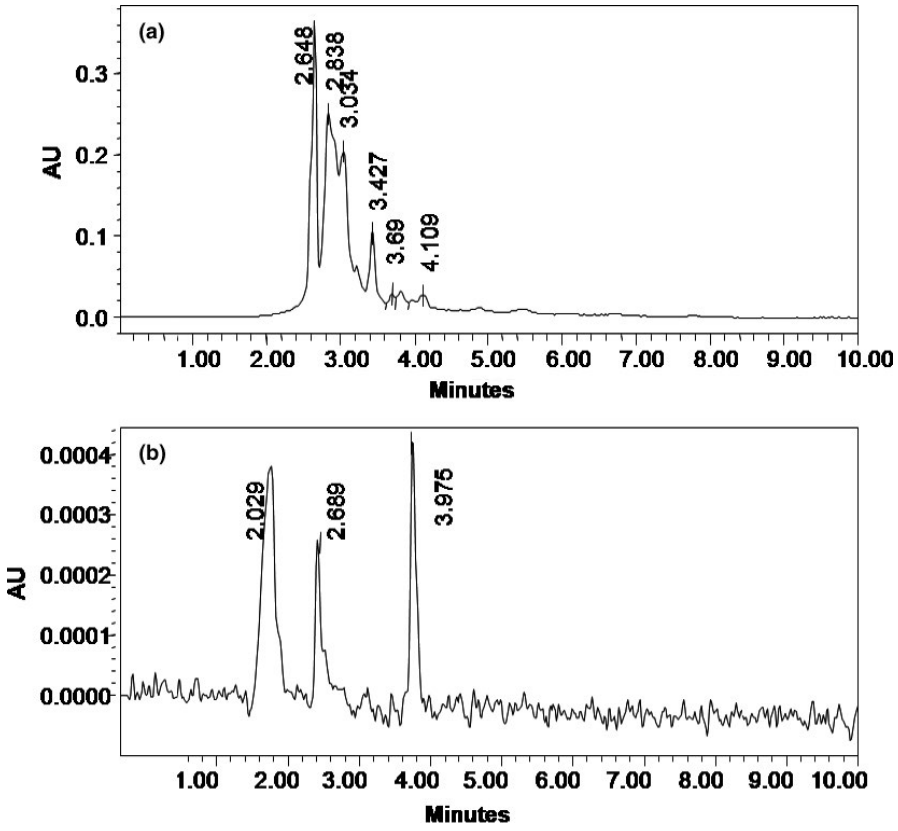


**FIGURE 3** (a) HPLC elution profile of paper mill effluent; (b) degradation metabolites of dye after bacterial treatment

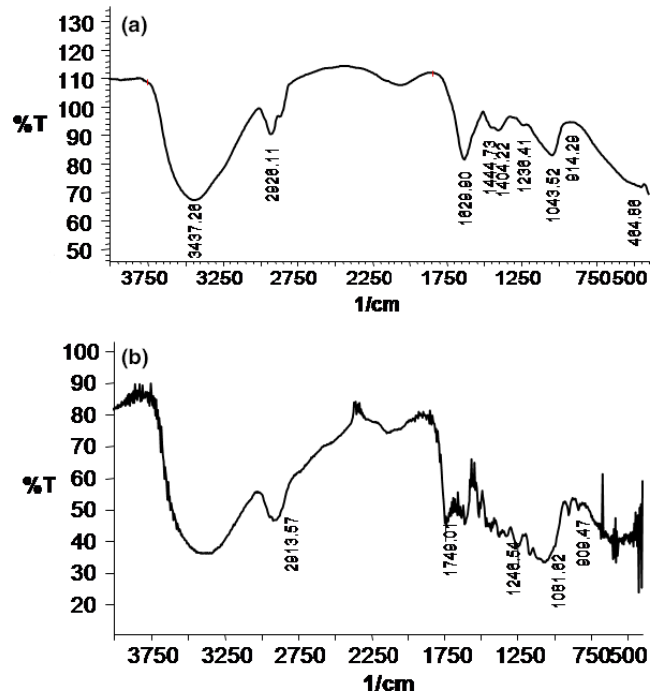


**FIGURE 4** (a) FTIR spectrum of paper mill effluent; (b) degradation metabolites of dye after bacterial treatment

(Figure 5). The pathway mainly follows azo cleavage and desulfonation rout for effluent biotransformation. The major metabolites traced were 1-amino-napthalene-2-ol (M.W. 158, mass peak 158), 3-(6-methylene-cyclohexa2-4 dienylidene)-propen-2ol (M.W. 142, mass peak 143) and 4 amino-2-(2, 5-dichloro-4 methyl-phenyl)-5-methyl-2H-pyrazol-3-ol (M.W. 236, mass peak 235). The first two metabolites would be possible cleavage products of Acid Orange 7, as predicted via the pathway (Figure 5a), whereas the last metabolite may be the cleavage product of Acid Yellow 17 (Figure 5b). The analytical results suggest the effluent was significantly transformed to different metabolites through microbial action.

## 4 | DISCUSSION

The present study provided evidence that *Rhizobium* sp. is a diazotrophic strain that can be effectively used for treating paper mill effluents. The use of *Rhizobium* sp. for bioremediation of industrial effluent could be adventitious as the strain is diazotrophic in nature. Thus, the process can also be effectively coupled to phytoremediation. Moreover, the isolate *Rhizobium* sp., not only degraded the effluent, but also detoxified it, as evidenced by various toxicological parameters.

Toxicity analysis was one of the major components in the present study, which has also been coupled with the biodegradation and biotransformation studies. The *Allium cepa* test of the effluent before and after biotreatment with *Rhizobium* sp. was used for toxicological judgement. Table 2 suggests that plants exposed to untreated effluent exhibited elevated levels to SOD ($26.55 \pm 2.63$ mg$^{-1}$ protein hr$^{-1}$) and CAT

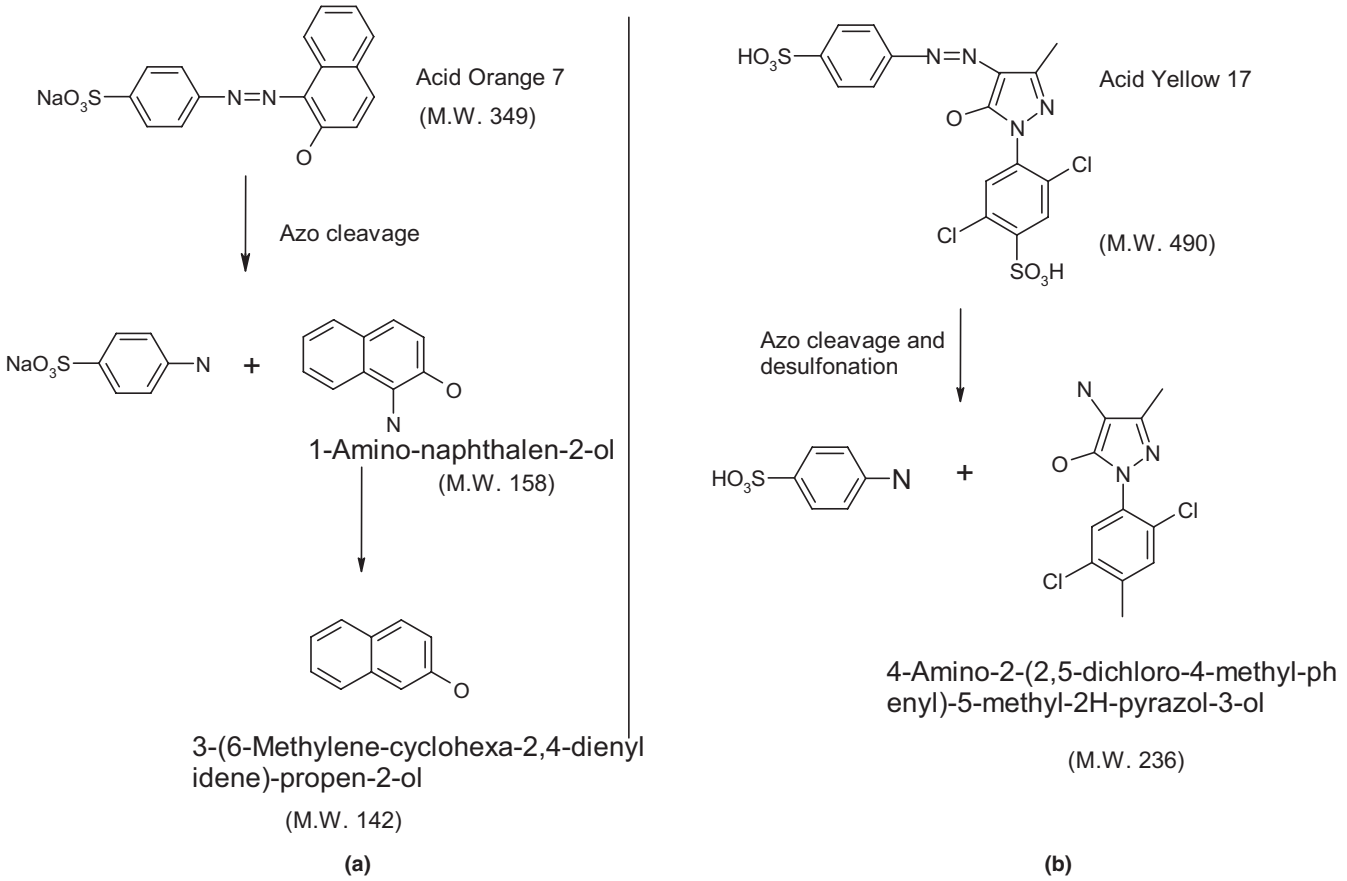


**FIGURE 5** Biodegradation pathway of effluent, predicted through GC-MS mass fragmentation pattern: (a) biodegradation pathway of Acid Orange 7; (b) biodegradation pathway of Acid Yellow 17

**TABLE 3** Antioxidant enzyme activities (SOD and CAT), protein oxidation, lipid peroxidation from root cells of *A. Cepa* exposed to treated and untreated paper mill effluent (values are mean of three experiments; *SEM* (±) is significantly different from the control at, $^*p < 0.001$, by one-way analysis of variance (ANOVA) with Tukey–Kramer comparison test)

| Parameter analysed | Control | Raw effluent | Treated effluent |
|---|---|---|---|
| SOD activity (inhibition of NBT reduction by 50%) $mg^{-1}$ protein $hr^{-1}$ | 3.12 ± 1.13 | 26.55 ± 2.63* | 9.85 ± 2.19 |
| CAT activity (nmol of $H_2O_2$ utilized) per mg protein | 35.40 ± 2.94 | 47.56 ± 1.19* | 39.75 ± 2.35* |
| Protein oxidation (Carbonyl content in nmol/mg protein) | 2.4 ± 0.87 | 7.57 ± 2.05* | 4.07 ± 1.31 |
| Lipid peroxidation (MDA nmol/g FW) | 0.20 ± 0.54 | 0.92 ± 0.80* | 0.25 ± 0.54 |

(47.56 ± 1.19 $mg^{-1}$ protein) activities, compared to plants exposed to treated effluent. Toxicity analysis on plant populations, therefore, is of great importance as they are important commercial products used in day-to-day human activities (Phugare et al. 2011). It was previously shown that industrial effluents can induce production of reactive oxygen species (ROS) in both plant and mammalian cells, which induce oxidative stress (Moore, 2008; Cross, Vliet, Louie, Thiele, & Halliwell, 1998; Phugare et al. 2011). Further, developed oxidative stress is harmful for the DNA, protein and lipid profile of the cells. The studies highlighted significant damage to protein and lipids of plant cell membrane, as evidenced by elevated values of protein oxidation and lipid peroxidation (Table 3). The protein oxidation and lipid peroxidation activities were significantly increased in untreated effluent, compared to treated effluent.

The genotoxic potential (DNA damaging) of the effluent was analysed through comet assay using *A. cepa* root tissue. The root meristem cells were used for electrophoretic analysis and for detecting subsequent DNA damage. The percentage of tail DNA (% of DNA in comet tail) and tail length (μm; Table 4) indicates significant DNA damage in untreated effluent, compared to control and treated effluents. The sensitivity of comet assay allows rapid prediction of the genotoxic potential of compounds, having been shown to be useful for both in vivo and in vitro biomonitoring of environmental pollutants (Saghirzadeh, Gharaati, Mohammadi, & Nejad, 2008). On the other hand, the *A. cepa* is among the plant

**TABLE 4** Detection of DNA damage in *Allium cepa* root meristem cells exposed to treated and untreated paper mill effluent using comet assay (values are mean of three experiments; *SEM* (±), significantly different from control (roots meristem germinated in water); *$p < 0.001$, by one-way analysis of variance (ANOVA) with Tukey–Kramer comparison test)

| | Samples | | |
|---|---|---|---|
| Analysis | Control | Raw effluent | Treated effluent |
| TL (μm) Mean ± *SD* | 12.44 ± 1.43 | 31.53 ± 2.97* | 21.40 ± 1.38 |
| % T Mean ± *SD* | 31.50 ± 2.62 | 52.14 ± 2.45* | 39.46 ± 1.47 |

bioassays reviewed by the US Environmental Protection Agency (EPA) Gene-Tox Program in 1980 (Bernhard, Tamara, Maria, & Siegfried, 2005; Grant, 1982). This plant has often been used to determine the cytotoxic and genotoxic effects of various substances (Ma, 1999; Grant, 1999; Bernhard et al., 2005). Overall, toxicological parameters illustrated that raw effluent could be extremely toxic if released untreated to the ecosystem. On the other hand, microbial treatment of such effluents could reduce the toxicity to a significant extent, thereby minimizing the environmental impacts. The results of the present study also reveal that microbes from alkaline, as well as acid lakes, can be used to treat industrial effluents before they are discharged into lakes, reservoirs and other water systems.

## ACKNOWLEDGEMENTS

The authors are thankful to the Management and Principal of Yashwantrao Chavan College of Science Karad and Smt. Kasturbai Walcahnd College, Sangli, for the help rendered in completion of the present study.

## ORCID

*Avinash A. Raut* http://orcid.org/0000-0001-9609-3704

## REFERENCES

Achary, V. M. M., Jena, S., Panda, K. K., & Panda, B. B. (2008). Aluminium induced oxidative stress and DNA damage in root cells of *Allium cepa* L. *Ecotoxicology and Environmental Safety*, *70*, 300–310. https://doi.org/10.1016/j.ecoenv.2007.10.022

Ali, M., & Sreekrishnan, T. R. (2001). Aquatic toxicity from pulp and paper mill effluent. *Advances in Environmental Research*, *5*, 175–196. https://doi.org/10.1016/S1093-0191(00)00055-1

Bajpai, P., Mehna, A., & Bajpai, P. K. (1993). Decolorization of Kraft bleach plant effluent with white-rot fungus *Tramates versicolor*. *Process Biochemistry*, *28*, 377–384. https://doi.org/10.1016/0032-9592(93)80024-B

Barua, S., Tripathi, S., Chakraborty, A., Ghosh, S., & Chakrabarti, K. (2011). Studies on non-symbiotic diazotrophic bacterial populations of coastal arable saline soils of India. *Indian Journal of Microbiology*, *51*, 369–376. https://doi.org/10.1007/s12088-011-0082-9

Bernhard, J. M., Tamara, G., Maria, U., & Siegfried, K. (2005). Use of plant bioassays forthe detection of genotoxins in the aquatic environment. *Acta Hydrochimica et Hydrobiologica*, *33*, 45–55.

Cross, C. E., Vliet, A. V., Louie, S., Thiele, J. J., & Halliwell, B. (1998). Oxidative stress and antioxidants at biosurfaces: Plants, skin, and respiratory tract surfaces. *Environmental Health Perspectives*, *106*, 1241–1251. https://doi.org/10.1289/ehp.98106s51241

Dhanve, R. S., Kalyani, D. C., Phugare, S. S., & Jadhav, J. P. (2009). Coordinate action of exiguobacterial oxidoreductive enzymes in biodegradation of reactive yellow 84A dye. *Biodegradation*, *20*, 245–255. https://doi.org/10.1007/s10532-008-9217-z

Esposito, E., Canhos, V. P., & Nelson, D. (1991). Screening of lignin-degrading fungi for removal of color from Kraft mill wastewater with no additional extra carbon-source. *Biotechnology Letters*, *13*, 571–576. https://doi.org/10.1007/BF01033412

Grant, W. F. (1982). Chromosome aberration assay in Allium. A report of U.S. Environmental Protection Agency Gene-Tox Program. *Mutation Research*, *99*, 273–229.

Grant, W. F. (1994). The present status of higher plant bioassays for detection of environmental mutagens. *Mutation Research*, *310*, 175–185. https://doi.org/10.1016/0027-5107(94)90112-0

Grant, W. F. (1999). Higher plant assays for the detection of chromosomal aberrations and gene mutations–a brief historical background on their use for screening and monitoring environmental chemicals. *Mutation Research*, *426*, 107–112. https://doi.org/10.1016/S0027-5107(99)00050-0

Haddadin, M. S., Al-Natour, R., Al-Qsous, S., & Robinson, R. K. (2002). Bio-degradation of lignin in olive pomace by freshly-isolated species of Basidiomycete. *Bioresource Technology*, *82*, 131–137. https://doi.org/10.1016/S0960-8524(01)00171-7

Jadhav, J. P., Phugare, S. S., Dhanve, R. S., & Jadhav, S. B. (2010). Rapid biodegradation and decolorization of Direct Orange 39 (Orange TGLL) by an isolated bacterium Pseudomonas aeruginosa strain BCH. *Biodegradation*, *21*, 453–463. https://doi.org/10.1007/s10532-009-9315-6

Ma, T. H. (1999). The international program on plant bioassays and the report of the follow-up study after the hands-on workshop in China. *Mutation Research*, *426*, 103–106. https://doi.org/10.1016/S0027-5107(99)00049-4

Ma, T., & Zuazaga, G. (1942). Micro-Kjeldahl determination of nitrogen. A new indicator and an improved rapid method. *Industrial and Engineering Chemistry Analytical Edition*, *14*, 280–282. https://doi.org/10.1021/i560103a035

Martin, C., & Manzanares, P. (1994). A study of the decolorization of straw soda pulping effluents by *Trametes versicolor*. *Bioresource Technology*, *47*, 209–214. https://doi.org/10.1016/0960-8524(94)90182-1

Modi, D. R., Chandra, H., & Garg, S. K. (1998). Decolorization of bagasse based paper mill effluent by white-rot fungus, *Trametes versicolor*. *Bioresource Technology*, *66*, 79–81. https://doi.org/10.1016/S0960-8524(97)00186-7

Moore, M. N. (2008). Autophagy as a second level protective process in conferring resistance to environmentally-induced oxidative stress. *Autophagy*, *16*, 254–256. https://doi.org/10.4161/auto.5528

Patil, P. S., Phugare, S. S., Jadhav, S. B., & Jadhav, J. P. (2010). Communal action of microbial cultures for Red HE3B degradation. *Journal of Hazardous Materials*, *181*, 263–270. https://doi.org/10.1016/j.jhazmat.2010.05.006

Patil, P. S., Phugare, S. S., Kalyani, D. C., Surwase, S. N., & Jadhav, J. P. (2012). Bioremediation perspective of Navy Blue Rx containing textile effluent by bacterial isolate. *Bioremediation*, *16*, 185–194. https://doi.org/10.1080/10889868.2012.731441

Phugare, S. S., Kalyani, D. C., Patil, A. V., & Jadhav, J. P. (2011). Textile dye degradation by bacterial consortium and subsequent toxicological analysis of dye and dye metabolites using cytotoxicity,

genotoxicity and oxidative stress studies. *Journal of Hazardous Materials*, *186*, 713–723. https://doi.org/10.1016/j.jhazmat.2010.11.049

Rehman, A., Bhatti, H. N., & Athar, H. R. (2009). Textile effluents affected seed germination and early growth of some winter vegetable crops: A case study. *Water Air Soil Pollution*, *198*, 155–163. https://doi.org/10.1007/s11270-008-9834-5

Saghirzadeh, M., Gharaati, M. R., Mohammadi, S., & Nejad, M. G. (2008). Evaluation of DNA damage in the root cells of *Allium cepa* seeds growing in soil of high background radiation areas of Ramsar, Iran. *Journal of Environmental Radioactivity*, *99*, 1698–1702. https://doi.org/10.1016/j.jenvrad.2008.03.013

Sullivan, J. T., Eardly, B. D., Berkum, P. V., & Ronson, C. W. (1996). Four unnamed species of nonsymbiotic Rhizobia isolated from the rhizosphere of *Lotus corniculatus*. *Applied and Environment Microbiology*, *62*, 2818–2825.

Tamura, K., Dudley, J., Nei, M., & Kumar, S. (2007). MEGA4: Molecular evolutionary genetics analysis (MEGA) software version 4.0. *Molecular Biology and Evolution*, *24*, 1596–1599. https://doi.org/10.1093/molbev/msm092

Thompson, G., Swain, J., Kay, M., & Forster, C. F. (2001). The treatment of pulp and paper mill effluent: A review. *Bioresource Technology*, *77*, 275–286. https://doi.org/10.1016/S0960-8524(00)00060-2

Wu, J., Xiao, Y. Z., & Yu, H. Q. (2005). Degradation of lignin in pulp mill wastewaters by white-rot fungi on biofilm. *Bioresource Technology*, *96*, 1357–1363. https://doi.org/10.1016/j.biortech.2004.11.019

Yin, C. F., Joyce, T. W., & Chang, H. M. (1989). Kinetics of bleach plant effluent decolorization by *Phanerochaete chrysosporium*. *Journal of Biotechnology*, *10*, 67–76. https://doi.org/10.1016/0168-1656(89)90092-8

Zouari, H., Labat, M., & Sayadi, S. (2002). Degradation of 4-chlorophenol by the white rot fungus *Phanerochaete chrysosporium* in free and immobilized cultures. *Bioresource Technology*, *84*, 145–150. https://doi.org/10.1016/S0960-8524(02)00032-9

**How to cite this article:** Raut AA, Phugare SS, Kalyani DC, Bajekal SS. Biotreatment of paper mill effluent using alkaliphilic *Rhizobium* sp. NCIM 5590 isolated from meteoric alkaline Lonar Lake, Buldhana District, Maharashtra, India. *Lakes & Reserv.* 2018;00:1–9. https://doi.org/10.1111/lre.12216