

Degradation of azo dye by bacterium, *Alishewanella* sp. CBL-2 isolated from industrial effluent and its potential use in decontamination of wastewater

Mehvish Ajaz, Amina Elahi and Abdul Rehman

ABSTRACT

In the present study, *Alishewanella* sp. CBL-2 was characterized on the basis of biochemical and 16S rRNA sequencing. The bacterium was found to decolorize 83% Sumifex Tourqi blue within a period of 6 days at 37 °C and pH 7. The predominant form of the enzyme was found to be extracellular (78%) as compared to intracellular (16%). The breakdown of azo bond was confirmed through thin layer chromatography (TLC) analysis as new bands and peaks were observed in chromatograms of extracted metabolites of decolorized samples. Growth of beneficial microbial fauna in the presence of bacterial decolorized wastewater indicates that this wastewater is safe for the survival of microorganisms. High bioremediation potential makes *Alishewanella* sp. CBL-2 an impending foundation for green chemistry to remove azo dyes from industrial wastewater.

Key words | *Alishewanella* sp. CBL-2, azo dye, decolorization, TLC

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INTRODUCTION

Among industrial wastewaters, dye wastewater from textile and dyestuff industries is one of the most difficult to treat. This is because dyes usually have a synthetic origin and complex aromatic molecular structures which make them more stable and more difficult to biodegrade (Seshadri *et al.* 1994). Azo dyes, which are aromatic compounds with one or more –N = N– groups, constitute the largest class of synthetic dyes used in commercial applications (Zollinger 1991). It is reported that there are over 100,000 commercially available dyes with a production of over 7×10^5 metric tons per year (Mishra & Tripathy 1993).

Azo dyes are used in different industries, including textiles, cosmetics, paper, leather, pharmaceuticals, and food,

with an annual consumption of about 0.7 million ton (Chen *et al.* 2003). The two major sources of release of dyes into the environment are the effluents from textile processing units and dyestuff manufacturing industries (O'Neill *et al.* 1999). Discharge of highly colored dye containing effluents from textile and dyestuff industries to neighboring water bodies is causing significant health concerns (Martins *et al.* 2002). During the past two decades, several physico-chemical decolorization techniques have been reported; few, however, have been accepted by the textile industries (Okazaki *et al.* 2002; da Silva & Faria 2003). Their lack of implementation has been largely due to high cost, low efficiency, and generation of toxic by-products (Selvam *et al.* 2003).

The ability of microorganisms to carry out dye decolorization has recently received much attention. Microbial decolorization of dyes is a cost-effective method to remove them from the environment (Moosvi *et al.* 2005; Aftab

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et al. 2011). The present day bioremediation relies upon the pollutant degrading capacities of naturally occurring microbial consortia in which bacteria play a central role (O'Neill *et al.* 2000). Microbial consortia are usually used without analyzing the constituent microbial populations for environmental remediation and complexity of the microbial consortium enables them to act on a variety of pollutants (Watanabe & Baker 2000). Recently, the aerobic decolorization of azo dyes has been reported by a number of investigators (Adedayo *et al.* 2004; Aftab *et al.* 2011).

A wide variety of microorganisms is capable of decolorization of a wide range of dyes including fungi: *Aspergillus niger* (Fu & Viraraghavan 2002), *Phanerochaete chrysosporium*, *Aspergillus terricola* (Saikia & Gopal 2004), *P. chrysosporium* (Fournier *et al.* 2004); yeasts: *Saccharomyces cerevisiae*, *Candida tropicalis*, *C. lipolytica* (Aksu & Dönmez 2003); algae: *Spirogyra* species (Gupta *et al.* 2006), *Chlorella vulgaris* (Acuner & Dilek 2004), *C. sorokiniana* (de-Bashan *et al.* 2002), *Lemna minuscula* (Valderama *et al.* 2002), *Scenedesmus obliquus*, *C. pyrenoidosa* and *Closterium lunula* (Yan & Pan 2004), and bacteria: *Escherichia coli* NO₃ (Chang & Kuo 2000), *Aeromonas hydrophila* (Chen *et al.* 2003), *Kurthia* species (Sani & Banerjee 1999).

The present study was aimed at isolating and characterizing potent bacterium from industrial wastes able to decolorize azo dyes. Decolorization products were tested against beneficial microbial flora and dye degraded products were also analyzed through thin layer chromatography (TLC).

EXPERIMENTAL

Wastewater sample collection

Wastewater samples were collected in sterilized (autoclaved at 121 °C, 15 lb/inch² pressure for 15 min) screw capped bottles. Some physiochemical parameters of wastewater, i.e., temperature, pH, color, and smell were also measured. About six wastewater samples were collected and brought to the laboratory and then proceeded to bacterial isolation. For isolation of azo dyes degrading bacteria, 100 µL of wastewater sample was spread on Luria-Bertani (LB) agar

plates containing 100 µL of dye stress in the medium. LB agar plates were prepared by dissolving 1 g NaCl, 1 g Tryptone and 0.5 g yeast extract in 100 mL distilled water, pH adjusted to 7.0–7.2 and then agar was added in 250 mL flasks. The medium was autoclaved at 121 °C and 15 lb/inch² for 15 min. The growth of bacterial colonies was observed after 24 h of incubation at 37 °C.

Isolation of azo dye degrading bacteria

Effect of dye on the growth of bacterial isolates was determined in minimum salt medium (MSM) which contained (g L⁻¹): KH₂PO₄, 0.2 g; MgSO₄·7H₂O, 0.02 g; Na₂CO₃ 1 g; yeast extract, 0.5 g; in 100 mL distilled water (pH 7.0), then agar 1.5 g was added, autoclaved, and supplemented with azo dyes when the temperature reached 50–55 °C. Medium was poured in autoclaved dried Petri plates under sterilized conditions. Plates were left to solidify, inoculated with bacterial isolates and then incubated again at 37 °C for 24 h. This experiment was repeated with successfully higher concentration of dye until the minimum inhibitory concentration (MIC) of the bacterial isolates was obtained.

Morphological, biochemical, and molecular characterization

The morphological parameters which were checked for bacterial isolates include size, shape, color, margin, elevation, motility, etc. Gram staining and various biochemical tests were performed according to the scheme given in Cappuccino & Sherman (2001). For molecular characterization, DNA was isolated (Masneuf-Pomarade *et al.* 2007) and 16S rRNA gene was amplified through polymerase chain reaction (PCR) by using universal bacterial primers 8F (5-AGAGTTTGATCCTGGCTCAG-3) and 1492R (5-GGTTACCTTGTACGACTT-3) (Turner *et al.* 1999). PCR was performed by initial denaturation at 95 °C for 5 min, followed by 35 cycles of denaturation at 95 °C for 1 min, annealing at 55 °C for 1 min and extension at 72 °C for 1 min, with a final extension at 72 °C for 10 min. Fermentas Gene Jet Gel Extraction kit (#K0691) was used for gene cleaning of PCR product and then sequenced from 1st Base, Malaysia. The sequence was then submitted to GenBank to obtain accession numbers.

Determinations of optimum growth conditions

For optimum growth of bacterial isolate two parameters, i.e., temperature and pH were considered. For determination of optimum temperature, LB broth was prepared in 100 mL distilled water. Five milliliters of LB broth was added in four sets, each of three sets of test tubes autoclaved and inoculated with 50 μ L of freshly prepared culture of bacterial isolate. Tubes were incubated at 20 °C, 30 °C, 37 °C, and 45 °C for overnight incubation of 24 h. Absorbance was measured at 600 nm using UV/Vic spectrophotometer (PerkinElmer, USA).

For optimum pH, test tubes having 5 mL LB broth were prepared in six sets, each containing three test tubes and their pH was adjusted to 5.0, 6.0, 7.0, 8.0, 9.0, and 10.0 by using 0.1 M NaOH and 0.1 M HCl and then autoclaved. The tubes were inoculated with 50 μ L freshly prepared culture of bacterial isolate and were incubated at 37 °C for 24 h and their absorbance was measured at 600 nm using spectrophotometer.

Effect of dye on bacterial growth

Growth curves of bacterial isolate were determined by using MSM. The flasks containing 100 μ L of dye were designated as 'treated' and flasks without dye were designated as 'control'. In this way, 100 mL MSM was taken into 250 mL flasks, autoclaved and then inoculated with 50 μ L of freshly prepared bacterial culture. The cultures were incubated at 37 °C in a shaker at 100 rpm. An aliquot of culture was taken out in an oven sterilized test tube at regular intervals of 0, 4, 8, 12, 16, 20, and 24 h.

Dye decolorization

Decolorization experiment was performed in 250 mL Erlenmeyer flasks containing 100 mL of MSM and was inoculated with 100 μ L of freshly prepared bacterial culture. The dye was added to the culturing medium 100 μ L and 200 μ L from stock solution after 24 h of growth and the flask containing no bacterial culture was used as control. The flasks were incubated at 37 °C, 3 ml culture medium was withdrawn after 1, 2, 3, 4, 5, and 6 days and centrifuged at 10,000 rpm for 15 min to separate the bacterial cell mass.

Decolorization was determined by measuring the absorbance of decolorization medium at 465 nm (Kalyani *et al.* 2008). Percentage decolorization was calculated as follows:

$$\text{Decolorization(\%)} = \frac{\text{Initial absorbance} - \text{Observed absorbance}}{\text{Initial absorbance}} \times 100$$

Enzyme assay

To prepare the crude cell-free extract, the bacterial cultures were grown with azo dyes (100 μ L from stock solution of dye) in 100 mL MSM for 96 h at 37 °C. Cells were harvested by centrifugation at 6,000 \times g for 15 min. For intracellular enzyme assay, pellet was washed with lysis buffer (Mercaptoethanol; SDS) and was suspended in 1 mL of the same buffer. Cells were disrupted by sonication for 15 s with 60 s interval (5 cycles) in cold conditions. The resultant homogenate was centrifuged at 10,000 \times g for 5 min. The supernatant was used as intracellular crude extract. While for extracellular crude extract, the supernatant was mixed with 60% ammonium salt, and the mixture was incubated overnight in a refrigerator at 4 °C. The overnight chilled mixture was then centrifuged at 10,000 rpm for 15 min. The aliquot was then used as extracellular crude enzyme. The activity of azoreductase was determined by a slightly modified method of Moutaouakkil *et al.* (2003). The reaction mixture consisted of: sodium acetate, 500 μ L (pH 5); autoclaved water, 500 μ L; ABTS, 100 μ L; and crude enzyme 100 μ L. The reaction was allowed to proceed for 30 min at 30 °C and optical density was measured at 465 nm. For control reaction, distilled water was used instead of crude enzyme.

Thin layer chromatography

After the enzyme reaction the products were used for TLC. For this purpose, a silica plate was used. The spotting was done with the help of a Pasteur pipette. The spots were dried and then allowed to run into a chromatographic tank through a mobile phase (chloroform, 30 mL; ethyl acetate, 30 mL; acetic acid, 0.6 mL). The silica plate was then observed under UV illuminator at wavelength 254 and 366 nm.

Microbial toxicity

The toxic effect of decolorized dye sample was checked on agriculturally important soil microflora, i.e., *Bacillus subtilis* (z-66), *Pseudomonas aeruginosa* (PA1), and *Bacillus cereus* (T358-2) (Mali *et al.* 2000). L-agar plates were swabbed with these bacteria and then a well of 2 mm was made which was filled by centrifuged decolorized broth. The plates were then incubated at 37 °C for 24 h. The zone surrounding the well showed the index of toxicity of the decolorized sample.

Statistical analysis

Observations were made and all the experiments run in triplicate. At least three separate flasks were usually maintained for one treatment. Each time three readings were taken, their mean, and standard error of the mean were calculated.

RESULTS AND DISCUSSION

Sampling and screening of dye decolorizing bacteria

A total of six wastewater samples with physiochemical parameters was collected (Table S1, available with the online version of this paper). Bacterial cultures were isolated, purified, and screened for the decolorization of azo dye. Of all the cultures tested, one bacterial isolate (CBL-2) was selected on the basis of dye tolerance, i.e., resistance in MSM containing 1% of reactive azo dye Sumifex Tourqi Blue (Figure 1).

Bioremediation is becoming important because it is cost-effective and environmentally friendly, and produces less sludge (Robinson *et al.* 2001; Chen *et al.* 2003). Microbial decolorization of dyes is a cost-effective procedure to remove them from the environment (Moosvi *et al.* 2005). The biodegradation ability of bacteria is assumed to be associated with the production of lignolytic enzymes such as lignin peroxidase and laccase. The presence of laccase-like enzymes has been reported in bacteria (Givaudan *et al.* 1993). The ability of bacteria to metabolize azo dyes has been investigated by a number of research groups.



Figure 1 | Change in color of Sumifex Tourqi blue as compared to the control in flask containing culture of *Alishewanella* sp. CBL-2.

Many microorganisms are capable of decolorizing the azo dyes, including Gram-positive and Gram-negative bacteria (Sani & Banerjee 1999; Kodam *et al.* 2005; Moosvi *et al.* 2005).

Biochemical and molecular characterization of bacterial isolate

Initially, the bacterial isolate was identified on the basis of morphological and biochemical characteristics (Table 1). The sequence of 16S rRNA gene of bacterial isolate showed 95% homology with the sequence of 16S rRNA gene of *Alishewanella* sp. The sequence was then submitted to GenBank under accession number JN546609.

Determination of growth conditions

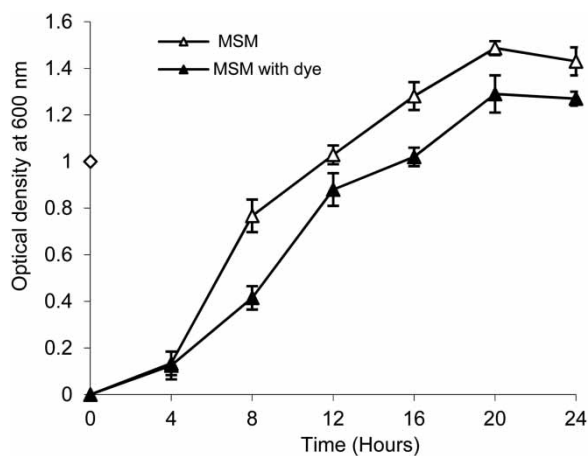
The most suitable temperature and pH of bacterial isolate were found to be 37 °C and 7 (Figure S1, available online). The strain CBL-2 showed continuous increase in growth rate until the stationary phase was reached, while the control showed a steady increase in the growth. The control was without dye and for the treated Turqi blue dye plus inoculum was used (Figure 2). Asada *et al.* (2006) isolated

Table 1 | Morphological and biochemical characteristics of *Alishewanella* sp. CBL-2

<i>Alishewanella</i> sp. CBL-2	
Morphological characteristics	
Shape	Rod
Motility	Non-motile
Color	Yellowish
Elevation	Raised
Size	2 μm
Margin	Smooth
Gram staining	Gram negative
Biochemical characteristics	
Indole production	-ve
Methyl red reaction	-ve
Voges Proskauer reaction	-ve
Citrate use	-ve
MacConkey agar	-ve
Catalase activity	+ve
Oxidase activity	+ve
EMB agar	-ve

+ve: positive, -ve: negative.

the azo dye decolorizing bacteria which were Gram-negative and showed maximum growth at 34 °C–37 °C. Banat *et al.* (1996) also observed that various textile and other dye effluents are produced at relatively high temperatures (50–60 °C), so temperature will be an important factor in the real application of biosorption by biomass in the future.

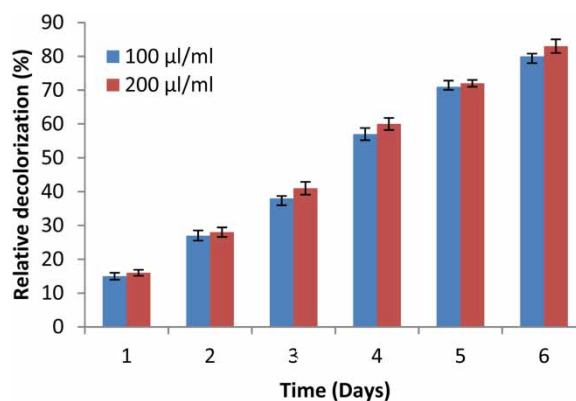
**Figure 2** | Effect of Sumifex Tourqi blue on the growth (OD) of *Alishewanella* sp. CBL-2 incubated at 37 °C for a period of 24 h.

Dye decolorization

The efficient removal of dye is necessary and significant for the protection of the environment. In the present study, bacterial strain CBL-2 was able to decolorize 80 and 83% of 100 and 200 $\mu\text{L mL}^{-1}$ dye stress, respectively (Figure 3). Sheth & Dave (2009) reported *P. aeruginosa* exhibited 91% decolorization of azo dye. Wu *et al.* (2009) reported that *Shewanella oneidensis* WL-7 was capable of decolorizing the Reactive Black 5 from the medium containing 100 μM after 12 h. Decolorization of azo dyes by bacterial isolates has been reported by many researchers (Hong *et al.* 2007; Xu *et al.* 2007; Khalid *et al.* 2008).

Enzyme assay

Bacterial degradation of azo dyes is generally considered a specific reaction by azo reductase under aerobic condition or a nonspecific reduction process under anaerobic conditions. In the present study, both extra- and intracellular enzyme activities were determined by ABTS method. *Alishewanella* sp. CBL-2 showed high extracellular enzyme activity (78%) as opposed to intracellular enzyme activity (16%), so the predominant form of the enzyme was extracellular (Figure 4). Maier *et al.* (2004) reported that a thermoalkalophilic *Bacillus* sp. was able to reduce a large structural variety of azo dyes. Kolekar *et al.* (2013) reported that *Alishewanella* sp.

**Figure 3** | *Alishewanella* sp. CBL-2 potential to decolorize Sumifex Tourqi blue incubated at 37 °C for a period of 6 days.

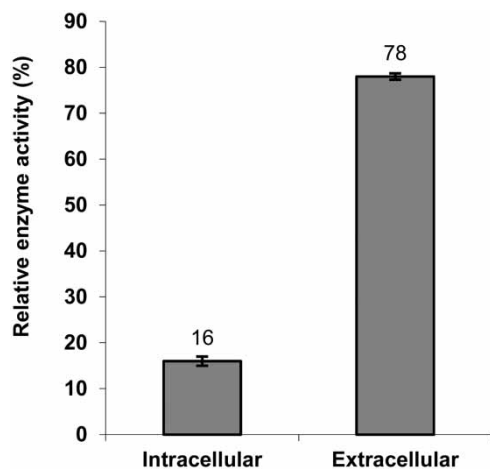


Figure 4 | Comparison between extra- and intracellular enzyme (azoreductase) activities of *Alishewanella* sp. CBL-2 using ABTS as a substrate.

strain KMK6 was able to degrade efficiently a mixture of textile dyes ($0.5\text{--}2.0\text{ g L}^{-1}$) within 8 h. An initial 28% reduction in COD was observed immediately after decolorization at static anoxic conditions, which on further incubation at shaking conditions, reduced by 90%.

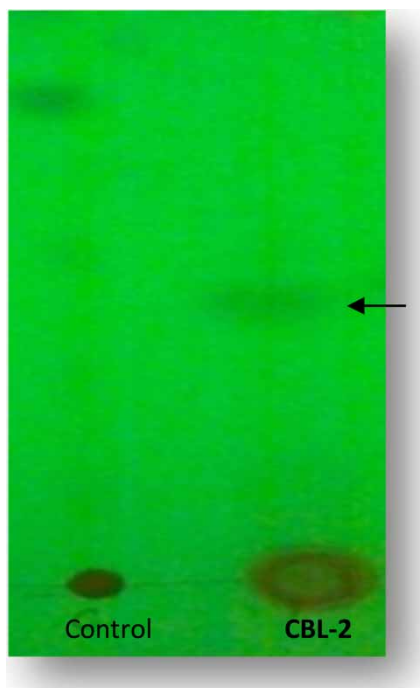


Figure 5 | TLC chromatogram of extracted metabolites of *Alishewanella* sp. CBL-2 decolorized dye sample visualizes UV range of 254 and 366 nm.

Partially purified azoreductase was able to utilize different azo dyes as substrates.

Dye degraded products analysis

The presence of bands with different Rf value in the control and treated sample clearly revealed that dye was degraded into its products (Figure 5). Another study from this laboratory showed the presence of bands with Rf value of 0.96 and 0.94 when observed under UV range of 254 and 366 nm while the Rf value of control dye was 0.83 (unpublished data). Singh *et al.* (2015) reported that two additional bands of Rf value 0.63 and 0.965 were observed in the TLC chromatogram of a decolorized sample of Direct orange 16 by *Micrococcus luteus* strain SSN2 when observed under UV range of 254 nm while the dye had a band of Rf value 0.74. Similarly, Sahasrabudhe *et al.* (2014) reported a band of Rf value 0.71 in the TLC chromatogram of extracted metabolites from a decolorized sample of Direct red by *Enterococcus faecalis* YZ66 while the original dye had a band of Rf value 0.97. Likewise, a single band of Rf value 0.83 was observed in TLC chromatogram of mono azo dye Amarnath while the extracted metabolites from *Acinetobacter calcoaceticus* NCIM 2890 decolorized sample had three bands of Rf value 0.93, 0.82, and 0.79 (Ghodake *et al.* 2011).

Microbial toxicity

There was no zone of inhibition found around the well which was a clear indication that the biodegraded wastewater is safe for the growth of microbial species (Figure 6). Ilyas & Rehman (2013) reported two fungal species, i.e., *Aspergillus niger* and *Nigrospora* sp. whose decolorized sample was proved safe for the microbial species. Similarly, the dye degradation products by *Alishewanella* sp. strain KMK6 were non-toxic compared to the dye mixture in genotoxicity assessment assay (Kolekar *et al.* 2013).

CONCLUSIONS

Alishewanella sp. CBL-2, characterized on the basis of biochemical tests and 16S rRNA sequencing, has promising

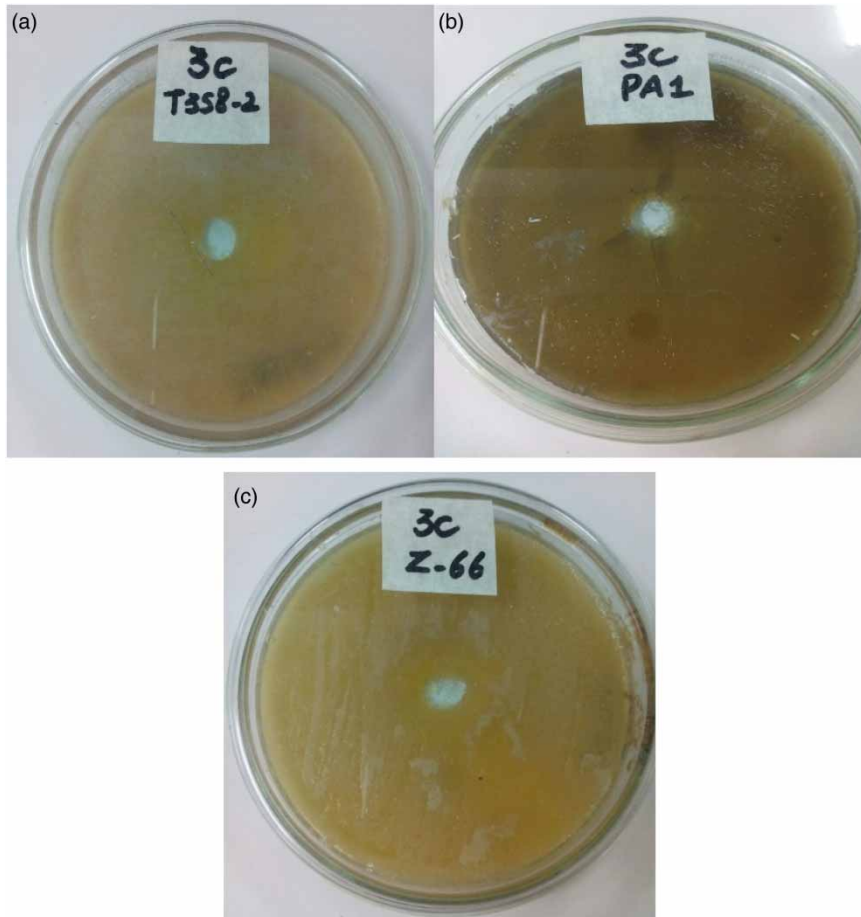


Figure 6 | Bacterial decolorized broth used for incubation of (a) *B. cereus* (T358-2), (b) *P. aeruginosa* (PA1), and (c) *B. subtilis* (z-66) at 37 °C for 48 h. No zone of inhibition was observed in any case.

potential to decolorize Sumifex Tourqi blue (83%) after 6 days of incubation at 37 °C and pH 7. *Alishewanella* sp. CBL-2 had shown maximum enzyme activity in extracellular assay (78%) as compared to the intracellular assay (16%). This degradation was confirmed through TLC. It was also proved that microbially treated dye wastewater is safe for growth of beneficial microbial flora. *Alishewanella* sp. CBL-2 can be used to ameliorate wastewater containing azo dyes by utilizing the decolorization potential ability of the bacterium.

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