

Isolation and Characterization of a Molybdenum-reducing and the Congo Red Dye-decolorizing *Pseudomonas putida* strain Neni-3 in soils from West Sumatera, Indonesia

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ABSTRACT

The elimination of heavy metals and organic contaminants, such as phenols, hydrocarbons, and amides, by bioremediation, is the most effective choice for the foreseeable future. This is especially true at low levels, where other methods, such as physical or chemical methods, may not be successful. Each year, a few million tons of these contaminants are emitted. In this study, we examined the ability of a molybdenum-reducing bacteria that were isolated from polluted soil to decolorize azo dyes independently of its ability to reduce molybdenum. The ideal conditions for the bacterium to convert molybdate to molybdate blue are a pH range of 6.0 to 6.5 and a temperature range of 25 to 37 degrees Celsius. After glucose, fructose and galactose were the most effective donors of electrons to enable the reduction of molybdate. Galactose was the least effective supplier of electrons. There are a few other prerequisites that need to be met as well, such as a phosphate concentration of between 2.5 and 7.5 mM and a molybdate concentration of between 10 and 15 mM. Its absorption spectra were identical to that of the phosphomolybdate reduction process and to that of the earlier Mo-reducing bacterium. At a concentration of 2 ppm, the heavy metals Ag (I), Hg (II), and Cu (II) each inhibited the reduction of molybdenum by a percentage of 62.8, 61.1, and 36.8 per cent, respectively. We put the bacterium through a test to see if it can remove the color from a variety of dyes. The Congo Red dye was able to lose its color when exposed to the bacterium. Based on the results of the biochemical study, the bacterium has been provisionally identified as *Pseudomonas putida* strain Neni-3. This bacteria's ability to detoxify various toxicants is a desirable quality, as it makes the bacterium an efficient bioremediation approach. As a result, this bacterium is in high demand. Purification of the molybdenum-reducing enzyme that was produced by this bacterium is presently being studied in order to characterize decolorization research in a more accurate manner.

INTRODUCTION

Heavy metals and organic pollutants can be removed most efficiently and affordably through bioremediation, especially at low concentrations when physical or chemical approaches may not be effective. Bioremediation is the most cost-effective method for getting rid of the massive amounts of heavy metals and organic contaminants that are produced every year [1]. Molybdenum is one of the critical heavy metals needed for trace amounts and is toxic at high levels to a number of species [2]. It

is used widely in industries such as alloying agents, car engines, corrosion resistant steel, and molybdenum disulphide as lubricant. Molybdenum is commonly used in the industry and causes many instances of water poisoning worldwide such as in Malaysia [3], the Tokyo Bay, Tyrol in Austria and the Black Sea [4,5]. Toxicity to molybdenum has been recorded as low as a few parts per million resulting in the inhibition of spermatogenesis and embryonic in a number of species including catfish and mouse [6,6–9]. In addition, molybdenum is extremely poisonous

to ruminants, with cows being the most susceptible to its effects at several parts per million [10,11].

In addition to heavy metals, the organic pollutant known as azo dyes is frequently found to be present as a co-pollutant in water, soils, and wastewater treatment plants throughout Indonesia [12]. Any of these dyes were unfortunately typically substantially toxic to marine animals and organisms as stated by The Ecological and Toxicological Association of the Dyestuff Manufacturing Industry (ETAD) [13]. Since they are reactive and include acid, these brightly colored, water-soluble reactive and acid dyes are not eliminated by conventional water treatment. It is well known that one of the most important contributions of water contamination is made by the textile finishing industry as 10-15% of dyes are wasted in the course of dyeing processes during the effluent treatment process. In addition, a significant threat to ecology occurs due to the high Biological Oxidation Demand (BOD), Chemical Oxygen Demand (COD), pH, color, and the occurrence of toxic metals [14–20].

A range of xenobiotics can be broken down by certain microorganisms, and at the same time, these microbes can also detoxify heavy metals [22–24]. In contaminated areas, where the presence of several pollutants is usual, the adaptability of these microbes is in high demand because of the requirement for their services. It has been reported that heavy metal reduction can be linked with azo dye decolorization [24], and this includes a few different Mo-reducing bacteria [25–27].

In this investigation, we are investigating whether or not a unique molybdenum-reducing bacteria that was isolated from polluted soil has the capacity to change the color of a variety of azo dyes. Static growth or circumstances are used on purpose, and they are easily achieved in a microplate setting where the oxygen content is lower than under aerobic conditions (typically 0% to 10% ambient oxygen, EO) (20 per cent environmental oxygen, EO). This is due to the fact that, among other ecological considerations, the bulk of bioremediation conditions will need to be carried out in aquatic bodies or soils where the EO level is less than 20 per cent. We report in this study that we were successful in isolating a molybdenum-reducing bacteria from polluted soil that was able to decolorize the Congo Red azo dye (Fig. 1). Because of its unique characteristics, this bacterium is going to be an excellent candidate for potential bioremediation work involving the heavy metal molybdenum as well as dye as organic contaminants.

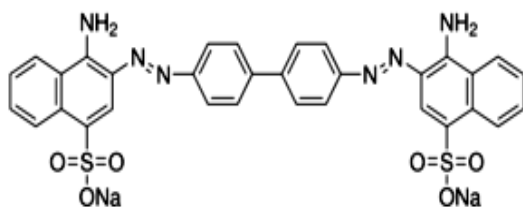


Fig. 1. The structure of Congo Red [21].

MATERIALS AND METHODS

Isolation of molybdenum-reducing bacterium

Soil samples were taken in January 2009 from a polluted area in the Indonesian province of Payakumbuh on the island of Sumatera. Five centimetres below the surface is where the samples were collected. Suspending 1 g of soil in sterile water, 0.1 ml of the resulting soil suspension was spread across agar of

low phosphate media at pH 7.0. After that, we gave the mixture 48 hours to incubate at room temperature. Components of low phosphate medium (LPM): glucose (1 per cent), $MgSO_4 \cdot 7H_2O$ (0.05 per cent), yeast extract (0.5 per cent), $(NH_4)_2SO_4$ (0.3 per cent), sodium chloride (0.5 per cent), sodium molybdate (0.242 per cent or 10 mM), and sodium dihydrogen phosphate (0.071 per cent or 5 mM) [28]. Molybdate has been reduced by molybdenum-reducing bacteria, as evidenced by the formation of blue colony forms. The most blue-strong colony was isolated and reseeded on low-phosphate substrate to produce a genetically pure population (LPM). A 250 mL shake flask culture was incubated at room temperature with a pH of 7.0 and 100 mL of the media indicated above, but the phosphate concentration needs to be increased to 100 mM, for 48 h on an orbital shaker, which was set at 120 rpm to reduce molybdenum in liquid media. When looking into the absorption spectrum of molybdenum blue (Mo-blue), 1.0 mL of the molybdenum blue was taken from the liquid culture and centrifuged at 10,000 g for ten min at room temperature.

To analyze the supernatant, a UV-spectrophotometer was utilized to scan the spectrum from 400 to 900 nm (Shimadzu 1201). We selected the medium with the least amount of phosphate as a reference point for the other conditions. The Bergey's Manual of Determinative Bacteriology [28] was utilized to identify the bacterium. Then the ABIS online system was utilized to interpret the results carried out via [29]. Preparation of resting cells for molybdenum reduction characterization using microplate or microtiter format resting cells as before [30]. To monitor Mo-blue production, the plate was first incubated at room temperature or designated temperatures when the temperature was optimized and then read at 750 nm using a microplate reader with the specific extinction coefficient of $11.69 \text{ mM}^{-1} \cdot \text{cm}^{-1}$ at 750 nm [31].

Effect of heavy metals on molybdenum reduction

This study made use of stock solutions derived from commercial salts as well as atomic absorption spectrometry standard solutions manufactured by MERCK in order to investigate the impact of heavy metals. Seven different heavy metals, including lead (II), copper (II), arsenic (V), silver (I), mercury (II), chromium (VI), and cadmium (II), were put through their paces during the experiment. In the microplate format, the bacterium was allowed to interact with heavy metals at a concentration of 1 ppm. As in the previous experiment, the amount of Mo-blue generation was evaluated at 750 nm.

The detection of bacterial decolorization of azo dyes

Following the aforementioned microplate format, we tested the bacteria's capacity to remove colors from the sample. Many different azo dyes had their final concentrations fixed to 100 mg/L. The following is a list of the dyes that were purchased from Sigma-Aldrich (located in St. Louis, United States of America), together with their maximum wavelengths, as shown by parentheses: Congo Red (C.I. 22120) has a wavelength of 498 nanometers, Evans Blue (C.I. 23860) has a wavelength of 594 nanometers, Fast Green FCF (C.I. 42053) has a wavelength of 620 nanometers, Metanil Yellow (C.I. 13065) has a wavelength of 414 nanometers, Methyl Red (C.I. 13020) has a wavelength of 493 nanometers (427 nm). The following is a list of the components that made up the growth medium, expressed as a per centage of the total volume: Glucose (1 per cent), sodium lactate (1 per cent), $(NH_4)_2SO_4$ (0.3 per cent), $NaNO_3$ (0.2 per cent), $MgSO_4 \cdot 7H_2O$ (0.05 per cent), yeast extract (0.05 per cent), $NaCl$ (0.5 per cent), Na_2HPO_4 (0.705 per cent or 50 mM).

The pH of the medium was brought up to 7.0. Because the color of some of the dyes can shift depending on the pH of the solution, the phosphate content was raised to 50 mM at a pH of 7.0 in order to counteract this. After an incubation period of 48 hours, the difference in absorbance values from the start measurements were subtracted from the final data, and a percentage of decolorization was determined.

Statistical analysis

Graphpad Prism, version 5.0, which can be downloaded for free at www.graphpad.com, was used to perform the analysis on the data. For the purpose of comparing two or more groups, either a one-way analysis of variance followed by a post hoc analysis using Tukey's test or a Student's t-test was carried out. When P was less than 0.05, statistical significance was assumed.

RESULTS AND DISCUSSION

Isolation of a bacterium that reduces molybdenum

The bacterium is a Gram-negative bacterium that looked like short rods. It could potentially migrate in different directions due to its flagella. The bacterium was identified by comparing the results of many culture, morphological, and biochemical tests with those listed in Bergey's Manual of Determinative Bacteriology [29] and by using the ABIS online software (Table 1) [29]. The software gave three suggestions for the bacterial identity with the highest homology (81%) and accuracy at 85% as *Pseudomonas putida*. In the future, however, further work is required to further identify this species, especially the molecular identification technique by comparing the 16s rRNA gene. The bacteria has been provisionally named *Pseudomonas putida* strain Neni-3 in memory of Dr. Neni Gusmanizar. Two Mo-reducing bacteria from this genus; *Pseudomonas* sp. strain DRY2 [32] and the Antarctic bacterium *Pseudomonas* sp. strain DRY1 [33] have been reported previously.

Table 1. Biochemical tests for *Pseudomonas putida* strain Neni-3.

Biochemical Test	Result	Biochemical Test	Result
Motility	+	Utilization of:	
Hemolysis	+	L-Arabinose	+
Growth at 4 °C	-	Citrate	+
Growth at 41 °C	+	Fructose	+
Growth on MacConkey agar	-	Glucose	+
Arginine dihydrolase (ADH)	+	meso-Inositol	-
Alkaline phosphatase (PAL)	+	2-ketogluconate	+
Indole production	-	Mannose	+
Nitrates reduction	-	Mannitol	-
Lecithinase	-	Sorbitol	-
Lysine decarboxylase (LDC)	-	Sucrose	+
Ornithine decarboxylase (ODC)	-	Trehalose	-
ONPG (beta-galactosidase)	-	Xylose	-
Esculin hydrolysis	-		
Gelatin hydrolysis	-		
Starch hydrolysis	-		
Urea hydrolysis	+		
Oxidase reaction	+		

Note: + positive result, - negative result, d indeterminate result

The use of resting cells under static conditions appears to be a viable approach in studying the characterization of Mo-reducing bacterium as the reduction is best carried out under static conditions as elevated oxygen appears to arrest the growth and reduction of the bacterium [34]. The use of resting cells is not new since it has been used in studying heavy metals reduction such as in selenate [35], chromate [36], vanadate [37] reductions and xenobiotics biodegradation such as diesel [38], SDS [39], phenol [40], amides [41] and pentachlorophenol [42].

Molybdenum absorbance spectrum

Shoulder at around 700 nm and greatest peak near the infra-red region of between 860 and 870 nm with a median at 865 nm can be seen in the absorption spectrum of Mo-blue generated by *Pseudomonas putida* strain Neni-3 (Fig. 2). Due to the Mo-natural blue's diversity and the fact that it is composed of multiple distinct species, its identification is not easily accessible [43].

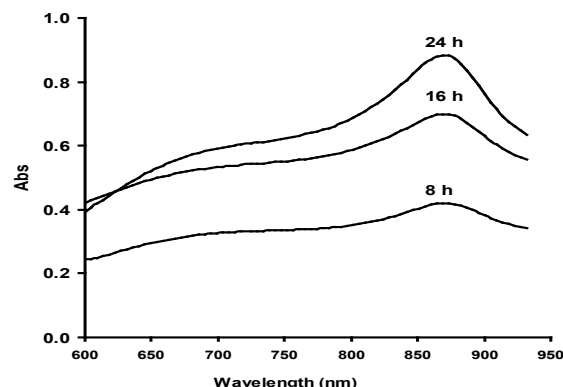


Fig. 2. Scanning absorption spectrum of Mo-blue from *Pseudomonas putida* strain Neni-3 at different time intervals.

Molybdate reduction: the role of pH and temperature

The *Pseudomonas putida* Neni-3 strain was used in incubations with Bis-Tris and Tris.Cl acts as a buffer between acidic (5.5) and basic (8.0) conditions (20 mM). According to the findings of an ANOVA study, the optimal range for pH during the reduction process was between 6.0 and 6.5. When the pH was lower than 5, there was a significant inhibition of reduction (Fig. 3). From 20 to 60 degrees Celsius, the temperature effect (shown in Fig. 4) was seen, with an ideal temperature between 25 and 37 degrees Celsius. The results of the analysis of variance showed that the values were not significantly different from one another ($p > 0.05$). Inhibition of Mo-blue production by the *Pseudomonas putida* strain Neni-3 at temperatures over 37 degrees Celsius was shown to be quite significant.

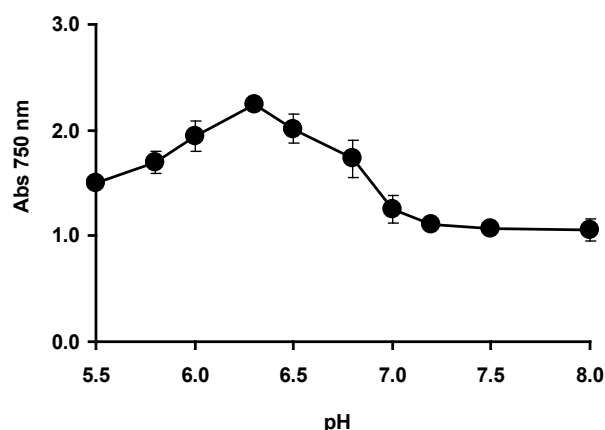


Fig. 3. The influence of pH on *Pseudomonas putida* strain Neni-3's ability to reduce molybdenum. The bacterium was allowed to rest for 72 hours in an optimal microtiter plate environment. (N=3) Error bars reflect the mean standard deviation.

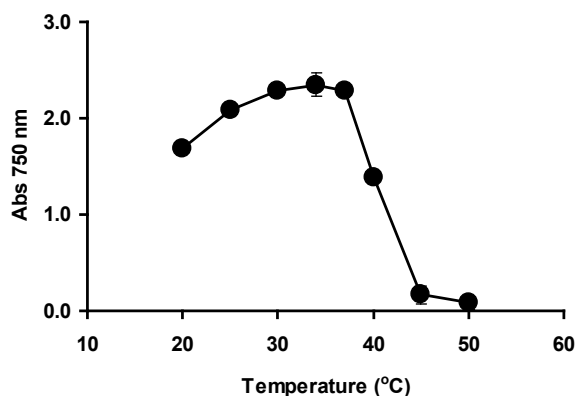


Fig. 4. The ability of the *Pseudomonas putida* strain Neni-3 to decrease molybdenum was observed to be temperature dependent. The bacterium was placed in a microtiter plate, and gave three days to incubate in ideal conditions.

Temperature and pH both play significant roles in the molybdenum reduction process. Because this reaction is enzyme-mediated, these parameters influence the folding of proteins as well as the activity of the enzyme, which is what ultimately leads to molybdenum reduction being inhibited. In a tropical nation like Malaysia, where annual average temperatures range from 25 to 35 degrees Celsius, bioremediation will profit from the appropriate conditions, which will be a benefit in and of themselves [44]. As a result, the *Pseudomonas putida* strain Neni-3 could be a contender for molybdenum soil bioremediation not just in this tropical region but also in other tropical nations. The ideal temperature for the majority of the reducers is somewhere between 25 and 37 degrees Celsius [32,44–54] as they are isolated from tropical soils with The only psychrotolerant reducer that can survive in isolation in the Antarctic, demonstrating at an optimal temperature that promotes molybdenum reduction between 15 and 20 °C [33].

The fact that *Pseudomonas putida* strain Neni-3 is a neutrophile is reflected in the ideal pH range that the strain demonstrates for sustaining molybdenum reduction. One of the characteristics of neutral flora is their capacity to flourish at pH levels ranging from 5.5 to 8.0. An important discovery regarding molybdenum reduction in bacteria was made regarding the best pH reduction, which was found to be mildly acidic, with optimal pHs ranging from pH 5.0 to pH 7.0 [32–34,44–46,48–56]. Because *Pseudomonas putida* strain Neni-3 is a neutrophile, the ideal pH range that it exhibits for sustaining molybdenum reduction is reflective of this feature of the bacterium. One of the characteristics of neutrophiles is their capacity to thrive in an environment with a pH range of 5.5 to 8.0. Understanding that slightly acidic pHs, specifically those between 5.0 and 7.0, are ideal for molybdenum reduction in bacteria is crucial [43].

Effect of electron donor on molybdate reduction

The findings of this investigation confirmed the findings of earlier studies, which found that glucose was the most effective electron donor for enabling molybdate reduction out of all the electron donors that were investigated. In descending order of their effectiveness, these are followed by sucrose, adonitol, mannose and finally xylose (Fig. 5). Other carbon sources did not support molybdenum reduction. Studies done before by Shukor et al. showed that several different kinds of Mo-reducing bacteria prefer glucose and sucrose [32–34,44–46,48–56].

Through metabolic pathways such as glycolysis, the Krebs cycle, and the electron transport chain, bacteria are able to produce electron-donating substrates, NADH and NADPH, when there are carbon sources present in the medium. NADH and NADPH are both responsible for molybdenum reducing-enzyme as the electron-donating substrates [54,57].

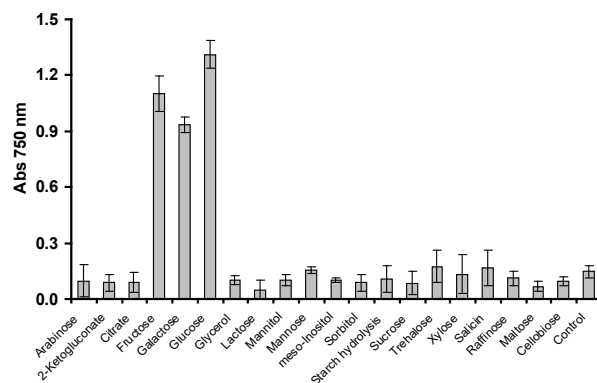


Fig. 5. The effect of various electron donor sources (1 per cent weight-per-volume) on the reduction of molybdenum. *Pseudomonas putida* strain Neni-3 was cultivated in low-phosphate media that included 10 mM molybdate in addition to a variety of electron donors. The bacterium's dormant cells were placed in a microtiter plate and allowed to grow for a total of three days in an environment that had been optimized. The error bars show the mean value as well as the standard deviation (n = 3).

Effect of phosphate and molybdate concentrations to molybdate reduction

It is essential to determine the optimal quantities of phosphate and molybdate in order to promote optimal molybdenum reduction because it has been demonstrated that both phosphate and molybdate limit the growth of Mo-blue in bacteria [32,33,44,46–49,52,54,56]. The optimal concentration of phosphate was found to be between 2.5 and 7.5 mM, whereas values that were greater than that were found to be very inhibitory to reduction (Fig. 6). A high quantity of phosphate can lead to phosphomolybdate instability, which is why acidic conditions are necessary for the combination.

A higher concentration of phosphate in a buffer will enhance its buffering capacity, as the buffering capacity of phosphate buffers is proportional to the concentration of phosphate in the buffer. Also, for reasons that have not been fully elucidated, the phosphomolybdate complex is inherently unstable in the presence of high concentrations of phosphate [58–60]. In order to function at their peak, all of the isolated molybdenum-reducing bacteria require a phosphate concentration of no more than 5 mM [32–34,44–46,48–56]. Phosphate concentrations more than 5 millimoles per liter (mM) are toxic to the molybdenum-reducing bacteria that have been discovered to far (Fig. 7). The lowest optimal concentration of molybdenum reported is 15 mM in *Pseudomonas* sp strain Dr.Y2 [32], whilst the highest molybdenum required for optimal reduction was 80 mM in *E. coli* K12 [55] and *Klebsiella oxytoca* strain hkeem [49]. In actuality, the highest environmental pollutant concentration of molybdenum is approximately 2000 ppm, which is equivalent to approximately 20 mM, and the utilization of this strain to remediate such a high molybdenum concentration in soil ought to be carried out [61].

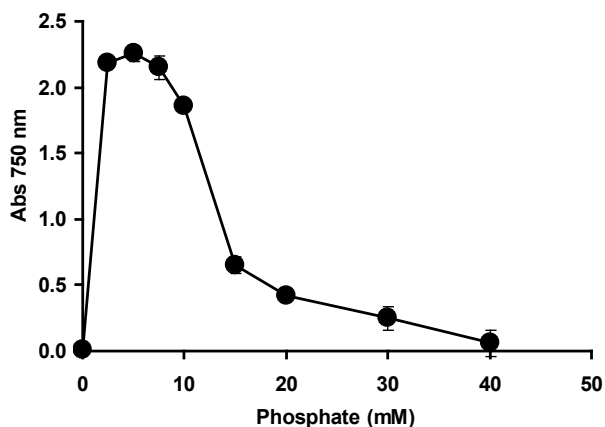


Fig. 6. The influence that the concentration of phosphate has on the amount of molybdenum that is reduced by the *Pseudomonas putida* strain Neni-3. The bacterium's dormant cells were placed in a microtiter plate and allowed to grow for a total of three days in an environment that had been optimized. The error bars show the mean value as well as the standard deviation (n = 3).

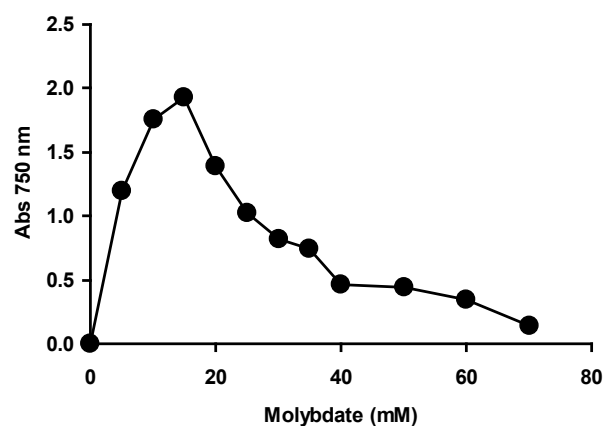


Fig. 7. *Pseudomonas putida* strain Neni-3's ability to reduce molybdenum depends on the concentration of molybdate. The bacterium's dormant cells were placed in a microtiter plate and allowed to grow for a total of three days in an environment that had been optimized. The error bars show the mean value as well as the standard deviation (n = 3).

Effect of heavy metals

At a concentration of 1 ppm, the reduction of molybdenum was slowed down by 62.8, 61.1, and 36.8 per cent, respectively, when Ag (I), Hg (II), and Cu (II) were present (**Fig. 8**). Inhibition brought on by the presence of other metal ions and heavy metals presents a significant obstacle for bioremediation. In addition to this, it is essential to search for and isolate microorganisms that have the similar ability to withstand metals. According to what was stated earlier [62], mercury is a physiological inhibitor to molybdate reduction. Bioremediation faces a substantial challenge in the form of inhibition, which is caused by the presence of various metal ions and heavy metals in the environment. In addition to this, it is vital to seek for and isolate microorganisms that have a comparable capacity to survive metals. According to the information that was provided earlier (**Table 2**). Mercury, cadmium, silver and copper usually target sulfhydryl group of enzymes [63]. Chromate is notorious for its ability to block a variety of enzymes, including glucose oxidase [64] and enzymes of nitrogen metabolism in plants [65]. The binding of heavy metals to the catalytic site of the enzyme rendered that enzyme's ability to reduce metals inactively. This rendered the enzyme (or enzymes) responsible for the reduction ineffective.

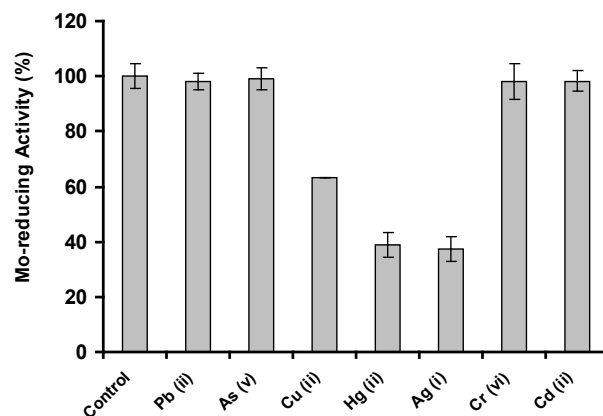


Fig. 8. The influence that various metals have on the synthesis of mo blue by the *Pseudomonas putida* strain Neni-3. The bacterium's dormant cells were placed in a microtiter plate and allowed to grow for a total of three days in an environment that had been optimized. The error bars show the mean value as well as the standard deviation (n = 3).

Table 2. Inhibition of Mo-reducing bacteria by heavy metals.

Bacteria	Heavy Metals that inhibit reduction	Author
<i>Bacillus pumilus</i> strain lbna	As ³⁺ , Pb ²⁺ , Zn ²⁺ , Cd ²⁺ , Cr ⁶⁺ , Hg ²⁺ , Cu ²⁺	[50]
<i>Bacillus</i> sp. strain A.rzi	Cd ²⁺ , Cr ⁶⁺ , Cu ²⁺ , Ag ⁺ , Pb ²⁺ , Hg ²⁺ , Co ²⁺ , Zn ²⁺	[52]
<i>Serratia</i> sp. strain Dr.Y8	Cr, Cu, Ag, Hg	[46]
<i>S. marcescens</i> strain Dr.Y9	Cr ⁶⁺ , Cu ²⁺ , Ag ⁺ , Hg ²⁺	[47]
<i>Serratia</i> sp. strain Dr.Y5	n.a.	[45]
<i>Pseudomonas</i> sp. strain DRY2	Cr ⁶⁺ , Cu ²⁺ , Pb ²⁺ , Hg ²⁺	[32]
<i>Pseudomonas</i> sp. strain DRY1	Cd ²⁺ , Cr ⁶⁺ , Cu ²⁺ , Ag ⁺ , Pb ²⁺ , Hg ²⁺	[33]
<i>Enterobacter</i> sp. strain Dr.Y13	Cr ⁶⁺ , Cd ²⁺ , Cu ²⁺ , Ag ⁺ , Hg ²⁺	[56]
<i>Acinetobacter calcoaceticus</i> strain Dr.Y12	Cd ²⁺ , Cr ⁶⁺ , Cu ²⁺ , Pb ²⁺ , Hg ²⁺	[48]
<i>Serratia marcescens</i> strain DRY6	Cr ⁶⁺ , Cu ²⁺ , Hg ²⁺ *	[44]
<i>Enterobacter cloacae</i> strain 48	Cr ⁶⁺ , Cu ²⁺	[34]
<i>Escherichia coli</i> K12	Cr ⁶⁺	[55]
<i>Klebsiella oxytoca</i> strain hkeem	Cu ²⁺ , Ag ⁺ , Hg ²⁺	[49]

Azo dye-decolorizing ability of the molybdenum-reducing bacterium

Nearly all of the molybdenum-reducing bacteria that have been identified to this point are capable, under static conditions, of reducing molybdenum into Mo-blue. Research is being done on the bacteria to determine whether or not it has the potential to decolorize a variety of azo dyes. With the help of the bacterium, nearly all of the azo dyes were able to be degraded including Congo Red, Ponceau S, Metanil Yellow, Remazol Black B, Tartrazine, Evans Blue, Naphthol Blue Black, Sudan Black B, Methyl Red and Fast Green FCF giving 88.08, 49.82, 48.89, 46.61, 42.12, 41.45, 41.09, 35.75, 32.41 and 10.1 % degradation

respectively in descending order (Fig. 9). Because of this, the bacterium has an increased capacity to breakdown Congo red. Under typical environmental circumstances, azo dyes exhibit a high level of resistance to biodegradation; yet, the azo bond is susceptible to reductive cleavage. Species of bacteria that have been shown to be able to break down this dye have been reported. They are *Serratia marcescens* [66], *Pseudomonas luteola* [67], *Bacillus* sp. [68], *Citrobacter* sp. [69], *Stenotrophomonas maltophilia* [70], *Acinetobacter baumannii* [71], *Staphylococcus* sp. [72] and *Enterobacter* sp. [73] and the bacterial species *Hafnia alvei*, *Enterobacter cloacae* and *Klebsiella pneumonia* [66]. The dye could also be efficiently decolorized by bacterial consortia [74,75].

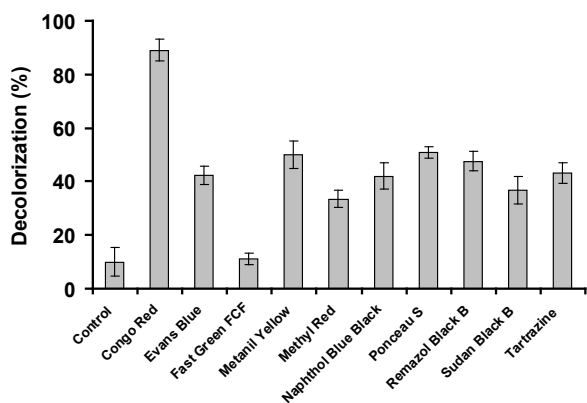


Fig. 9. Decolorization of various azo dyes by *Pseudomonas putida* strain Neni-3. Resting cells of the bacterium were incubated in a microtiter plate under optimized conditions for 72 hours. Error bars represent mean \pm standard deviation (n = 3).

CONCLUSION

An indigenous Mo-reducing bacteria isolate that has the potential to decolorize Congo Red has been discovered and isolated. This is the first proof that a bacteria may reduce molybdenum levels and so have the ability to decolorize Congo Red. Following glucose as the best electron donor for aiding molybdate reduction came fructose, and then galactose in declining order. Galactose was the least effective electron donor. The ideal conditions for the bacterium to convert molybdate to molybdate blue are a pH range of 6.0 to 6.5 and a temperature range of 25 to 37 degrees Celsius. A phosphate content of between 2.5 and 7.5 mM and a molybdate concentration of between 10 and 15 mM are two additional conditions that must be met. The Mo-blue bacterium produced an absorption spectrum that was comparable to that of the earlier Mo-reducing bacterium and that was very similar to the spectrum produced by the phosphomolybdate reduction reaction. Heavy metals that are known to be hazardous have an inhibiting effect on molybdenum reduction. We test the bacterium to see if it has the ability to decolorize a number of different azo dyes. The bacterium was able to decolorize a number of different azo dyes, however the dye Congo Red showed the greatest degree of decolorization. This bacteria's ability to detoxify various toxicants is a desirable quality, as it makes the bacterium an efficient bioremediation approach. As a result, this bacterium is in high demand. Purification of the molybdenum-reducing enzyme that was produced by this bacterium is presently being studied in order to characterize decolorization research in a more accurate manner.

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REFERENCES

1. Rieger PG, Meier HM, Gerle M, Vogt U, Groth T, Knackmuss HJ. Xenobiotics in the environment: Present and future strategies to obviate the problem of biological persistence. *J Biotechnol.* 2002;94(1):101–23.
2. AhmadPanahi H, Hosseinzadeh M, Adinehlo H, Moniri E, Manoochehri M. Removal of molybdenum from environmental sample by adsorption using modified aniline-formaldehyde with salicylic acid. *World Appl Sci J.* 2014;30(12):1892–8.
3. Yakasai HM, Rahman MF, Yasid NA, Ahmad SA, Halmi MIE, Shukor MY. Elevated Molybdenum Concentrations in Soils Contaminated with Spent Oil Lubricant. *J Environ Microbiol Toxicol.* 2017;5(2):1–3.
4. Davis GK. Molybdenum. In: Merian E, editor. *Metals and their Compounds in the Environment, Occurrence, Analysis and Biological Relevance.* VCH Weinheim, New York; 1991. p. 1089–100.
5. Neunhuserer C, Berreck M, Insam H. Remediation of soils contaminated with molybdenum using soil amendments and phytoremediation. *Water Air Soil Pollut.* 2001;128(1–2):85–96.
6. Bi CM, Zhang YL, Liu FJ, Zhou TZ, Yang ZI, Gao SY, et al. The effect of molybdenum on the in vitro development of mouse preimplantation embryos. *Syst Biol Reprod Med.* 2013;59(2):69–73.
7. Meeker JD, Rossano MG, Protas B, Diamond MP, Puscheck E, Daly D, et al. Cadmium, lead, and other metals in relation to semen quality: Human evidence for molybdenum as a male reproductive toxicant. *Environ Health Perspect.* 2008;116(11):1473–9.
8. Zhai XW, Zhang YL, Qi Q, Bai Y, Chen XL, Jin LJ, et al. Effects of molybdenum on sperm quality and testis oxidative stress. *Syst Biol Reprod Med.* 2013;59(5):251–5.
9. Zhang YL, Liu FJ, Chen XL, Zhang ZQ, Shu RZ, Yu XL, et al. Dual effects of molybdenum on mouse oocyte quality and ovarian oxidative stress. *Syst Biol Reprod Med.* 2013;59(6):312–8.
10. Kincaid RL. Toxicity of ammonium molybdate added to drinking water of calves. *J Dairy Sci.* 1980;63(4):608–10.
11. Underwood EJ. Environmental sources of heavy metals and their toxicity to man and animals. *Prog Water Technol.* 1979;11(4–5):33–45.
12. Meitiniarti VI, Soetarto ES, Timotius KH, Sugiharto E. Products of orange II biodegradation by *Enterococcus faecalis* ID6017 and *Chryseobacterium indologenes* ID6016. *Microbiol Indones.* 2007;1:51–4.
13. Hadibarata T, Adnan LA, Yusoff ARM, Yuniarto A, Rubiyatno, Zubir MMFA, et al. Microbial decolorization of an azo dye reactive black 5 using white-rot fungus *Pleurotus eryngii* F032. *Water Air Soil Pollut.* 2013;224(6).
14. Adnan LA, Mohd Yusoff AR, Hadibarata T, Khudhair AB. Biodegradation of bis-azo dye reactive black 5 by white-rot fungus *Trametes gibbosa* sp. WRF 3 and its metabolite characterization. *Water Air Soil Pollut.* 2014;225(10).
15. Chaudhuri M, Elmolla ES, Othman RB. Adsorption of reactive dyes Remazol Red F-3B and Remazol Blue from aqueous solution by coconut coir activated carbon. *Nat Environ Pollut Technol.* 2011;10(2):193–6.
16. Cui D, Li G, Zhao D, Zhao M. Effect of quinoid redox mediators on the aerobic decolorization of azo dyes by cells and cell extracts from *Escherichia coli*. *Environ Sci Pollut Res.* 2014;
17. Hafshejani MK, Ougubue CJ, Morad N. Sequential microaerophilic-oxic phase mineralization of Azo dyes by a monoculture of *Pseudomonas aeruginosa* strain AWF isolated from textile wastewater. *Water Air Soil Pollut.* 2013;224(9).
18. Kanagaraj J, Velan TS, Mandai AB. Biological method for decolourisation of an azo dye: Clean technology to reduce pollution load in dye waste water. *Clean Technol Environ Policy.* 2012;14(4):565–72.

19. Zablocka-Godlewska E, Przystas W, Grabinska-Sota E. Decolourization of diazo evans blue by two strains of *Pseudomonas fluorescens* isolated from different wastewater treatment plants. *Water Air Soil Pollut.* 2012;223(8):5259–66.
20. Zin KM, Effendi Halmi MI, Abd Gani SS, Zaidan UH, Samsuri AW, Abd Shukor MY. Microbial Decolorization of Triazo Dye, Direct Blue 71: An Optimization Approach Using Response Surface Methodology (RSM) and Artificial Neural Network (ANN) [Internet]. Vol. 2020, *BioMed Research International*. Hindawi; 2020 [cited 2020 Sep 10]. p. e2734135. Available from: <https://www.hindawi.com/journals/bmri/2020/2734135/>
21. Zollinger H. *Color Chemistry: Syntheses, Properties, and Applications of Organic Dyes and Pigments*. John Wiley & Sons; 2003. 656 p.
22. Anu M, Gnana TVS, Reshma JK. Simultaneous phenol degradation and chromium (VI) reduction by bacterial isolates. *Res J Biotechnol.* 2010;5(1):46–9.
23. Bhattacharya A, Gupta A, Kaur A, Malik D. Efficacy of *Acinetobacter* sp. B9 for simultaneous removal of phenol and hexavalent chromium from co-contaminated system. *Appl Microbiol Biotechnol.* 2014;98(23):9829–41.
24. Chaudhari AU, Tapase SR, Markad VL, Kodam KM. Simultaneous decolorization of reactive Orange M2R dye and reduction of chromate by *Lysinibacillus* sp. KMK-A. *J Hazard Mater.* 2013;262:580–8.
25. 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.
26. Gusmanizar N, Halmi MIE, Rusnam M, Rahman MFA, Shukor MS, Azmi NS, et al. Isolation and characterization of a molybdenum-reducing and azo-dye decolorizing *Serratia marcescens* strain Neni-1 from Indonesian soil. *J Urban Environ Eng.* 2016;10(1):113–23.
27. Mansur R, Gusmanizar N, Roslan MAH, Ahmad SA, Shukor MY. Isolation and characterisation of a molybdenum-reducing and Metanil yellow dye-decolourising *Bacillus* sp. strain Neni-10 in soils from West Sumatera, Indonesia. *Trop Life Sci Res.* 2017 Jan;28(1):69–90.
28. Holt JG, Krieg NR, Sneath PHA, Staley JT, Williams ST. *Bergey's Manual of Determinative Bacteriology*. 9th ed. Lippincott Williams & Wilkins; 1994.
29. Costin S, Ionut S. ABIS online - bacterial identification software, http://www.tgw1916.net/bacteria_logare.html, database version: Bacillus 022012-2.10, accessed on Mar 2015. 2015.
30. Shukor MS, Shukor MY. A microplate format for characterizing the growth of molybdenum-reducing bacteria. *J Environ Microbiol Toxicol.* 2014;2(2):42–4.
31. Shukor MY, Lee CH, Omar I, Karim MIA, Syed MA, Shamaan NA. Isolation and characterization of a molybdenum-reducing enzyme in *Enterobacter cloacae* strain 48. *Pertanika J Sci Technol.* 2003;11(2):261–72.
32. Shukor MY, Ahmad SA, Nadzir MMM, Abdullah MP, Shamaan NA, Syed MA. Molybdate reduction by *Pseudomonas* sp. strain DRY2. *J Appl Microbiol.* 2010;108(6):2050–8.
33. Ahmad SA, Shukor MY, Shamaan NA, Mac Cormack WP, Syed MA. Molybdate reduction to molybdenum blue by an antarctic bacterium. *BioMed Res Int.* 2013;2013.
34. Ghani B, Takai M, Hisham NZ, Kishimoto N, Ismail AKM, Tano T, et al. Isolation and characterization of a Mo6+-reducing bacterium. *Appl Environ Microbiol.* 1993;59(4):1176–80.
35. Losi ME, Jr WTF. Reduction of selenium oxyanions by *Enterobacter cloacae* strain SLD1a-1: Reduction of selenate to selenite. *Environ Toxicol Chem.* 1997;16(9):1851–8.
36. Llovera S, Bonet R, Simon-Pujol MD, Congregado F. Chromate reduction by resting cells of *Agrobacterium radiobacter* EPS-916. *Appl Environ Microbiol.* 1993;59(10):3516–8.
37. Carpentier W, Smet LD, Beeumen JV, Brigé A. Respiration and growth of *Shewanella oneidensis* MR-1 using vanadate as the sole electron acceptor. *J Bacteriol.* 2005;187(10):3293–301.
38. Auffret MD, Yergeau E, Labbé D, Fayolle-Guichard F, Greer CW. Importance of *Rhodococcus* strains in a bacterial consortium degrading a mixture of hydrocarbons, gasoline, and diesel oil additives revealed by metatranscriptomic analysis. *Appl Microbiol Biotechnol.* 2015 Mar;99(5):2419–30.
39. Chaturvedi V, Kumar A. Diversity of culturable sodium dodecyl sulfate (SDS) degrading bacteria isolated from detergent contaminated ponds situated in Varanasi city, India. *Int Biodeterior Biodegrad.* 2011;65(7):961–71.
40. Sedighi M, Vahabzadeh F. Kinetic Modeling of cometabolic degradation of ethanethiol and phenol by *Ralstonia eutropha*. *Biotechnol Bioprocess Eng.* 2014;19(2):239–49.
41. Raj J, Prasad S, Sharma NN, Bhalla TC. Bioconversion of Acrylonitrile to Acrylamide using Polyacrylamide Entrapped Cells of *Rhodococcus rhodochrous* PA-34. *Folia Microbiol (Praha).* 2010;55(5):442–6.
42. Steiert JG, Pignatello JJ, Crawford RL. Degradation of chlorinated phenols by a pentachlorophenol-degrading bacterium. *Appl Environ Microbiol.* 1987;53(5):907–10.
43. Shukor Y, Adam H, Ithnin K, Yunus I, Shamaan NA, Syed A. Molybdate reduction to molybdenum blue in microbe proceeds via a phosphomolybdate intermediate. *J Biol Sci.* 2007;7(8):1448–52.
44. Shukor MY, Habib SHM, Rahman MFA, Jirangon H, Abdullah MPA, Shamaan NA, et al. Hexavalent molybdenum reduction to molybdenum blue by *S. marcescens* strain Dr. Y6. *Appl Biochem Biotechnol.* 2008;149(1):33–43.
45. Rahman MFA, Shukor MY, Suhaili Z, Mustafa S, Shamaan NA, Syed MA. Reduction of Mo(VI) by the bacterium *Serratia* sp. strain DRY5. *J Environ Biol.* 2009;30(1):65–72.
46. Shukor MY, Rahman MF, Suhaili Z, Shamaan NA, Syed MA. Bacterial reduction of hexavalent molybdenum to molybdenum blue. *World J Microbiol Biotechnol.* 2009;25(7):1225–34.
47. Yunus SM, Hamim HM, Anas OM, Aripin SN, Arif SM. Mo (VI) reduction to molybdenum blue by *Serratia marcescens* strain Dr. Y9. *Pol J Microbiol.* 2009;58(2):141–7.
48. Shukor MY, Rahman MF, Suhaili Z, Shamaan NA, Syed MA. Hexavalent molybdenum reduction to Mo-blue by *Acinetobacter calcoaceticus*. *Folia Microbiol (Praha).* 2010;55(2):137–43.
49. Lim HK, Syed MA, Shukor MY. Reduction of molybdate to molybdenum blue by *Klebsiella* sp. strain hkeem. *J Basic Microbiol.* 2012;52(3):296–305.
50. Abo-Shakeer LKA, Ahmad SA, Shukor MY, Shamaan NA, Syed MA. Isolation and characterization of a molybdenum-reducing *Bacillus pumilus* strain lbna. *J Environ Microbiol Toxicol.* 2013;1(1):9–14.
51. Halmi MIE, Zuhainis SW, Yusof MT, Shaharuddin NA, Helmi W, Shukor Y, et al. Hexavalent molybdenum reduction to Mo-blue by a Sodium-Dodecyl-Sulfate-degrading *Klebsiella oxytoca* strain DRY14. *BioMed Res Int.* 2013;2013:e384541.
52. Othman AR, Bakar NA, Halmi MIE, Johari WLW, Ahmad SA, Jirangon H, et al. Kinetics of molybdenum reduction to molybdenum blue by *Bacillus* sp. strain A.rzi. *BioMed Res Int.* 2013;2013.
53. Khan A, Halmi MIE, Shukor MY. Isolation of Mo-reducing bacterium in soils from Pakistan. *J Environ Microbiol Toxicol.* 2014;2(1):38–41.
54. Shukor MY, Halmi MIE, Rahman MFA, Shamaan NA, Syed MA. Molybdenum reduction to molybdenum blue in *Serratia* sp. strain DRY5 is catalyzed by a novel molybdenum-reducing enzyme. *BioMed Res Int.* 2014;2014.
55. Campbell AM, Del Campillo-Campbell A, Villaret DB. Molybdate reduction by *Escherichia coli* K-12 and its chl mutants. *Proc Natl Acad Sci U S A.* 1985;82(1):227–31.
56. Shukor MY, Rahman MF, Shamaan NA, Syed MS. Reduction of molybdate to molybdenum blue by *Enterobacter* sp. strain Dr.Y13. *J Basic Microbiol.* 2009;49(SUPPL. 1):S43–54.
57. Shukor MY, Rahman MFA, Shamaan NA, Lee CH, Karim MIA, Syed MA. An improved enzyme assay for molybdenum-reducing activity in bacteria. *Appl Biochem Biotechnol.* 2008;144(3):293–300.
58. Glenn JL, Crane FL. Studies on metalloflavoproteins. V. The action of silicomolybdate in the reduction of cytochrome c by aldehyde oxidase. *Biochim Biophys Acta.* 1956;22(1):111–5.
59. Shukor MY, Shamaan NA, Syed MA, Lee CH, Karim MIA. Characterization and quantification of molybdenum blue production in *Enterobacter cloacae* strain 48 using 12-molybdophosphate as the reference compound. *Asia-Pac J Mol Biol Biotechnol.* 2000;8(2):167–72.

60. Sims RPA. Formation of heteropoly blue by some reduction procedures used in the micro-determination of phosphorus. *The Analyst*. 1961;86(1026):584–90.
61. Runnells DD, Kaback DS, Thurman EM. Geochemistry and sampling of molybdenum in sediments, soils, and plants in Colorado. In: Chappel WR, Peterson KK, editors. *Molybdenum in the environment*. New York: Marcel and Dekker, Inc.; 1976.
62. Shukor MY, Syed MA, Lee CH, Karim MIA, Shamaan NA. A method to distinguish between chemical and enzymatic reduction of molybdenum in *Enterobacter cloacae* strain 48. *Malays J Biochem*. 2002;7:71–2.
63. Sugiura Y, Hirayama Y. Structural and electronic effects on complex formation of copper(II) and nickel(II) with sulfhydryl-containing peptides. *Inorg Chem*. 1976;15(3):679–82.
64. Zeng GM, Tang L, Shen GL, Huang GH, Niu CG. Determination of trace chromium (VI) by an inhibition-based enzyme biosensor incorporating an electropolymerized aniline membrane and ferrocene as electron transfer mediator. *Int J Environ Anal Chem*. 2004;84(10):761–74.
65. Sangwan P, Kumar V, Joshi UN. Effect of chromium(VI) toxicity on enzymes of nitrogen metabolism in clusterbean (*Cyamopsis tetragonoloba* L.). *Enzyme Res*. 2014;2014:784036.
66. Raj DS, Prabha RJ, Leena R. Analysis of bacterial degradation of azo dye congo red using HPLC. *J Ind Pollut Control*. 2012;28(1):57–62.
67. Hsueh CC, Chen BY. Comparative study on reaction selectivity of azo dye decolorization by *Pseudomonas luteola*. *J Hazard Mater*. 2007;141(3):842–9.
68. Gopinath KP, Sahib HAM, Muthukumar K, Velan M. Improved biodegradation of Congo red by using *Bacillus* sp. *Bioresour Technol*. 2009;100(2):670–5.
69. 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;24(12):1037–40.
70. Galai S, Limam F, Marzouki MN. A new *Stenotrophomonas maltophilia* strain producing laccase. use in decolorization of synthetic dyes. *Appl Biochem Biotechnol*. 2009;158(2):416–31.
71. Ning XA, Yang C, Wang Y, Yang Z, Wang J, Li R. Decolorization and biodegradation of the azo dye Congo red by an isolated *Acinetobacter baumannii* YNWH 226. *Biotechnol Bioprocess Eng*. 2014;19(4):687–95.
72. Park EH, Jang MS, Cha IH, Choi YL, Cho YS, Kim CH, et al. Decolorization of a sulfonated azo dye, Congo Red, by *Staphylococcus* sp. EY-3. *J Microbiol Biotechnol*. 2005;15(1):221–5.
73. Prasad SS, Aikat K. Optimization of medium for decolorization of Congo red by *Enterobacter* sp. SXCR using response surface methodology. *Desalination Water Treat*. 2013;52(31–33):6166–74.
74. Bafana A, Chakrabarti T, Devi SS. Azoreductase and dye detoxification activities of *Bacillus velezensis* strain AB. *Appl Microbiol Biotechnol*. 2008;77(5):1139–44.
75. Isik M, Sponza DT. Decolorization of Azo Dyes under Batch Anaerobic and Sequential Anaerobic/Aerobic Conditions. *J Environ Sci Health - Part Toxic/Hazardous Subst Environ Eng*. 2004;39(4):1107–27.