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.

**KEYWORDS**

Mo-reducing bacterium
*Pseudomonas putida*
Mo-blue
Azo dye-degrading
Congo red

**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 thymes 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 an 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 azo dyes. Static growth or circumstances are used on purpose, and 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), MgSO4.7H2O (0.05 per cent), yeast extract (0.5 per cent), (NH4)2SO4 (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 mM⁻¹cm⁻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), (NH4)2SO4 (0.3 per cent), NaNO3 (0.2 per cent), MgSO4.7H2O (0.05 per cent), yeast extract (0.05 per cent), NaCl (0.5 per cent), Na2HPO4 (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 per centage 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 ºC              | –      | Citrate                   | +      |
| Growth at 41 ºC             | +      | Fructose                  | +      |
| Growth on MacConkey agar    | –      | Glucose                   | +      |
| Arginine dihydroase (ADH)   | +      | meso-Inositol             | –      |
| Alkaline phosphatase (PAL)  | +      | 2-ketoglucuronate         | –      |
| Indole production           | –      | Mannose                   | +      |
| Nitrates reduction          | –      | Mannitol                  | –      |
| Lecithinase                 | –      | Sorbitol                  | –      |
| Lysine decarboxylase (LDC)  | –      | Succrose                  | +      |
| Ornithine decarboxylase (ODC)| –    | Trehalose                 | –      |
| ONPG (beta-galactosidase)   | –      | Xyllose                   | –      |
| 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 of 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.
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 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 Kreb's 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].

**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 phosphomolybate 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 phosphomolybate 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].
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 a report that was provided earlier [32], 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.

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, Metalin 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 putida strain Nen-3. Resting cells of the bacterium were incubated in a microtiter plate under optimized conditions for 72 hours. Error bars represent mean ± 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 bacterium'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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