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Characterization of a molybdenum-reducing bacterium with the ability to degrade phenol, isolated in soils from Egypt

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Abstract

Polluted sites often contain both heavy metals and organic xenobiotic contaminants. This warrants the use of either a great number of bacterial degraders or bacteria having the ability to detoxify several toxicants simultaneously. In this research, the ability of a molybdenum-reducing (Mo-reducing) bacterium isolated from contaminated soil to decolorize various phenolics independent of Mo reduction was screened. Studies showed that this bacterium was able to grow on 4-nonylphenol and reduced molybdate to Mo-blue. The optimal condition for this activity was pH between 6.3 and 6.8 and temperature of 34°C. Glucose proved to be the best electron donor for supporting molybdate reduction followed by galactose, fructose, and citrate in descending order. Other requirements included a phosphate concentration between 2.5 mM and 7.5 mM and a molybdate concentration between 20 and 30 mM. The absorption spectrum of the Mo-blue produced was similar to numerous previously described Mo-reducing bacteria, closely resembling a spectrum of the reduced phosphomolybdate. Mo reduction was inhibited by mercury (II), silver (I), copper (II), cadmium (II), and chromium (VI) at 2 ppm by 79.6%, 64.2%, 51.3%, 28.1%, and 25.0%, respectively. The biochemical analysis resulted in a tentative identification of the bacterium as *Pseudomonas aeruginosa* strain Amr-11. The ability of this bacterium to detoxify Mo and grow on nonylphenol makes this bacterium an important tool for bioremediation.

Key words: bioremediation, electron donor, molybdenum, nonylphenol, phenol

Introduction

Polluted sites are often contaminated with both heavy metals and organic compounds. This requires the use of either a multitude of bacterial degraders specific for each toxicant or bacteria having the capacity to detoxify numerous toxicants simultaneously. Molybdenum is one of the essential heavy metals that is required at trace amounts as a cofactor for numerous enzymes while it is toxic to a variety of organisms at elevated levels (Ahmad Panahi et al., 2014). This metal has many applications in industries in which it can be used as an alloying agent, an automobile engine anti-freeze component, a component of corrosion resistant steel, or a lubricant in the form of Mo disulfide. The wide application of Mo in industry has resulted in several water pollutions all over the world, including the Tokyo Bay, the Tyrol in Austria, and the Black Sea, where the Mo level counts in hundreds of parts per million (Davis, 1991; Neunhäuserer et al., 2001). In addition, terrestrially, levels of Mo above the Maximum Permissible Limit have been found in sewage, posing a health hazard as some of the reclaimed water from sewage were used in agriculture in the 1970s (Lahann, 1976). The toxicity of Mo in inhibiting spermatogenesis and arresting embryogenesis in organisms such as catfish and mice at levels as low as several parts per million have been reported (Meeker et al.,

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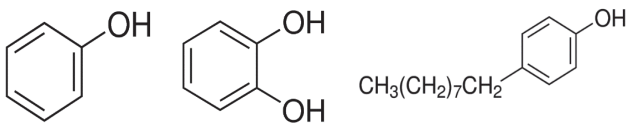
2008; Bi et al., 2013; Bi et al., 2013; Zhai et al., 2013; Zhang et al., 2013). Furthermore, Mo is very toxic to ruminants at levels of several parts per million, and cows are affected the most (Underwood, 1979). In addition to heavy metals, hydrocarbons in the form of oil, grease, and phenolics are ranked as the number one scheduled waste generated from industries (Achmadi, 1996). Other sources of pollutants are accidents. For instance, an accident causing the 533 ton Indonesian tanker MV Endah Lestari carrying 18 tons of diesel and 600 tons of phenol to capsize in 2001 polluted the coastal waters of Indonesia and Malaysia, killing thousands of fish and cockles reared in 85 offshore cages (Dahalan et al., 2014).

Phenol and phenolic compounds (Fig. 1) are not only toxic to humans but also to many other organisms as well (Gami et al., 2014). Toxicity is due to the hydrophobicity of those compounds and to a certain extent, the formation of phenoxyl radicals (Hansch et al., 2000). Nonylphenol (NP) is one of the major degradation products when nonylphenol ethoxylates (NPEs), widely used as non-ionic surfactants, are discharged into sewage treatment plants or directly released into natural ecosystems. Large amounts of NPEs are used globally and have resulted in the accumulation of NP in the environment. The half-lives of NP in the environment can be as long as 100 days, which, coupled with its toxicity and endocrine activity (Gu et al., 2010), makes NP a potential threat to human health and to the aquatic and terrestrial ecosystems. It was reported that NP at environmental levels of between 0.022 ng/l and 220 ng/l could affect cytokine secretion in human placenta (Bechi et al., 2006) making its removal a priority. At this level, bioremediation is the plausible option since chemical and physical methods are noneconomic and ineffective (Watanabe et al., 2012).



(A) Phenol (B) Catechol (C) 4-Nonylphenol

Fig. 1. The structure of some toxic phenolic compounds

Some microbes are able to degrade a variety of xenobiotic and detoxify heavy metals at the same time. Owing to their versatility, these microbes are in great demand in polluted sites where the presence of several contaminants is the norm. Specific examples of their application include the reduction of chromate coupled with phenol biodegradation (Sun et al., 2011; Bhattacharya et al., 2014). In the present study, the ability of a novel Mo-reducing bacterium isolated from contaminated soil to grow on various phenolic compounds including phenol was screened. The study was conducted under static growth conditions, which were easy to obtain in a microplate environment where the oxygen concentration is between 0% and 10% of environmental oxygen (EO) whereas the EO under aerobic conditions is about 20%. The microplate conditions mimic most of the bioremediation conditions, which are carried out in aquatic bodies or soils where the EO level is less than ~20% EO. This paper reports on a novel Mo-reducing bacterium with the capacity to grow on NP isolated from contaminated soil. The characteristics of this bacterium would make it suitable for future bioremediation works involving both the heavy metal Mo and dye as organic contaminants.

Materials and methods

Isolation of a molybdenum-reducing bacterium

Soil samples were taken (5 cm deep from topsoil) from the grounds of the contaminated land in Sadat City, Egypt, in 2014. About 1 g of a soil sample was suspended in sterile tap water. A total of 0.1 ml aliquot of the soil suspension was pipetted and spread onto agar of low phosphate media (LPM) (pH 7.0) and incubated for 48 h at room temperature. The composition of the LPM was as follows: glucose anhydrous (55 mM), $(NH_4)_2 \cdot SO_4$ (22.7 mM), $MgSO_4 \cdot 7H_2O$ (2.03 mM), yeast extract (0.5%), NaCl (85.56 mM), $Na_2MoO_4 \cdot 2H_2O$ (10 mM) and Na_2HPO_4 (5 mM) (Yunus et al., 2009). The formation of blue colonies indicates molybdate reduction by Mo-reducing bacteria. A colony with most intense blue color was isolated and re-streaked on LPM to obtain a pure culture. Molybdenum reduction in liquid media (at pH 7.0) was carried out in 100 ml of the LPM. The bacterial culture was grown at room temperature for 48 h on an orbital shaker set at 120 rpm. To analyze the Mo blue (Mo-blue) absorption spectrum 1.0 ml of the Mo-blue formed from the liquid culture above was centrifuged at 10,000 × g for 10 min at room temperature. Scanning of the supernatant was carried out from 400 to 900 nm using a UV-spectrophotometer (Shimadzu 1201). The LPM were utilized as the baseline correction.

Mo-reducing bacterium identification

The bacterium was biochemically and phenotypically characterized using standard methods such as colony shape, gram staining, size and color on nutrient agar plate, motility, oxidase (24 h), ONPG (beta-galactosidase), catalase production (24 h), ornithine decarboxylase (ODC), arginine dihydrolase (ADH), lysine decarboxylase (LDC), nitrate reduction, Methyl red, indole production, Voges-Proskauer (VP), hydrogen sulfide (H_2S), acetate utilization, malonate utilization, citrate utilization (Simmons), esculin hydrolysis, tartrate (Jordans), gelatin hydrolysis, urea hydrolysis, deoxyribonuclease, lipase (corn oil), phenylalanine deaminase, gas production from glucose, and the production of acids from various sugars were carried out according to the Bergey's Manual of Determinative Bacteriology (Holt et al., 1994). Interpretation of the results was carried out via the ABIS online system (Costin and Ionut, 2015).

Preparation of resting cells for molybdenum reduction characterization

The characterization works such as the effects of pH, temperature, phosphate, and molybdate concentrations to Mo-blue production were carried out utilizing resting cells in a microplate or microtiter format as previously developed. (Shukor and Shukor, 2014). Cells from a 1l overnight culture were grown in high phosphate media (HPM) at room temperature on orbital shaker (150 rpm) with the only difference between the LPM and HPM being the phosphate concentration that was fixed at 100 mM for the HPM. Cells were harvested by centrifugation at 15,000 × g for 10 min, and the pellet was washed several times to remove residual phosphate and was subsequently resuspended in 20 ml of LPM (LPM without glucose) to OD_{600} of approximately 1.00. In the LPM, the concentration of 5 mM phosphate has been found optimal for all of the Mo-reducing bacteria isolated so far, and hence this concentration was used in this work. Higher concentrations were found to be strongly inhibitory to molybdate reduction (Campbell et al., 1985; Ghani et al., 1993; Shukor et al., 2007; Shukor et al., 2008a; Rahman et al., 2009; Shukor et al., 2009a; Yunus et al., 2009; Shukor et al., 2009b; Shukor et al., 2010a; Shukor et al., 2010b; Lim et al., 2012; Abo-Shakeer et al., 2013; Ahmad et al., 2013; Halmi et al., 2013; Othman et al., 2013; Khan et al., 2014). Then, 180 µl was pipetted into each well of a sterile microplate. An amount of 20 µl of glucose from a stock solution was then added to each well to initiate Mo-blue production. A sealing tape that allows gas exchange (Corning® microplate) was used for sealing the tape. The microplate was incubated at room temperature. At defined time points, the absorbance at 750 nm was read in a BioRad (Richmond, CA) Microtiter Plate reader (Model No. 680). The production of Mo-blue from the media in a microplate format was measured using the specific extinction coefficient of 11.69/mM/cm at 750 nm as the maximum filter wavelength available for the microplate unit was 750 nm (Shukor et al., 2003).

Effect of heavy metals on molybdenum reduction

Seven heavy metals, namely, lead (II), arsenic (V), copper (II), mercury (II), silver (I), chromium (VI), and cadmium (II) were prepared from commercial salts or from Atomic Absorption Spectrometry standard solutions from MERCK. The bacterium was incubated with heavy metals, at various concentrations, in the microplate. The plate was incubated for 72 h at 30 °C. The amount of Mo-blue production was measured at 750 nm as before.

Screening of molybdenum reduction and independent growth using phenolics

The ability of phenolics such as phenol, 2,4-dinitrophenol, pentachlorophenol, 2-chlorophenol, 4-chlorophenol, catechol, salicylic acid, 4-nonylphenol, p-hydroxybenzoic acid, benzoate, and 2-napthol to support molybdenum reduction as electron donors was tested using the microplate as described above. In this case, glucose was replaced in the low phosphate medium with the phenolic compounds. The phenolic compounds, each at the final concentration of 200 mg/l and in a volume of 50 µl, were then mixed with 150 µl of LPM (Arif et al., 2013). Then, 200 µl of this preparation was placed into the microplate wells and mixed with 50 µl of resting cell suspension. The microplate was incubated at room temperature for three days, and the amount of Mo-blue production was measured at 750 nm as before. The ability of the phenolics to support growth of this bacterium independent of molybdenum-reduction was tested using the LPM minus molybdate and replacing glucose with the phenolics at the final concentration of 200 mg/l in a volume of 50 µl. Then, 200 µl of the media was added into the microplate wells with 50 µl of resting cell suspension. The ingredients of the growth media (LPM)

Table 1. Biochemical tests for *Pseudomonas aeruginosa* strain Amr-11

Motility	+	Utilization of:	
Hemolysis	+	L-Arabinose	d
Growth at 4 °C	–	Citrate	+
Growth at 41 °C	+	Fructose	+
Growth on MacConkey agar	+	Glucose	+
Arginine dihydrolase (ADH)	+	meso-Inositol	–
Alkaline phosphatase (PAL)	d	2-Ketogluconate	+
H_2S production	–	Mannose	–
Indole production	–	Mannitol	+
Nitrates reduction	+	Sorbitol	–
Lecithinase	–	Sucrose	–
Lysine decarboxylase (LDC)	–	Trehalose	–
Ornithine decarboxylase (ODC)	–	Xylose	–
ONPG (beta-galactosidase)	–	Starch hydrolysis	–
Esculin hydrolysis	–		
Gelatin hydrolysis	+		
Starch hydrolysis	–		
Oxidase reaction	+		

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

were as follows: $(NH_4)_2 \cdot SO_4$ (22.7 mM), $NaNO_3$ (0.2%), $MgSO_4 \cdot 7H_2O$ (2.03 mM), yeast extract (0.01%), NaCl (85.56 mM), and Na_2HPO_4 (50 mM). The media were adjusted to pH 7.0 with 1 M HCl. The increase of bacterial growth was measured at 600 nm after three days of incubation at room temperature.

Statistical analysis

Values are means ± SE. Data analyses were carried out using the Graphpad Prism version 3.0 and Graphpad InStat version 3.05 available from www.graphpad.com.

Results and discussion

Identification of Mo-reducing bacterium

The bacterium was a short rod-shaped, motile, Gram-negative bacterium. It was identified by comparing the results of cultural, morphological, and various biochemical tests (Table 1) to the Bergey's Manual of Determinative Bacteriology (Holt et al., 1994) and using the ABIS online software (Costin and Ionut, 2015). The computational analyses suggested the bacterial identity as *Pseudomonas aeruginosa* with a high homology score of 98% and accuracy score at 90%. However, additional analyses, especially a molecular identification technique through comparison of the 16srRNA gene, are needed to characterize this species further. However, at this juncture, the bacterium is tentatively identified as *P. aeruginosa* strain Amr-11. Two Mo-reducing bacteria from this genus such as *Pseudomonas* sp. strain DRY2 (Shukor et al., 2010a) and the Antarctic bacterium *Pseudomonas* sp. strain DRY1 (Ahmad et al., 2013) have been reported previously. Bacterial strains from this genus have been known to degrade NP and NPEs (Yuan et al., 2004; Chakraborty and Dutta, 2006; Liu et al., 2006; Watanabe et al., 2012).

In the present work using this bacterium, a rapid and simple high throughput method involving microplate format was used to speed up the characterization works and obtain more data than in the ordinary shake-flask approach (Iyamu et al., 2008; Shukor and Shukor, 2014). The use of resting cells under static conditions to characterize Mo reduction in bacterium was initiated by Ghani et al., 1993. Resting cells have been used for studying

heavy metal reduction such as selenate (Losi and Jr, 1997), chromate (Llovera et al., 1993), vanadate (Carpentier et al., 2005) reduction and xenobiotic biodegradation such as diesel (Auffret et al., 2014), SDS (Chaturvedi and Kumar, 2011), phenol (Sedighi and Vahabzadeh, 2014), amides (Raj et al., 2010), and pentachlorophenol (Steiert et al., 1987).

Molybdenum absorbance spectrum

The absorption spectrum of Mo-blue produced by *P. aeruginosa* strain Amr-11 exhibited a shoulder at approximately 700 nm and a maximum peak near the infra-red region of between 860 and 870 nm with a median at 865 nm (Fig. 2A). Mo-blue production was increased approximately in a linear fashion up to 24 h of incubation. Between 24 and 48 h, Mo-blue production appeared to be slowed down (Fig. 2B). The identification of the Mo-blue is not easily ascertained as it is complex in structure and has many species (Shukor et al., 2007). Basically, Mo-blue is a reduced product of two types of Mo complexes as follows: isopolymolybdate and heteropolymolybdate. It has been suggested by Campbell et al. (Campbell et al., 1985) that the Mo-blue observed after the reduction of Mo by *E. coli* K12 is a reduced form of phosphomolybdate, but these authors did not provide a plausible mechanism. The Mo-blue spectrum from the phosphate determination method normally showed a maximum absorption around 880 to 890 nm and a shoulder around 700 to 720 nm (Hori et al., 1988). We have shown previously that the entire Mo-blue spectra from other bacteria obey this requirement (Shukor et al., 2007). In this work, the result from the absorption spectrum clearly implies a similar spectrum, and thus confirms such hypothesis. Due to the complex structure of the compound, accurate identification of the phosphomolybdate species must be carried out using Nuclear Magnetic Resonance (NMR) and Electron Spin Resonance (ESR) (Chae et al., 1993). However, the spectrophotometric characterization of heteropolymolybdate species via analyzing the scanning spectroscopic profile is a less cumbersome and accepted method (Glenn and Crane, 1956; Sims, 1961; Kazansky and Fedotov, 1980; Yoshimura et al., 1986). Although the maximum absorption wavelength for Mo-blue was 865 nm, the measurement at 750 nm, although approximately 30% lower, was precise enough for routine monitoring of Mo-blue production as the absorbance value obtained have not interfered with bacterial cell absorption of between 600 and 620 nm (Shukor and Shukor, 2014). Previous determination of Mo-blue production was carried out using wavelengths such as 710 nm (Ghani et al., 1993) and 820 nm (Campbell et al., 1985).

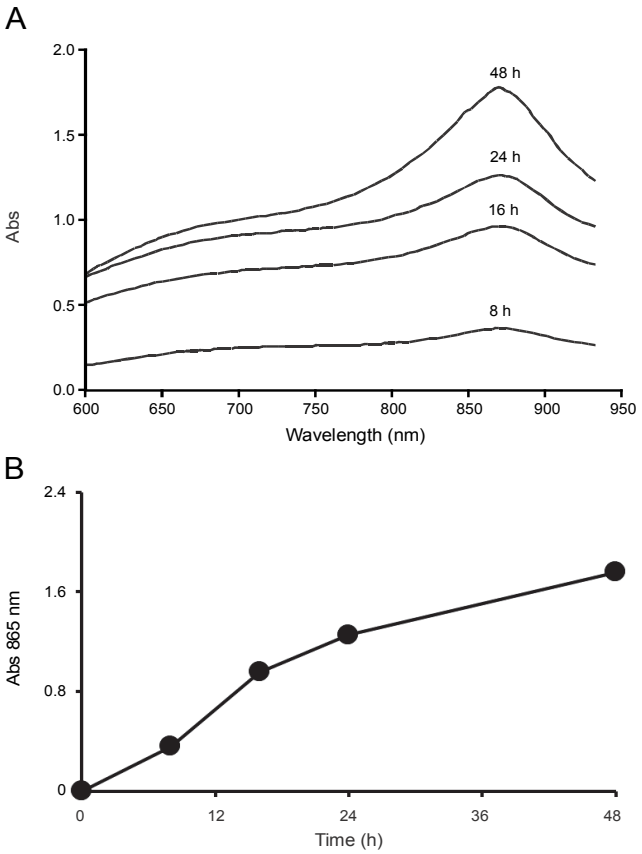


Fig. 2. A) Scanning absorption spectrum of Mo-blue from *Pseudomonas aeruginosa* strain Amr-11 at different time intervals, and B) profile of Mo-blue production over time measured at 865 nm

Effect of pH and temperature on molybdenum reduction

P. aeruginosa strain Amr-11 was incubated at different pH ranging from 5.5 to 8.0 using Bis-Tris and Tris-Cl buffers (20 mM). The optimum pH for molybdenum reduction was between 6.3 and 6.5 (Fig. 3). The effect of temperature (Fig. 4) was observed over a wide range of temperatures (20-60 °C) with an optimum temperature ranging from 25 to 37 °C. Temperatures higher than 37 °C were strongly inhibitory to Mo-blue production from *P. aeruginosa* strain Amr-11.

Temperature and pH play important role in Mo reduction, and since this process is enzyme mediated, both parameters affect protein folding and enzyme activity causing the inhibition of Mo reduction. The optimum

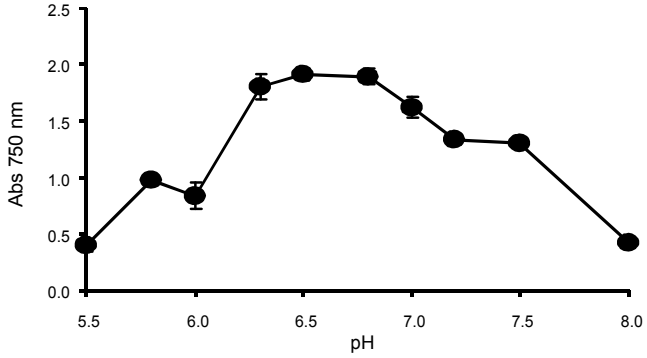


Fig. 3. Effect of pH on molybdenum reduction by *Pseudomonas aeruginosa* strain Amr-11. Resting cells of the bacterium were incubated in a microtiter plate under optimized conditions for 72 h. Error bars represent mean ± SD (n = 3)

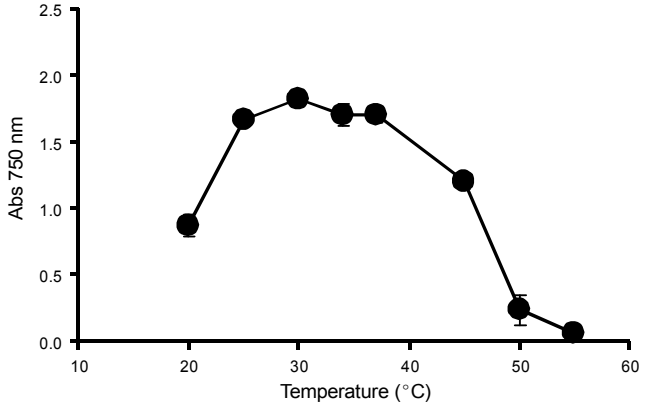


Fig. 4. Effect of temperature on molybdenum reduction by *Pseudomonas aeruginosa* strain Amr-11. Resting cells of the bacterium were incubated in a microtiter plate under optimized conditions for 72 h. Error bars represent mean ± SD (n = 3)

conditions for bioremediation would be found in a tropical country like Malaysia where average yearly temperature ranges from 25 to 35°C (Shukor et al., 2008a). Therefore, *P. aeruginosa* strain Amr-11 could be a candidate for soil bioremediation of Mo locally and in other tropical countries. The majority of the molybdenum-reducing (Mo-reducing) bacteria show optimal temperatures supporting reduction between 25 and 37°C (Shukor et al., 2008a; Rahman et al., 2009; Yunus et al., 2009; Shukor et al., 2009b; Shukor et al., 2010a; Shukor et al., 2010b; Lim et al., 2012; Abo-Shakeer et al., 2013; Halmi et al., 2013; Othman et al., 2013; Khan et al., 2014; Shukor et al., 2014) as these bacteria are isolated from tropical soils. The only psychrotolerant Mo-reducing bacterium was isolated from Antarctica, and it shows optimal temperature supporting reduction between 15 and 20°C (Ahmad et al., 2013).

The optimal pH range exhibited by *P. aeruginosa* strain Amr-11 for supporting Mo reduction reflects the property of the bacterium as a neutrophile. The characteristics of neutrophiles include their ability to grow between pH 5.5 and 8.0. An important observation regarding molybdenum reduction in bacteria is that a majority of the Mo-reducing bacteria exhibit optimal pH for molybdenum reduction under sub-neutral pHs, with optimal pH ranging from pH 5.0 to 7.0 (Campbell et al., 1985; Ghani et al., 1993; Shukor et al., 2008a; Rahman et al., 2009; Shukor et al., 2009a; Shukor et al., 2009b; Shukor et al., 2010a; Shukor et al., 2010b; Lim et al., 2012; Abo-Shakeer et al., 2013; Ahmad et al., 2013; Halmi et al., 2013; Othman et al., 2013; Khan et al., 2014; Shukor et al., 2014). It has been suggested previously that acidic pH plays an important role in the formation and stability of phosphomolybdate before it is being reduced to Mo-blue. Thus, optimal molybdenum reduction occurs at pH that is optimal for enzyme activity and at pH that maintains substrate stability (Shukor et al., 2007).

Effect of electron donor on molybdate reduction

Among the electron donors tested, glucose was the best electron donor for supporting molybdate reduction followed by galactose, fructose, and citrate in descending order (Fig. 5). Other carbon sources tested did not support Mo reduction. Previous works by Shukor et al. demonstrated that several of Mo-reducing bacteria such as *Enterobacter cloacae* strain 48 (Ghani et al., 1993), *Serratia* sp. strain Dr.Y5 (Rahman et al., 2009), *S. marcescens* strain Dr.Y9 (Yunus et al., 2009), and *Serratia marcescens* strain DRY6 (Shukor et al., 2008a) used sucrose as the best carbon source. Other Mo reducers such as *Escherichia coli* K12 (Campbell et al., 1985), *Serratia* sp. strain Dr.Y5 (Rahman et al., 2009), *Pseudomonas* sp. strain DRY2 (Shukor et al., 2010a), *Pseudomonas* sp. strain DRY1 (Ahmad et al., 2013), *Enterobacter* sp. strain Dr.Y13 (Shukor et al., 2009a), *Acinetobacter calcoaceticus* strain Dr.Y12 (Shukor et al., 2010b), *Bacillus pumilus* strain lbna (Abo-Shakeer et al., 2013), and *Bacillus* sp. strain A.rzi (Othman et al., 2013) prefer glucose as the carbon source while *Klebsiella oxytoca* strain hkeem prefers fructose (Lim et al., 2012). In the presence of carbon sources in the media, the bacteria could produce electron-donating substrates such as NADH and NADPH through metabolic pathways such as glycolysis,

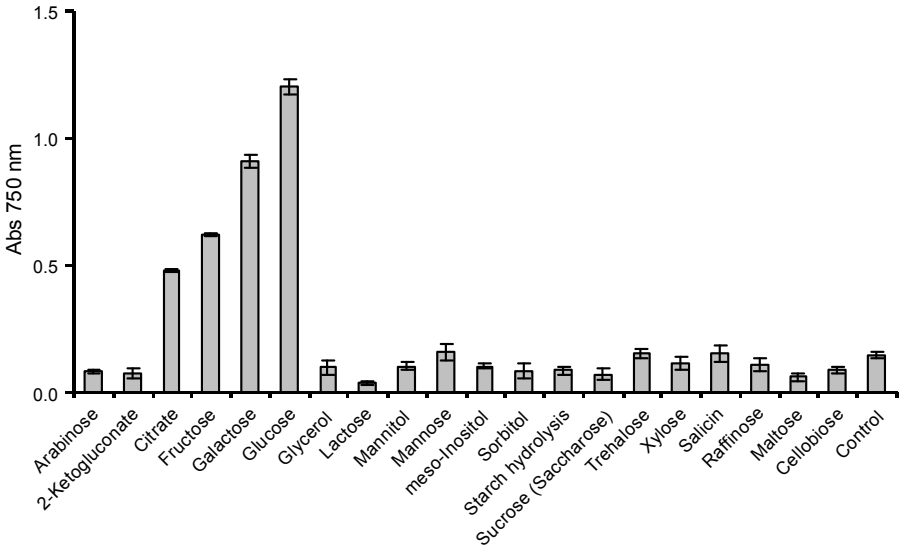


Fig. 5. Effect of different electron-donor sources (1% w/v) on molybdenum reduction. *Pseudomonas aeruginosa* strain Amr-11 was grown in LPM containing 10 mM molybdate and various electron donors. Resting cells of the bacterium were incubated in a microtiter plate under optimized conditions for 72 h. Error bars represent mean ± SD (n = 3)

Krebs cycle, and electron transport chain. Both NADH and NADPH serve as the electron donors for Mo-reducing enzymes (Shukor et al., 2008b; Shukor et al., 2014).

Effect of phosphate and molybdate concentrations to molybdate reduction

The determination of phosphate and molybdate concentrations supporting optimal Mo reduction is important because both anions have been shown to inhibit Mo-blue production in bacteria (Shukor et al., 2008a; Shukor et al., 2009a; Yunus et al., 2009; Shukor et al., 2009b; Shukor et al., 2010a; Shukor et al., 2010b; Lim et al., 2012; Ahmad et al., 2013; Othman et al., 2013; Shukor et al., 2014). The optimum concentration of phosphate has been found between 2.5 and 7.5 mM with higher concentrations being strongly inhibitory to molybdenum reduction (Fig. 6). High phosphate was suggested to inhibit phosphomolybdate stability as the complex requires acidic conditions of which the higher the phosphate concentration the stronger buffering power of the phosphate buffer used. In addition, the phosphomolybdate complex itself is unstable in the presence of high phosphate through an unknown mechanism (Glenn and Crane, 1956; Sims, 1961; Shukor et al., 2000). All the Mo-reducing bacteria isolated so far tolerate phosphate concentrations not higher than 5 mM for optimal reduction (Campbell et al., 1985; Ghani et al., 1993; Shukor et al., 2008a; Rahman et al., 2009; Shukor et al., 2009a;

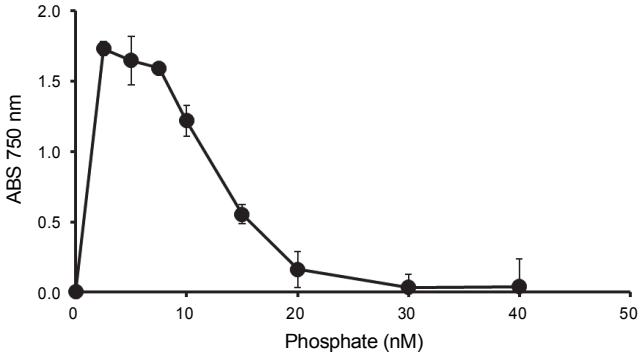


Fig. 6. The effect of phosphate concentration on molybdenum reduction by *Pseudomonas aeruginosa* strain Amr-11. Resting cells of the bacterium were incubated in a microtiter plate under optimized conditions for 72 h. Error bars represent mean ± SD (n = 3)

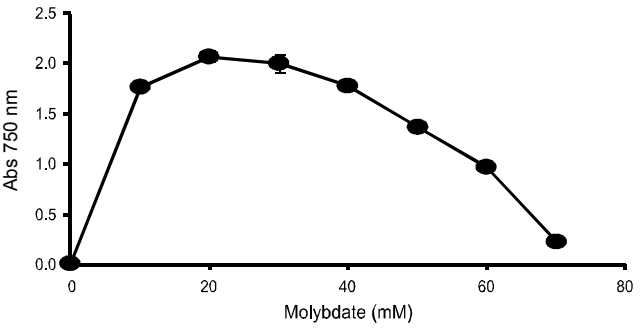


Fig. 7. The effect of molybdate concentration on molybdenum reduction by *Pseudomonas aeruginosa* strain Amr-11. Resting cells of the bacterium were incubated in a microtiter plate under optimized conditions for 72 h. Error bars represent mean ± SD (n = 3)

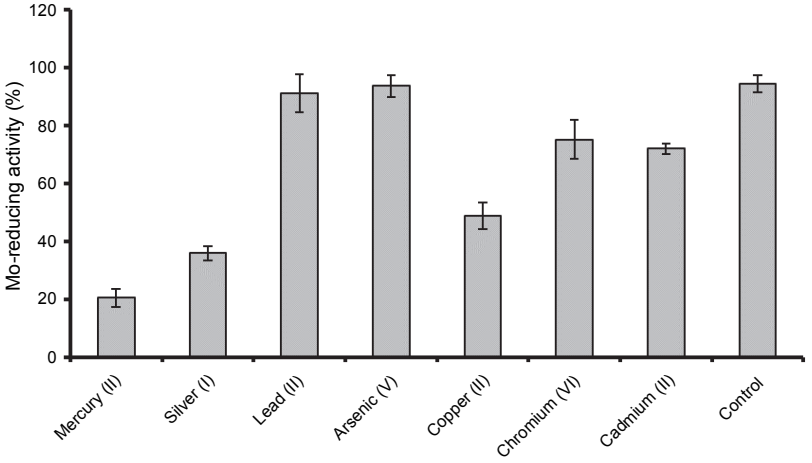


Fig. 8. The effect of metals on Mo-blue production by *Pseudomonas aeruginosa* strain Amr-11. Resting cells of the bacterium were incubated in a microtiter plate under optimized conditions for 72 h. Error bars represent mean ± SD (n = 3)

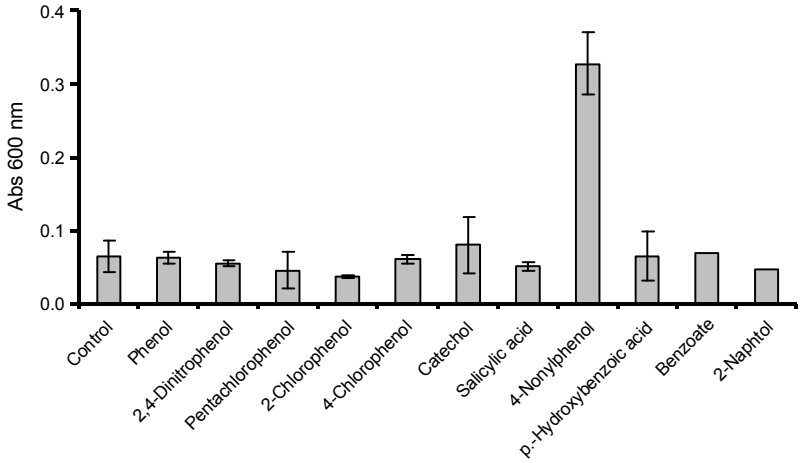


Fig. 9. Growth of *Pseudomonas aeruginosa* strain Amr-11 on phenolic compounds. Resting cells of the bacterium were incubated in a microtiter plate under optimized conditions for 72 h. Error bars represent mean ± SD (n = 3)

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

Bacteria	Heavy Metals that inhibit reduction	Author
Bacillus pumilus strain lbna	As^{3+}, Pb^{2+}, Zn^{2+}, Cd^{2+}, Cr^{6+}, Hg^{2+}, Cu^{2+}	(Abo-Shakeer et al., 2013)
Bacillus sp. strain A.rzi	Cd^{2+}, Cr^{6+}, Cu^{2+},Ag^{+}, Pb^{2+}, Hg^{2+}, Co^{2+},Zn^{2+}	(Othman et al., 2013)
Serratia sp. strain Dr.Y8	Cr, Cu, Ag, Hg	(Shukor et al., 2009b)
S. marcescens strain Dr.Y9	Cr^{6+}, Cu^{2+}, Ag^{+}, Hg^{2+}	(Yunus et al., 2009)
Serratia sp. strain Dr.Y5	n.a.	(Rahman et al., 2009)
Pseudomonas sp. strain DRY2	Cr^{6+}, Cu^{2+}, Pb^{2+}, Hg^{2+}	(Shukor et al., 2010a)
Pseudomonas sp. strain DRY1	Cd^{2+}, Cr^{6+}, Cu^{2+},Ag^{+}, Pb^{2+}, Hg^{2+}	(Ahmad et al., 2013)
Enterobacter sp. strain Dr.Y13	Cr^{6+}, Cd^{2+}, Cu^{2+}, Ag^{+}, Hg^{2+}	(Shukor et al., 2009a)
Acinetobacter calcoaceticus strain Dr.Y12	Cd^{2+}, Cr^{6+}, Cu^{2+}, Pb^{2+}, Hg^{2+}	(Shukor et al., 2010b)
Serratia marcescens strain DRY6	Cr^{6+}, Cu^{2+}, Hg^{2+}*	(Shukor et al., 2008a)
Enterobacter cloacae strain 48	Cr^{6+}, Cu^{2+}	(Ghani et al., 1993)
Escherichia coli K12	Cr^{6+}	(Campbell et al., 1985)
Klebsiella oxytoca strain hkeem	Cu^{2+}, Ag^{+}, Hg^{2+}	(Lim et al., 2012)

Shukor et al., 2009b; Shukor et al., 2010a; Shukor et al., 2010b; Lim et al., 2012; Abo-Shakeer et al., 2013; Ahmad et al., 2013; Halmi et al., 2013; Othman et al., 2013; Khan et al., 2014; Shukor et al., 2014). Our studies on the effect of Mo concentration on Mo reduction showed that the newly isolated bacterium was able to reduce Mo at concentrations as high as 60 mM but with reduced Mo-blue production. The optimal reduction range was at Mo concentrations between 20 and 30 mM (Fig. 7). The reduction of Mo at such high concentrations, into an insoluble form, would allow the strain to decontaminate highly Mo-polluted sites. The lowest optimal concentration of Mo required for optimal reduction by bacteria reported so far is 15 mM for *Pseudomonas* sp strain Dr.Y2 (Shukor et al., 2010a), while the highest was 80 mM for *E. coli* K12 (Campbell et al., 1985) and *Klebsiella oxytoca* strain hkeem (Lim et al., 2012). Other Mo-reducing bacteria such as EC48 (Ghani et al., 1993), *S. marcescens* strain Dr.Y6 (Shukor et al., 2008a), *S. marcescens*. Dr.Y9 (Yunus et al., 2009), *Pseudomonas* sp. strain Dr.Y2 (Shukor et al., 2010a), *Serratia* sp. strain Dr.Y5 (Rahman et al., 2009), *Enterobacter* sp. strain Dr.Y13 (Shukor et al., 2009a), and *Acinetobacter calcoaceticus* (Shukor et al., 2010b) could produce optimal Mo-blue using the optimal molybdate concentrations of 50, 25, 55, 30, 30, 50, and 20 mM, respectively. In fact the highest concentration of Mo as a pollutant in the environment is around 2000 ppm, which is about 20 mM (Runnells et al., 1976).

Effect of heavy metals

Molybdenum reduction was inhibited by mercury (II), silver (I), copper (II), cadmium (II), and chromium (VI) at 2 mg/l by 79.6%, 64.2%, 51.3%, 28.1%, and 25.0%, respectively (Fig. 8). The inhibition effects by other metal ions and heavy metals present a major problem for bioremediation. Therefore, it is important to screen for and isolate bacteria with as many metal-resistance capabilities as possible. As described previously (Shukor et al., 2002), mercury is a physiological inhibitor to molybdate reduction. A summary of the type of heavy metals that inhibited Mo-reducing bacteria showed that almost all of the reducers are inhibited by toxic heavy metals (Table 2). Heavy metals such as mercury, cadmium, silver, and copper usually target sulfhydryl groups of enzymes (Sugiura and Hirayama, 1976). Chromate is known to inhibit certain enzymes such as glucose oxidase (Zeng et al., 2004) and enzymes of nitrogen metabolism in plants (Sangwan et al., 2014).

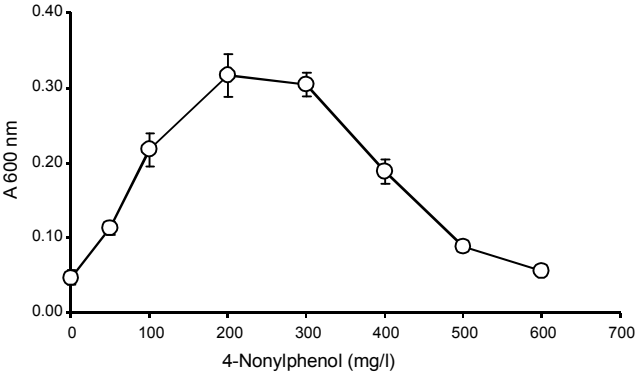


Fig. 10. Growth of *Pseudomonas aeruginosa* strain Amr-11 on 4-nonylphenol. Resting cells of the bacterium were incubated in a microtiter plate under optimized conditions for 72 h. Error bars represent mean ± SD (n = 3)

Phenolics as electron-donor sources for molybdenum reduction and independent growth

The screening for phenolics that could serve as electron donors supporting Mo reduction failed to give positive results. However, this bacterium was able to grow on the phenolic compound 4-nonylphenol (Fig. 9) with optimal growth efficiency between 200 and 300 mg/l (Fig. 10). Bacterial species and genera that have been reported to degrade NP and NPEs include *Ochrobactrum* sp., *Staphylococcus* sp., *Achromobacter* sp., *Alcaligenes* sp. (Yu et al., 2012), *Sphingomonas cloacae* (Pluemsab et al., 2007), the genera *Pseudomonas, Sphingomonas, Sphingobium, Cupriavidus, Ralstonia, Achromobacter*, *Staphylococcus* (Gu et al., 2010), and *Pseudomonas* spp. (Yuan et al., 2004; Chakraborty and Dutta, 2006; Liu et al., 2006; Watanabe et al., 2012). The existence of multitude of bacteria with NP-degrading ability(ties) makes bioremediation the most useful method for NP degradation. However, very few bacteria have been reported to be able to degrade phenol and detoxify heavy metals. The ability of the bacterium described here, to do both, suggest that this bacterium may be very useful as a bioremediation agent in polluted sites co-contaminated with xenobiotic and heavy metals.

Conclusions

A local isolate of Mo-reducing bacterium with a novel ability to biodegrade the phenolic compound 4-nonylphenol has been isolated. This is the first time that

a Mo-reducing bacterium with the ability to grow on this compound has been reported. The bacterium reduced molybdate to Mo-blue optimally in pH between 6.3 and 6.8 and at 34 °C. Glucose followed by galactose, fructose, and citrate (in descending order) has been found to be the best electron donors supporting molybdate reduction. Other requirements included a phosphate concentration between 2.5 and 7.5 mM and a molybdate concentration between 20 and 30 mM. The absorption spectrum of the Mo-blue produced was similar to previously described Mo-reducing bacteria, closely resembling this of a reduced phosphomolybdate. Mo reduction by this bacterium was inhibited by mercury (II), silver (I), copper (II), cadmium (II), and chromium (VI). The ability of isolated bacterium to detoxify multiple toxicants is a sought-after property, and this makes the bacterium an important tool for bioremediation. Currently, work is underway to purify the Mo-reducing enzyme from this bacterium and to characterize 4-nonylphenol biodegradation properties in more detail.

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