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ISOLATION AND CHARACTERIZATION OF A MOLYBDENUM-REDUCING AND AZO-DYE DECOLORIZING *SERRATIA MARCESCENS* STRAIN NENI-1 FROM INDONESIAN SOIL

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Abstract: Heavy metals and organic xenobiotics including dyes are important industrial components with their usage amounting to the millions of tonnes yearly. Their presence in the environment is a serious pollution issue globally. Bioremediation of these pollutants using microbes with multiple detoxification capacity is constantly being sought. In this work we screen the ability of a molybdenum-reducing bacterium isolated from contaminated soil to decolorize various azo and triphenyl methane dyes. The bacterium reduces molybdate to molybdenum blue (Mo-blue) optimally at pH 6.0, and temperatures of between 25 and 40°C. Glucose was the best electron donor for supporting molybdate reduction followed by sucrose, trehalose, maltose, d-sorbitol, d-mannitol, d-mannose, myo-inositol, glycerol and salicin in descending order. Other requirements include a phosphate concentration of between 5.0 and 7.5 mM and a molybdate concentration between 10 and 20 mM. The absorption spectrum of the Mo-blue produced was similar to previous Mo-reducing bacterium, and closely resembles a reduced phosphomolybdate. Molybdenum reduction was inhibited by copper, silver and mercury at 2 ppm by 43.8%, 42.3% and 41.7%, respectively. We screen for the ability of the bacterium to decolorize various dyes. The bacterium was able to decolorize the dye Congo Red. Biochemical analysis resulted in a tentative identification of the bacterium as *Serratia marcescens* strain Neni-1. The ability of this bacterium to detoxify molybdenum and decolorize azo dye makes this bacterium an important tool for bioremediation.

Keywords: molybdenum; bioremediation; azo dye; Congo Red; decolorization

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INTRODUCTION

Bioremediation of heavy metals and organic pollutants making use of bioremediation is often a less costly solution in the long run as compared to physical or chemical approaches, particularly at low concentrations (Rieger *et al.*, 2002). Molybdenum is an important cofactor in biological system at trace amount but it is toxic to several organisms at certain levels (AhmadPanahi *et al.*, 2014). For instance, molybdenum inhibits spermatogenesis and arrests embryogenesis in organisms such as catfish and mice at levels as low as several parts per million (Bi *et al.*, 2013, 2013; Meeker *et al.*, 2008; Zhai *et al.*, 2013; Zhang *et al.*, 2013). Furthermore, molybdenum is very toxic to ruminants being the most affected are cows at the similar low levels (Kincaid, 1980; Underwood, 1979). It has many uses in industries. The wide application of molybdenum in industry has resulted in several water pollution cases all around the world such as in the Tokyo Bay, Tyrol in Austria and the Black Sea, where molybdenum level reaches in the hundreds of ppm (Davis, 1991; Neunhäuserer et al., 2001). In addition, it is a significant pollutant in sewage sludge that poses health threats (Lahann, 1976).

Aside from heavy metals, the organic pollutant azo dyes are often present as a co-pollutant. Basic and diazo direct dyes including Congo Red (**Fig. 1**) are being produced in the orders of nearly one million of tons yearly. Unfortunately, they have the highest rates of toxicity (LD50) as reported by The Ecological and Toxicological Association of the Dyestuff Manufacturing Industry (ETAD) (Shore, 1996). Being brightly colored and water soluble, these reactive and acid dyes tend to pass through conventional water treatment systems unscathed. Due to this it is well known that the textile finishing industries generates one of the largest contribution of water pollution as 10-15% of dyes are lost in the effluent during the dyeing processes. In addition, the high Chemical Oxygen Demand (COD), Biological Oxidation Demand (BOD), color, pH and the presence of toxic metal ions making them a significant threat to ecology (Moran *et al.*, 1997).

Some microbes are able to degrade a variety of xenobiotics and detoxify heavy metals at the same time (Anu *et al.*, 2010; Bhattacharya *et al.*, 2014; Chaudhari *et al.*, 2013) and the versatility of these microbes are in great demand in polluted sites where the presence of several contaminants are the norm (Ahmad *et al.*, 2014). Heavy metals reduction coupled with azo dye decolorization have been reported (Chaudhari *et al.*, 2013).

In the present work, we screen for the ability of a novel molybdenum reducing bacterium isolated from contaminated soil to decolorize several azo dyes. Here we report on a novel molybdenum-reducing bacterium with the capacity to decolorize the azo dye Congo Red

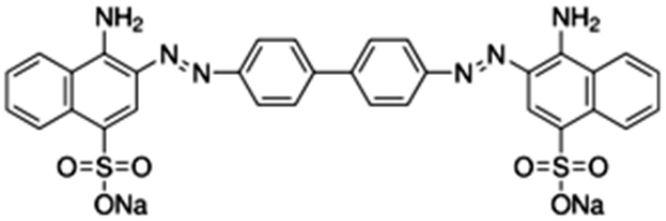

Fig. 1 The structure of Congo Red (Zollinger).

isolated from a contaminated soil. The characteristics of this bacterium would make it suitable for future bioremediation works involving both the heavy metal molybdenum and dye as an organic contaminant.

MATERIALS AND METHODS

Isolation of molybdenum-reducing bacterium

Soil samples were taken (5 cm deep from topsoil) from the grounds of a contaminated land in the province of Padang, Sumatera, Indonesia in January 2009. One gram of soil sample was suspended in sterile tap water. 0.1 mL aliquot of the soil suspension was pipetted and spread onto agar of low phosphate media (pH 7.0), and incubated for 24 hours at room temperature. The composition of the low phosphate media (LPM) were as follows: glucose (1%), $(NH_4)_2.SO_4$ (0.3%), $MgSO_4.7H_2O$ (0.05%), yeast extract (0.5%), NaCl (0.5%), $Na_2MoO_4.2H_2O$ (0.242% or 10 mM) and Na_2HPO_4 (0.071% or 5 mM) (Yunus *et al.*, 2009). The formations of blue colonies indicate molybdate reduction by molybdenum-reducing bacteria. Colony forming the strongest blue intensity was isolated and restreaked on low phosphate media (LPM) to obtain pure culture. Molybdenum reduction in liquid media (at pH 7.0) was carried out in 100 mL of the above media in a 250 mL shake flask culture at room temperature for 48 hours on an orbital shaker set at 120 rpm with the same media above but the phosphate concentration increased to 100 mM. Molybdenum blue (Mo-blue) absorption spectrum was studied by taking out 1.0 mL of the Mo-blue formed from the liquid culture above and then centrifuged at 10 000 x g for 10 minutes at room temperature. The supernatant was scanned from 400 to 900 nm using a UV-spectrophotometer (Shimadzu 1201) with low phosphate media as the baseline correction.

Morphological, physiological and biochemical characterization of the isolated strain

The isolated strain was phenotypically and biochemically characterized using standard techniques such as gram staining, colony shape, size and color on nutrient agar plate, motility, catalase production (24 h), oxidase (24 h), ONPG (beta-galactosidase), arginine dihydrolase (ADH), lysine decarboxylase (LDC),

ornithine decarboxylase (ODC), nitrates reduction, Methyl red, Voges-Proskauer (VP), indole production, hydrogen sulfide (H2S), acetate utilization, malonate utilization, citrate utilization (Simmons), tartrate (Jordans), esculin hydrolysis, gelatin hydrolysis, urea hydrolysis, deoxyribonuclease, lipase (corn oil), phenylalanine deaminase, gas production from glucose and production of acids from various sugars were carried out according to Bergey's Manual of Determinative Bacteriology (Holt *et al.*, 1994). Interpretation of the results was carried out via the ABIS online system (Costin & Ionut, 2015)

Preparation of resting cells for molybdenum reduction characterization

Characterization works on molybdenum reduction to Mo-blue such as the effects of pH, temperature, phosphate and molybdate concentrations were carried out statically using resting cells in a microplate or microtiter format as previously developed (Shukor & Shukor, 2014). Cells from a 1 L overnight culture grown in High Phosphate media (HPM) at room temperature on orbital shaker (150 rpm) with the only difference between the LPM and HPM was the phosphate concentration which was fixed at 100 mM for the HPM. Cells were harvested by centrifugation at 15 000 x g for 10 minutes and the pellet was washed several times to remove residual phosphate and resuspended in 20 mls of low phosphate media (LPM) minus glucose to an absorbance at 600 nm of approximately 1.00. In the low phosphate media, a concentration of 5 mM phosphate was 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 (Ahmad *et al.*, 2013; Campbell *et al.*, 1985; Ghani *et al.*, 1993; Halmi *et al.*, 2013; Khan *et al.*, 2014; Lim *et al.*, 2012; Othman *et al.*, 2013; Rahman *et al.*, 2009; Abo-Shakeer *et al.*, 2013; Shukor *et al.*, 2008a, 2009a, 2009b, 2010a, 2010b, 2007; Yunus *et al.*, 2009). Then 180 µL was sterically pipetted into each well of a sterile microplate. 20 µL of sterile glucose from a stock solution was then added to each well to initiate Mo-blue production. A sterile sealing tape that allows gas exchange (Corning® microplate) was used for sealing the tape. The microplate was incubated at room temperature. At defined times 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 $mM.^{-1}.cm^{-1}$ 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

Ten heavy metals namely chromium (vi), zinc (ii), mercury (ii), cadmium (ii), lead (ii), nickel (ii), copper (ii), arsenic (v), cobalt (ii) and silver (i) were prepared from commercial salts or from Atomic Absorption Spectrometry standard solutions from MERCK. The bacterium was incubated with heavy metals in the microplate format at 2 ppm (mg/L). The plate was incubated for 24 hours at 30ºC. The amount of Mo-blue production was measured at 750 nm as before.

Screening for bacterial decolorization of dyes

The ability of the bacterium to decolorize dyes was tested using the microplate format above with the dyes added to the final concentration of 100 mg/L. Dyes were sourced from Sigma-Aldrich (St. Loius, U.S.A.) and the list with maximum wavelength in parentheses were as follows: Congo Red (C.I. 22120) (498 nm), Cresol Red (C.I. 1733-12-6) (570 nm), Crocein Orange G (C.I. 15970) (482 nm), Evans Blue (C.I. 23860) (594 nm), Fast Green FCF (C.I. 42053) (620 nm), Fuchsin Basic (C.I. 42510) (625 nm), Crystal Violet (C.I. 42555) (590 nm), Metanil Yellow (C.I. 13065) (414 nm), Methyl Green (C.I. 42590) (635 nm), Methyl Orange (C.I. 13025) (505 nm), Methyl Red (C.I. 13020) (493 nm), Methylene Blue (C.I. 52015) (590 nm), Naphthol Blue Black (C.I. 20470) (618 nm), Nigrosin (C.I. 50415) (570 nm), Orange G (C.I. 16230) (476 nm), Orange II sodium salt (C.I. 15510) (483 nm), Ponceau 2R (C.I. 16150) (388 nm), Ponceau S (C.I. 27195) (352 nm), Remazol Black B (C.I. 20505) (597 nm), Rhodamine B (C.I. 45170) (554 nm), Safranin O (C.I. 50240) (530 nm), Direct Blue 71 (C.I. 34140) (586 nm), Sudan Black B (C.I. 26150) (600 nm), Tartrazine (C.I. 19140) (427 nm), Toluidine Blue (C.I. 52040) (626 nm) and Trypan Blue (C.I. 23850) (607 nm).

The ingredients of the growth media (% w/v) (LPM) were as follows: Glucose (1%), sodium lactate (1%), $(NH_4)_2.SO_4$ (0.3%), $NaNO_3$ (0.2%), $MgSO_4.7H_2O$ (0.05%), yeast extract (0.05%), NaCl (0.5%), Na_2HPO_4 (0.705% or 50 mM). The media was adjusted to pH 7.0. Some of the dyes change in color as the pH changes and the phosphate concentration was increased to 50 mM at pH 7.0 to prevent this. Decolorization was monitored using three standard wavelengths which were 405, 490 and 595 nm to cover maximum absorption values for specific dyes as these wavelengths were available in the BioRad 680 microplate reader. In addition the use of these preset wavelengths takes into account that the maximum absorption spectrums of water soluble dyes were generally shallow and a ± difference of 20 nm from the maximum absorption wavelength does not give dramatic reduction of absorbance values. The difference of absorbance values from the initial measurements were subtracted from the final measurements after an incubation period of 48 hours and a percentage decolorization was calculated.

RESULTS AND DISCUSSION

Identification of molybdenum reducing bacterium

The bacterium was a short rod-shaped, motile, Gram-negative and facultative anaerobe bacterium. The colonies were between 1 to 3 mm in diameter, shiny, cream white, smooth and circular in shape. The bacterium 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 & Ionut, 2015). The software gave three suggestions for the bacterial identity with the highest homology (90%) and accuracy at 97% as *Serratia marcescens*.

More work in the future especially molecular identification technique through comparison of the 16srRNA gene is needed to identify this species further. However, at this juncture the bacterium is tentatively identified as *Serratia marcescens* strain Neni-1 in honor of the late Dr. Neni Gusmanizar.

Table 1. Biochemical tests for *Serratia marcescens* strain Neni-1

		Acid production from	
Gram positive staining	+		
Motility	+	N-Acetyl-D-Glucosamine	d
Hemolysis	+	L-Arabinose	+
Growth at 45 ºC	+	Cellobiose	+
Growth at 65 ºC	–	Fructose	+
Growth at pH 5.7	+	D-Glucose	+
Growth on 7% NaCl media	+	Glycerol	+
Anaerobic growth	–	Glycogen	+
Casein hydrolysis	+	meso-Inositol	+
Esculin hydrolysis	+	Lactose	d
Gelatin hydrolysis	+	Mannitol	+
Starch hydrolysis	+	D-Mannose	+
Tyrosine degradation	–	Maltose	+
Beta-galactosidase (ONPG)	+	Melezitose	–
Catalase	+	Melibiose	d
Oxidase	d	Raffinose	+
Urease	–	Rhamnose	–
Arginine dehydrolase (ADH)	–	Ribose	+
Lysine decarboxylase (LDC)	–	Salicin	+
Ornithine decarboxylase (ODC)	–	Sorbitol	+
Citrate utilization	+	Sucrose	+
Egg-yolk reaction	–	Starch	+
Nitrates reduction	+	Trehalose	+
Voges-Proskauer test (VP)	+	D-Xylose	+

Note: + positive result; − negative result; d indeterminate result

Previous works have shown that the *Serratia* genus is a dominant genus for molybdenum reduction with species isolated so far includes *Serratia* sp. strain DR.Y5 (Rahman *et al.*, 2009), *Serratia marcescens*. DR.Y6 (Shukor *et al.*, 2008a) and *Serratia marcescens* DR.Y9 (Yunus *et al.*, 2009). The first Mo-blue reduction in *Serratia* sp. were first reported by Jan (Jan, 1939) followed by *S. marcescens* strain Dr.Y6 (Shukor *et al.*, 2008a), *Serratia* sp. Dr.Y5 (Rahman *et al.*, 2009) and (IJAB paper) and *S. marcescens* DR.Y9 (Yunus *et al.*, 2009). The species of this genus such as have also been reported to be able to decolorize azo dyes including congo red (Liang *et al.*, 2013; Mahmood *et al.*, 2013; Raj *et al.*, 2012; Verma & Madamwar, 2003).

In this work using this bacterium, a rapid and simple high throughput method involving microplate format was used to speed up characterization works and obtaining more data than the normal shake-flask approach (Iyamu *et al.*, 2008; Shukor & Shukor, 2014). The use of resting cells under static conditions to characterize molybdenum reduction in bacterium was initiated by Ghani *et al.* (Ghani *et al.*, 1993). Resting cells have been used in studying heavy metals reduction such as in selenate (Losi & Jr, 1997), chromate (Llovera *et al.*, 1993), vanadate (Carpentier *et al.*, 2005) reductions and xenobiotics biodegradation such as dyes (Chen *et al.*, 2011; Uddin *et al.*, 2007), diesel (Auffret *et al.*, 2014), SDS (Chaturvedi & Kumar, 2011), phenol (Sedighi & Vahabzadeh, 2014), amides (Raj *et al.*, 2010) and pentachlorophenol (Steiert *et al.*, 1987).

Molybdenum absorbance spectrum

The absorption spectrum of Mo-blue produced by *Serratia marcescens* strain Neni-1 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. 2**). The identity of the Mo-blue is not easily ascertained as it is complex in structure and has many species (Shukor *et al.*, 2007). Briefly Mo-blue is a reduced product of two type of molybdenum complexes-isopolymolybdate and heteropolymolybdate. It has been suggested by Campbell *et al.* (1985) that the Mo-blue observed in the reduction of molybdenum by *E. coli* K12 is a reduced form of phosphomolybdate but did not provide a plausible mechanism.

Formation of isopoly Mo-blue from molybdate itself is not possible using biological-based reducing agents as the conversion requires strong reducing agents and under acidic conditions. The formation of heteropoly Mo-blue by biologically-based reducing agents such as ascorbic acids or enzymatic reduction is more plausible as seen in the phosphate determination method using ascorbic acid (Hori *et al.*, 1988). We hypothesize that microbial molybdate reduction in media containing molybdate and phosphate must proceed via the

phosphomolybdate intermediate and the conversion from molybdate to this structure occurs due to the reduction of pH during bacterial growth, in other words the reduction of molybdenum to Mo-blue requires both chemical and biological processes. The absorption spectrum of the Mo-blue from this bacterium if it goes through this mechanism should show a spectrum closely resembling the phosphate determination method. To be exact, the spectrum observed showed a maximum absorption in between 860 and 870 nm and a shoulder at approximately 700 nm. 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 provides evidence for the hypothesis. Exact identification of the phosphomolybdate species must be carried out using n.m.r and e.s.r. due to the complex structure of the compound (Chae *et al.*, 1993). However, spectrophotmetric characterization of heteropolymolybdate species via analyzing the scanning spectroscopic profile is a less cumbersome and accepted method (Glenn & Crane, 1956; Kazansky & Fedotov, 1980; Sims, 1961; Yoshimura *et al.*, 1986). Although the maximum absorption wavelength for Mo-blue was 865 nm, measurement at 750 nm, although was approximately 30% lower, was enough for routine monitoring of Mo-blue production as the intensity obtained was much higher than cellular absorption at 600–620 nm (Shukor & Shukor, 2014). Previous monitoring of Mo-blue production uses several wavelengths such as 710 nm (Ghani *et al.*, 1993) and 820 nm (Campbell *et al.*, 1985).

Data analyses were carried out using Graphpad Prism version 5.0 available from www.graphpad.com. A one-way analysis of variance with post hoc analysis by the Tukey's test or a Student's t-test was utilized for between groups comparison. $P < 0.05$ was considered statistically significant.

Effect of pH and temperature on molybdate reduction

Serratia marcescens strain Neni-1 was incubated at different pH ranging from 5.5 to 8.0 using Bis-Tris and Tris.Cl buffers (20 mM). Analysis by ANOVA showed that the optimum pH for reduction was at 6.0. Inhibition of reduction was dramatic at pH lower than 5 (**Fig. 3**). The effect of temperature (**Fig. 4**) was observed over a wide range of temperature (20 to 60°C) with an optimum temperature ranging from 25 to 40°C with no significant different ($p > 0.05$) among the values measured as analyzed using ANOVA. Temperatures higher than 40°C were strongly inhibitory to Mo-blue production from *Serratia marcescens* strain Neni-1.

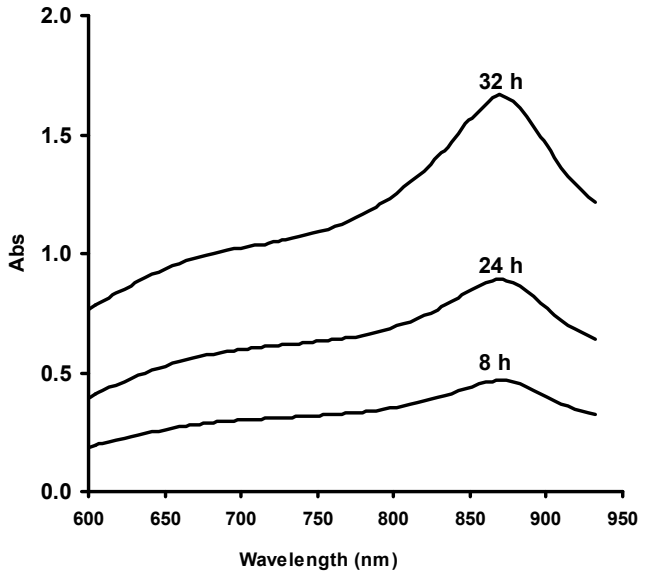

Fig. 2 Scanning absorption spectrum of Mo-blue from *Serratia marcescens* strain Neni-1 at different time intervals.

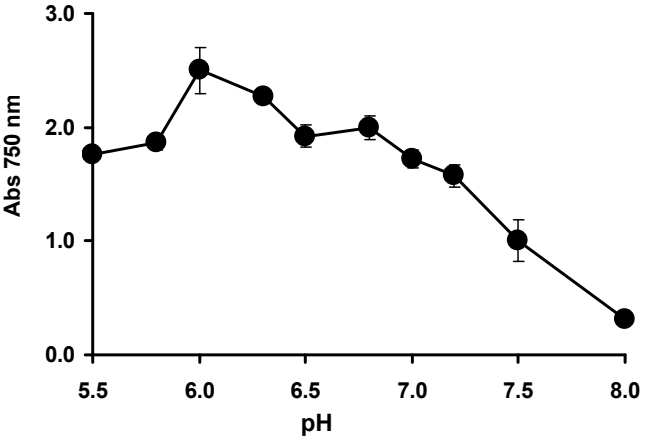

Fig. 3 Effect of pH on molybdenum reduction by *Serratia marcescens* strain Neni-1. Resting cells of the bacterium was incubated in a microtiter plate at room temperature for 48 hours. Resting cells of the bacterium were incubated in a microtiter plate under optimized conditions for 48 hours. Error bars represent mean ± standard deviation (n = 3).

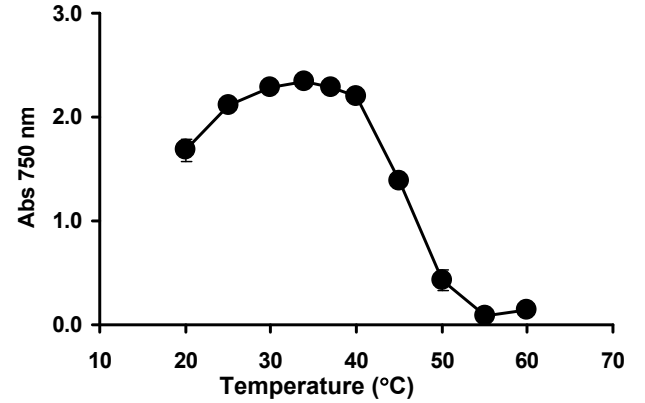

Fig. 4 Effect of temperature on molybdenum reduction by *Serratia marcescens* strain Neni-1. Resting cells of the bacterium was incubated in a microtiter plate at room temperature for 48 hours. Resting cells of the bacterium were incubated in a microtiter plate under optimized conditions for 48 hours. Error bars represent mean ± standard deviation (n = 3).

Temperature and pH play important roles in molybdenum reduction, since this process is enzyme mediated, both parameters affect protein folding and enzyme activity causing the inhibition of molybdenum reduction. The optimum conditions would be an advantage for bioremediation in a tropical country like Malaysia which have average yearly temperature ranging from 25 to 35°C (Shukor *et al.*, 2008a). Therefore, *Serratia marcescens* strain Neni-1 could be a candidate for soil bioremediation of molybdenum locally and in other tropical countries. The majority of the reducers shows an optimal temperature of between 25 and 37ºC (Halmi *et al.*, 2013; Khan *et al.*, 2014; Lim *et al.*, 2012; Othman *et al.*, 2013; Rahman *et al.*, 2009; Abo-Shakeer *et al.*, 2013; Shukor *et al.*, 2008a, 2009b, 2010a, 2010b, 2014; Yunus *et al.*, 2009) as they are isolated from tropical soils with the only psychrotolerant reducer isolated from Antarctica showing an optimal temperature supporting reduction of between 15 and 20ºC (Ahmad *et al.*, 2013).

The optimal pH range exhibited by *Serratia marcescens* strain Neni-1 for supporting molybdenum reduction reflects the property of the bacterium as a neutrophile. The characteristics neutrophiles are their ability to grow between pH 5.5 and 8.0. An important observation regarding molybdenum reduction in bacteria is the optimal pH reduction is slightly acidic with optimal pHs ranging from pH 5.0 to 7.0 (Ahmad *et al.*, 2013; Campbell *et al.*, 1985; Ghani *et al.*, 1993; Halmi *et al.*, 2013; Khan *et al.*, 2014; Lim *et al.*, 2012; Othman *et al.*, 2013; Rahman *et al.*, 2009; Abo-Shakeer *et al.*, 2013; Shukor *et al.*, 2008a, 2009a, 2009b, 2010a, 2010b, 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, the optimal reduction occurs by balancing between enzyme activity and substrate stability (Shukor *et al.*, 2007).

Effect of electron donor on molybdate reduction

Among the electron donor tested, glucose was the best electron donor for supporting molybdate reduction followed by sucrose, adonitol, mannose, mannitol, myo-inositol, maltose, glycerol, d-sorbitol, salicin, trehalose and xylose (**Fig. 5**). Other carbon sources did not support molybdenum 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) showed sucrose as the best carbon source. Other molybdenum 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, NADH and NADPH thorough metabolic pathways such as glycolysis, Kreb's cycle and electron transport chain. Both NADH and NADPH are responsible as the electron donating substrates for molybdenum reducing-enzyme (Shukor *et al.*, 2008b, 2014).

Effect of phosphate and molybdate concentrations to molybdate reduction

The determination of phosphate and molybdate concentrations supporting optimal molybdenum reduction is important because both anions have been shown to inhibit Mo-blue production in bacteria (Ahmad *et al.*, 2013; Lim *et al.*, 2012; Othman *et al.*, 2013; Shukor *et al.*, 2008a, 2009a, 2009b, 2010a, 2010b, 2014; Yunus *et al.*, 2009). The optimum concentration of phosphate occurred at 5 mM with higher concentrations were strongly inhibitory to 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 & Crane, 1956; Shukor *et al.*, 2000; Sims, 1961). All of the molybdenum-reducing

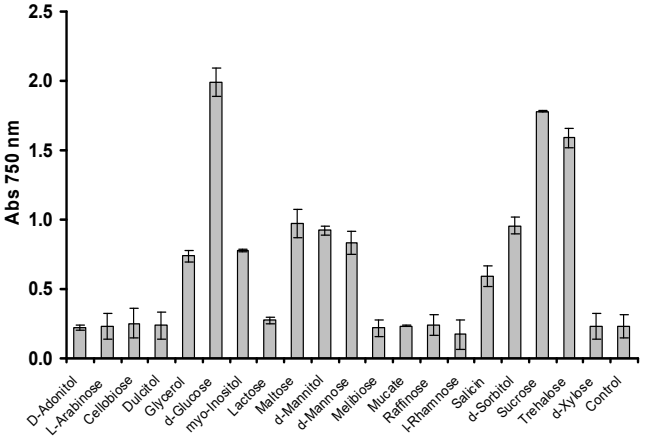

Fig. 5 Effect of different electron donor sources (1% w/v) on molybdenum reduction. *Serratia marcescens* strain Neni-1 was grown in low phosphate media containing 10 mM molybdate and various electron donors. Resting cells of the bacterium was incubated in a microtiter plate at room temperature for 48 hours. Resting cells of the bacterium were incubated in a microtiter plate under optimized conditions for 48 hours. Error bars represent mean ± standard deviation (n = 3).

bacterium isolated so far requires phosphate concentration not higher than 5 mM for optimal reduction (Ahmad *et al.*, 2013; Campbell *et al.*, 1985; Ghani *et al.*, 1993; Halmi *et al.*, 2013; Khan *et al.*, 2014; Lim *et al.*, 2012; Othman *et al.*, 2013; Rahman *et al.*, 2009; Abo-Shakeer *et al.*, 2013; Shukor *et al.*, 2008a, 2009a, 2009b, 2010a, 2010b, 2014). Studies on the effect of molybdenum concentration on molybdenum reduction showed that the newly isolated bacterium was able to reduce molybdenum as high as 60 mM but with reduced Mo-blue production. The optimal reduction range was between 20 and 40 mM (**Fig. 7**). Reduction at this high concentration into an insoluble form would allow the strain to reduce high concentration of molybdenum pollution. The lowest optimal concentration of molybdenum reported is 15 mM in *Pseudomonas* sp. strain Dr.Y2 (Shukor *et al.*, 2010a), whilst the highest molybdenum required for optimal reduction was 80 mM in *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 at 50, 25, 55, 30, 30, 50 and 20 mM, respectively. In fact the highest concentration of molybdenum as a pollutant in the environment is around 2000 ppm or about 20 mM (Runnells *et al.*, 1976).

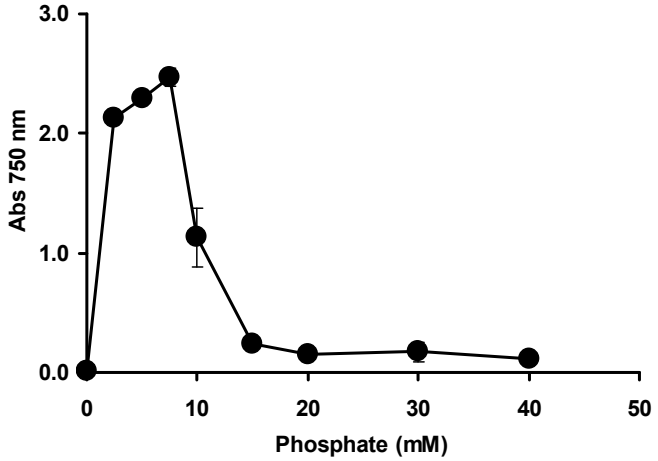

Fig. 6 The effect of phosphate concentration on molybdenum reduction by *Serratia marcescens* strain Neni-1. Resting cells of the bacterium was incubated in a microtiter plate at room temperature for 48 hours. Resting cells of the bacterium were incubated in a microtiter plate under optimized conditions for 48 hours. Error bars represent mean ± standard deviation (n = 3).

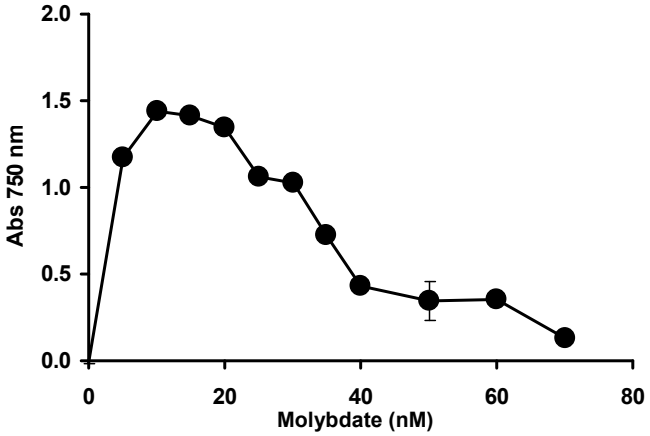

Fig. 7 The effect of molybdate concentration on molybdenum reduction by *Serratia marcescens* strain Neni-1. Resting cells of the bacterium was incubated in a microtiter plate at room temperature for 48 hours. Resting cells of the bacterium were incubated in a microtiter plate under optimized conditions for 48 hours. Error bars represent mean ± standard deviation (n = 3).

Effect of heavy metals

Inhibition of molybdenum reduction using several metals at 1 ppm showed that copper, mercury, silver and chromium showed 59.5, 78.9, 69.2 and 40.1% inhibition to Mo-reducing activity of *Serratia marcescens* strain Neni-1 (**Fig. 8**). The inhibition effects by others metal ions and heavy metals present a major problem for bioremediation. Therefore it is important to screen and isolate bacteria with as many metal resistance capability. 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 group of enzymes (Sugiura & Hirayama, 1976). Chromate is known to inhibit certain enzymes such as glucose

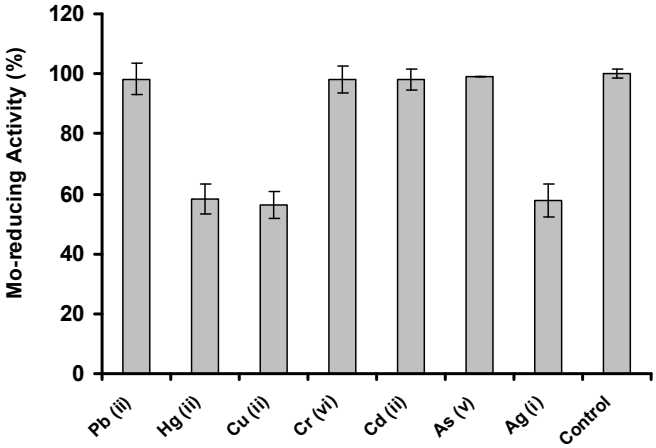

Fig. 8 The effect of metals on Mo-blue production by *Serratia marcescens* strain Neni-1. Resting cells of the bacterium was incubated in a microtiter plate at room temperature for 48 hours. Resting cells of the bacterium were incubated in a microtiter plate under optimized conditions for 48 hours. Error bars represent mean ± standard deviation (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)

oxidase (Zeng *et al.*, 2004) and enzymes of nitrogen metabolism in plants (Sangwan *et al.*, 2014). Binding of heavy metals inactivated metal-reducing capability of the enzyme(s) responsible for the reduction.

Azo dye-decolorizing ability of the molybdenum-reducing bacterium

We purposely use static growth or conditions, and is easily achieved in a microplate environment where the oxygen concentration (0~10% environmental oxygen, EO) is lower than under aerobic conditions (~20% environmental oxygen, EO) as most bioremediation conditions would have to be carried out in aquatic bodies or soils where the EO level is less than ~20% EO (Haley *et al.*, 2012) and other electron acceptors such as nitrate would started to be use. Almost all of the molybdenum-reducing bacteria isolated so far could reduce molybdenum into Mo-blue under static conditions. The ability of the bacterium to decolorize various azo dyes such as was explored. The bacterium was able to decolorize the dye Congo Red (**Fig. 9**). Azo dyes are strongly resistant to biodegradation under normal conditions but the azo bond is vulnerable to reductive cleavage. Bacterial species that have been reported to be able to degrade this dye are *Serratia marcescens* (Raj *et al.*, 2012) *Pseudomonas* luteola (Hsueh & Chen, 2007), *Bacillus* sp. (Gopinath *et al.* 2009), *Citrobacter* sp. (An *et al.*, 2002), *Stenotrophomonas maltophilia* (Galai *et al.*, 2009), *Pseudomonas luteola* (Hsueh & Chen, 2007), *Acinetobacter baumannii* (Ning *et al.*, 2014), *Staphylococcus* sp. (Park *et al.*, 2005) and *Enterobacter* sp. (Prasad & Aikat, 2013) and the bacterial species *Enterobacter cloacae*, Hafnia alvei, and *Klebsiella pneumonia* (Raj *et al.*, 2012). The mechanism of microbial degradation of azo dyes involves the reductive cleavage of azo bonds (–N=N–) with the help of azoreductase enzyme (Syed *et al.*, 2009).

CONCLUSION

A local isolate of Mo-reducing bacterium with the novel ability to decolorize azo dye has been isolated. This is the first report of a molybdenum reducing bacterium with the ability to decolorize dye. The bacterium reduces molybdate to Mo-blue optimally at pH 6.0, and temperatures of between 25 and 40°C. Glucose was the best electron donor for supporting molybdate reduction followed by sucrose, trehalose, maltose, d-sorbitol, d-mannitol, d-mannose, myo-inositol, glycerol and salicin in descending order. Other requirements include a phosphate concentration of between 5.0 and 7.5 mM and a molybdate concentration between 10 and 20 mM. The absorption spectrum of the Mo-blue produced was similar to previous Mo-reducing bacterium, and closely resembles a reduced phosphomolybdate. Molybdenum reduction was inhibited by Cu (ii), Ag (i) and Hg (ii) at 2 ppm by 43.8, 42.3 and 41.7%, respectively. We screen for the ability of the bacterium to decolorize various dyes. The bacterium was able to decolorize the dye Congo Red. Currently, efforts are underway to purify the molybdenum-reducing enzyme from this bacterium and to fully characterize the dye-decolorizing property. The ability of this bacterium to detoxify multiple toxicants is a sought after property, and this makes the bacterium an important tool for bioremediation.

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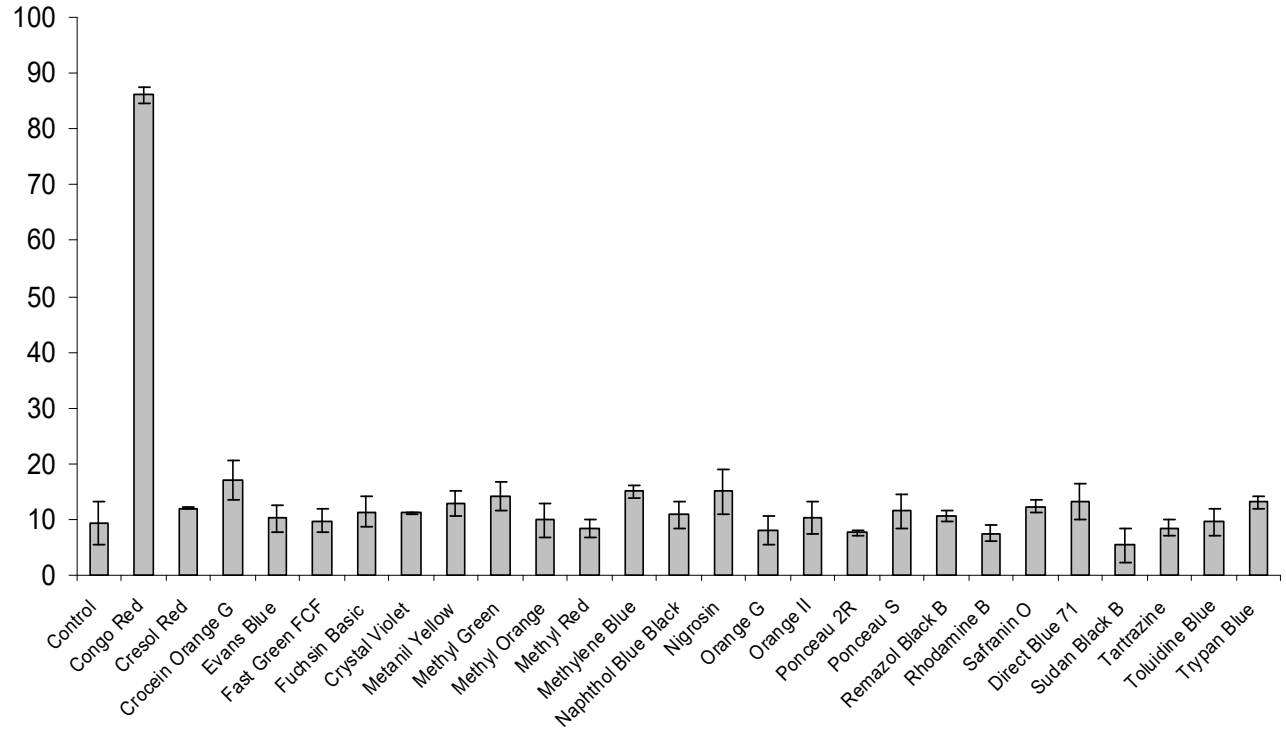

Fig. 9 Decolorization of various dyes by *Serratia marcescens* strain Neni-1. Resting cells of the bacterium was incubated in a microtiter plate at room temperature for 72 hours. Resting cells of the bacterium were incubated in a microtiter plate under optimized conditions for 48 hours. Error bars represent mean ± standard deviation (n = 3).

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