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

Neni Gusmanizar1,2, Mohd Izuan Effendi Halmi2, M. Rusnam3, Mohd Fadhil Abd Rahman2, Mohd Shukri Shukor4, Nina Suhaity Azmi3∗ and Mohd Yunus Shukor2,4

1Department of Animal Nutrition, Faculty of Animal Science, Andalas University, Padang, 25163, Indonesia
2Department of Biochemistry, Faculty of Biotechnology and Biomolecular Sciences, Universiti Putra Malaysia, UPM 43400 Serdang, Selangor, Malaysia
3Faculty of Industrial Sciences and Technology, University Malaysia Pahang, 26300 Gambang, Pahang, Malaysia
4Snoc International Sdn Bhd, Lot 343, Jalan 7/16 Kawasan Perindustrian Nilai 7, Inland Port, 71800, Negeri Sembilan, Malaysia
5Department of Agricultural Technology, Faculty of Agriculture, Padang, 25163, Indonesia

Received 24 June 2015; received in revised form 19 April 2016; accepted 20 April 2016

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

© 2016 Journal of Urban and Environmental Engineering (JUEE). All rights reserved.

* Correspondence to: Azmi, N.S. Tel.: +6095492404. Email: nina@ump.edu.my
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; 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 (AhmadPanahi et al., 2014). 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 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%), (NH4)2SO4 (0.3%), MgSO4.7H2O (0.05%), yeast extract (0.5%), NaCl (0.5%), Na2MoO4.2H2O (0.242% or 10 mM) and Na2HPO4 (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), phenylalana nine 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 placed over each well to initiate Mo-blue production. 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 30°C. 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. Louis, 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), 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 (C.I. 15510) (483 nm), Ponceau 2R (C.I. 16150) (388 nm), Ponceau S (C.I. 27195) (352 nm), Remazol Black B (C.I. 25055) (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%), (NH4)2SO4 (0.3%), NaNO3 (0.2%), MgSO4.7H2O (0.05%), yeast extract (0.05%), NaCl (0.5%), Na2HPO4 (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 16sRNA 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

<table>
<thead>
<tr>
<th>Acid production from</th>
<th>Gram positive staining</th>
<th>N-Acetyl-D-Glucosamine</th>
<th>d or +</th>
</tr>
</thead>
<tbody>
<tr>
<td>Motility</td>
<td>+</td>
<td>Glucosamine</td>
<td>d or +</td>
</tr>
<tr>
<td>Hemolysis</td>
<td>+</td>
<td>L-Arabinose</td>
<td>+</td>
</tr>
<tr>
<td>Growth at 45 °C</td>
<td>+</td>
<td>Cellobose</td>
<td>+</td>
</tr>
<tr>
<td>Growth at 65 °C</td>
<td>–</td>
<td>Fructose</td>
<td>+</td>
</tr>
<tr>
<td>Growth at pH 5.7</td>
<td>+</td>
<td>D-Glucose</td>
<td>+</td>
</tr>
<tr>
<td>Growth on 7% NaCl media</td>
<td>+</td>
<td>Glycerol</td>
<td>+</td>
</tr>
<tr>
<td>Anaerobic growth</td>
<td>–</td>
<td>Glycogen</td>
<td>+</td>
</tr>
<tr>
<td>Casein hydrolysis</td>
<td>+</td>
<td>meso-Inositol</td>
<td>+</td>
</tr>
<tr>
<td>Escolin hydrolysis</td>
<td>+</td>
<td>Lactose</td>
<td>d</td>
</tr>
<tr>
<td>Gelatin hydrolysis</td>
<td>+</td>
<td>Mannitol</td>
<td>+</td>
</tr>
<tr>
<td>Starch hydrolysis</td>
<td>+</td>
<td>D-Mannose</td>
<td>+</td>
</tr>
<tr>
<td>Tyrosine degradation</td>
<td>–</td>
<td>Maltose</td>
<td>+</td>
</tr>
<tr>
<td>Beta-galatosidase</td>
<td>–</td>
<td>d or +</td>
<td></td>
</tr>
<tr>
<td>(ONPG)</td>
<td>+</td>
<td>Melezitose</td>
<td>–</td>
</tr>
<tr>
<td>Catalase</td>
<td>+</td>
<td>Melibiose</td>
<td>d</td>
</tr>
<tr>
<td>Oxidase</td>
<td>d or +</td>
<td>Raffinose</td>
<td>+</td>
</tr>
<tr>
<td>Urease</td>
<td>–</td>
<td>Rhamnose</td>
<td>–</td>
</tr>
<tr>
<td>Arginine dehydrase</td>
<td>–</td>
<td>Ribose</td>
<td>+</td>
</tr>
<tr>
<td>ADH</td>
<td>–</td>
<td>Salicin</td>
<td>+</td>
</tr>
<tr>
<td>Lysine decarboxylase</td>
<td>(LDC)</td>
<td>–</td>
<td>Salicin</td>
</tr>
<tr>
<td>Ornithine</td>
<td>decarboxylase (ODC)</td>
<td>–</td>
<td>Sorbitol</td>
</tr>
<tr>
<td>Citrate utilization</td>
<td>+</td>
<td>Sucrose</td>
<td>+</td>
</tr>
<tr>
<td>Egg-yolk reaction</td>
<td>–</td>
<td>Starch</td>
<td>+</td>
</tr>
<tr>
<td>Nitrate reduction</td>
<td>+</td>
<td>Trehalose</td>
<td>+</td>
</tr>
<tr>
<td>Voges-Proskauer test</td>
<td>(VP)</td>
<td>+</td>
<td>D-Xylose</td>
</tr>
</tbody>
</table>

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, spectrophotometric 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.
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 37°C (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 20°C (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, 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 Ibna (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 molydate 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](image-url)  
*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; Ohman 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).

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

<table>
<thead>
<tr>
<th>Bacteria</th>
<th>Heavy Metals that inhibit reduction</th>
<th>Author</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bacillus pumilus strain lbnla</td>
<td>As⁴⁺, Pb⁴⁺, Zn⁴⁺, Cd⁰⁺, Cr⁶⁺, Hg⁰⁺</td>
<td>(Abo-Shakeer et al., 2013)</td>
</tr>
<tr>
<td>Bacillus sp. strain A.rzi</td>
<td>Cd⁵⁺, Cr⁶⁺, Cu²⁺, Ag⁺, Pb²⁺, Hg²⁺</td>
<td>(Othman et al., 2013)</td>
</tr>
<tr>
<td>Serratia sp. strain Dr.Y8</td>
<td>Cr, Cu, Ag, Hg</td>
<td>(Shukor et al., 2009b)</td>
</tr>
<tr>
<td>S. marcescens strain Dr.Y9</td>
<td>Cr⁶⁺, Cu²⁺, Ag⁺, Hg²⁺</td>
<td>(Yunus et al., 2009)</td>
</tr>
<tr>
<td>Serratia sp. strain Dr.Y5</td>
<td>n.a.</td>
<td>(Rahman et al., 2009)</td>
</tr>
<tr>
<td>Pseudomonas sp. strain DRY2</td>
<td>C₆H₅⁺, C₆H₅⁻, Pb²⁺</td>
<td>(Shukor et al., 2010a)</td>
</tr>
<tr>
<td>Pseudomonas sp. strain DRY1</td>
<td>C₆H₅⁺, C₆H₅⁻, Cu²⁺, Ag⁺, Pb²⁺, Hg²⁺</td>
<td>(Ahmad et al., 2013)</td>
</tr>
<tr>
<td>Enterobacter sp. strain Dr.Y13</td>
<td>C₆H₅⁺, C₆H₅⁻, Cu²⁺, Cu²⁺</td>
<td>(Shukor et al., 2009a)</td>
</tr>
<tr>
<td>Acinetobacter calcoaceticus strain Dr.Y12</td>
<td>C₆H₅⁺, C₆H₅⁻, Pb²⁺</td>
<td>(Shukor et al., 2009b)</td>
</tr>
<tr>
<td>Serratia marcescens strain DRY6</td>
<td>C₆H₅⁺, Cu²⁺, Hg²⁺</td>
<td>(Shukor et al., 2008a)</td>
</tr>
<tr>
<td>Enterobacter cloacae strain 48</td>
<td>C₆H₅⁺, Cu²⁺</td>
<td>(Ghanial et al., 1993)</td>
</tr>
<tr>
<td>Escherichia coli K12</td>
<td>C₆H₅⁺</td>
<td>(Campbell et al., 1985)</td>
</tr>
<tr>
<td>Klebsiella oxytoca strain hkeem</td>
<td>C₆H₅⁺, Ag⁺, Hg²⁺</td>
<td>(Lim et al., 2012)</td>
</tr>
</tbody>
</table>

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.

Acknowledgment This project was supported by funds from Snoc International Sdn Bhd.
REFERENCES


Auffret, M.D., Yergeau, E., Labbé, D., Fayolle-Guichard, F., & Ahmad, S.A., Shukor, M.Y., Sham aan, N.A., Mac Cormack, W.P., & Syed, M.A. (2014) Efficacy of Screening dyes by Serratia marcescens strain Neni-1. Resting cells of the bacterium were 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).


Halmi, M.I.E., Zuhaimis, S.W., Yusof, M.T., Shaharuddin, N.A., Helmi, W., Shukor, Y., Syed, M.A., & Ahmad, S.A. (2013) Hexavalent molybdenum reduction to Mo-blue by a Sodium-


Underwood, E.J. (1979) Environmental sources of heavy metals and their toxicity to man and animals. Progress in Water Technology 11, 33–45.


