MOLYBDATE-REDUCING AND SDS-DEGRADING *Enterobacter* sp. STRAIN NENI-13

M.F. RAHMAN[1], M. RUSNAM[2,*], N. GUSMANIZAR[1,3], N.A. MASDOR[4], C.H. LEE[1], M.S. SHUKOR[5], M.A.H. ROSLAN[1], M.Y. SHUKOR[1]

[1] *Department of Biochemistry, Faculty of Biotechnology and Biomolecular Sciences, Universiti Putra Malaysia, Serdang, UPM 43400, Selangor, Malaysia*
[2] *Department of Agricultural Engineering, Faculty of Agricultural Technology, Andalas University, Padang, 25163, Indonesia (*rusnam_ms@yahoo.com)*
[3] *Department of Animal Nutrition, Faculty of Animal Science, Andalas University, Padang, 25163, Indonesia*
[4] *Biotechnology Research Centre, MARDI, P. O. Box 12301, Kuala Lumpur, 50774, Malaysia*
[5] *Snoc International Sdn Bhd, Lot 343, Jalan 7/16 Kawasan Perindustrian Nilai 7, Inland Port, Negeri Sembilan, 71800, Malaysia*

Abstract: Toxicants removal through microorganism's action is intensely being sought due to economic reasons. The aim of this paper is to isolate a bacterium that is able to reduce molybdenum blue and at the same time can grow on the detergent Sodium Dodecyl Sulfate (SDS). Biochemical analysis resulted in a tentative identification of the bacterium as *Enterobacter* sp. strain Neni-13. Growth on SDS showed a 100 % removal at 800 mg/L SDS within 12 days. The removal of SDS from media was confirmed through Methylene Blue Active Substances Assay. Molybdenum reduction using sodium molybdate as a substrate was characterized using a microplate assay. The optimum pH and temperature for molybdenum reduction was between 6.0 and 6.5, and at 37 °C, respectively. Glucose was the best electron donor for molybdate reduction. Phosphate and molybdate concentrations of between 2.5 and 5.0 mM and at 15 mM, were optimal for molybdate reduction, respectively. Molybdate reduction was inhibited by the heavy metals mercury, silver, copper and chromium at 2 ppm. The ability of this bacterium to detoxify molybdate and degrade the SDS makes this bacterium an important tool for bioremediation of toxicants in soil.

Key words: molybdate-reducing, SDS-degrading, molybdenum blue, *Enterobacter* sp., heavy metals

1. Introduction

The improper disposal, industrial and mining activities and excessive use of agricultural chemicals have resulted in a global problem. Removing these types of pollutants through bioremediation is a less costly tactic in the long run particularly at low concentrations, where other strategies for example physical or chemical methods may not be effective. Industrial effluents and exhaust fumes are often the source of molybdenum pollution. For example, molybdenum in the exhaust fumes in industries in Tyrol, Austria are the cause of molybdenum pollution covering a 300-hectare agricultural land. Cattles grazing on this region suffered debilitating diseases including deaths due to hypocuprosis (NEUNHÄUSERER *et al.*, 2001).

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The Molycorp mine in New Mexico is a molybdenum mine. Its tailing wastes found their way into rivers near the mines including the Red River. Portions of the river exposed to high concentration of mine wastes are declared dead (JACOBS *et al.,* 2014). In Indonesia, the mine tailings from the Batu Hijau, Sumbawa, copper, gold and molybdenum mine are dumped into the ocean with an annual amount close to several million tonnes. The sea is gradually polluted resulting in a reported reduction in fish populations (ANGEL *et al*., 2013).

Bacteria are prime candidate for metal including molybdenum remediation. Bacteria have unique enzymes that can convert heavy metals to less toxic forms (AHMAD *et al.,* 2013b). In the case of molybdenum, bacteria can convert the soluble molybdenum into insoluble molybdenum disulphide (TUCKER *et al.,* 1997) or to the partially soluble and colloidal molybdenum blue (CAPALDI and PROSKAUER, 1896). Bioremoval columns packed with molybdenum-reducing sulphate-reducing bacteria showed good potential for the removal of molybdenum, but the toxic hydrogen sulphide gas emitted require another removal column packed with hydrogen sulphide-utilizing bacteria (TUCKER *et al.,* 1997). Another promising candidate is the Mo-reducing bacterial system. The system utilizes bacteria that reduce molybdenum into molybdenum blue. The end product can be trapped in membrane (HALMI *et al.,* 2014), and the process does not require strict anaerobic requirements (GHANI *et al.,* 1993). Numerous candidates have been isolated from the genera of *Klebsiella* (LIM *et al*., 2012; HALMI *et al.,* 2013b; MASDOR *et al.,* 2015), *Bacillus* (ABO-SHAKEER *et al.,* 2013; OTHMAN *et al.,* 2013), *Pseudomonas* (SHUKOR *et al*., 2010; AHMAD *et al*., 2013a), *Acinetobacter*, *Serratia* and *Enterobacter* (SHUKOR *et al.,* 2009).

Aside from heavy metals, detergents are often present as co pollutants, especially in water bodies. Detergents have detrimental effects to aquatic life (AMBILY and JISHA, 2012). The anionic surfactants such as Sodium Dodecylbenzene Sulfonate (SDBS) and Sodium Dodecyl Sulfate (SDS) show toxic effects to *Daphnia magna* at concentrations of between 0.0025 and 300 mg/L. Detergents main toxicity have been documented in invertebrates and crustaceans due to the massive amount of these detergents continuously being released into the aquatic environment (CHATURVEDI and KUMAR, 2011). Several microorganisms can degrade SDS and use it for growth. The enzyme alkylsulfatase is the first enzyme of the pathway to degrade and assimilate SDS for microbial growth (CHATURVEDI and KUMAR, 2011). To date numerous SDS-degrading bacteria have been isolated (ABBOUD *et al.,* 2007; HOSSEINI *et al.,* 2007; CHATURVEDI and KUMAR, 2011; AMBILY and JISHA, 2012; HALMI *et al.,* 2013a).

The current trend of xenobiotics detoxification research is to isolate multiple degraders or microorganisms showing multiple detoxification ability. This is especially important in polluted sites where multiple contaminants can be found (HUSAINI *et al.,* 2008). The versatility of such multiple-degrading microorganisms are constantly being sought. Previously, two Mo-reducing bacteria have been reported with ability to grow on the detergent SDS (HALMI *et al.,* 2013b; MASDOR *et al.,* 2015). Herein, we report on the isolation of another Mo-reducing bacterium that can

grow on SDS as a sole carbon source. We also report on the first utilization of the modified Gompertz model to obtain growth parameters of this bacterium on SDS.

2. Materials and Methods

2.1. Chemicals and apparatus

All of the chemicals utilized in this work are of analytical grade. The UV-spectrophotometer (Shimadzu 1201) was purchased from Shimadzu Corporation, Kyoto, Japan. The orbital shaker was purchased fromYihder Technology Co., Ltd., Taipei, Taiwan. The microplate reader utilized in this work is a BioRad 680 microplate model and was purchased from BioRad, Richmond, CA, USA. The centrifuge model used was the Beckman GS-15R and was purchased from Beckman Instruments Inc., Fullerton, CA, USA. Bacterial cells were ruptured using a sonicator (Biosonik 111TM, Bronwill Scientific, Rochester, N.Y)

2.2. Isolation of molybdenum-reducing bacteria

Water samples were taken from the Danau Maninjau Lake in the province of Padang, Sumatera, Indonesia in January 2009. Screening and isolation of potential molybdenum-reducing bacteria were carried in a minimal salts media with phosphate content fixed at 5 mM as most of the Mo-reducing bacteria isolated to date showed optimal molybdenum reduction at this concentration. The media was supplemented with sodium molybdate at 10 mM. About 0.1 mL of the water sample was spread onto a solid low phosphate agar (pH 6.5). The plates were incubated at room temperature (27 ^{o}C) for 48 hours. The composition (mM) of the reduction media is as follows: glucose anhydrous (55 mM), $(NH_4)_2.SO_4$ (22.7 mM), $MgSO_4.7H_2O$ (2.03 mM), yeast extract (0.5 %), NaCl (85.56 mM), $Na_2MoO_4.2H_2O$ (10 mM) and Na_2HPO_4 (5 mM). Solid medium was prepared by adding agar (1.5 %) (MASDOR *et al.*, 2015) This is a low phosphate molybdenum or LPM media. After the incubation period (48 hours), blue colonies were selected, and restreaked on the LPM agar several times to obtain pure culture. The Mo-reducing bacterial isolates were then grown in 20 mL of LPM liquid media to select for the best reducer. The bacterial isolates were grown for 48 hours at room temperature (27 ^{o}C)on an orbital shaker (120 rpm, Yihder, Taiwan). About 1 mL of the culture media from each bacterial isolate was centrifuged (Beckman GS-15R, Fullerton, CA, USA) at 10,000 x g for 5 min at room temperature. The amount of molybdenum blue produced in the extracellular liquid was quantified at 865 nm using the specific extinction coefficient of 16.3 $mM.^{-1}.cm^{-1}$. A portion of the blue supernatant from culture media for the best isolate was periodically sampled and scanned from 400 to 900 nm (UV-spectrophotometer, Shimadzu 1201) after undergoing the centrifugation step as before. Identification of the best isolate was carried out utilizing biochemical and phenotypical methods in accordance to the Bergey's Manual of Determinative Bacteriology (HOLT *et al.*,

1994), and followed up by using the ABIS online system software (COSTIN and IONUT 2015) as before (MASDOR *et al.,* 2015).

2.3. Bacterial resting cells preparation

The Mo-reducing bacterium was characterized in a microtiter plate format. The characterization include concentrations of molybdate, carbon sources, phosphate, effect of pH, heavy metals, temperature and screening of potential xenobiotics as sources of growth or as electron donors supporting molybdenum reduction (SHUKOR and SHUKOR, 2014; MASDOR *et al.,* 2015). A volume of 1 L of high phosphate molybdate media or HPM distributed in several smaller 250 mL conical flasks was utilized for growth. The HPM has the same composition to the LPM but with the phosphate concentration increased to 100 Mm. Briefly, bacterial cells were grown aerobically with shaking at 120 rpm on an orbital shaker (Yihder, Taiwan) at room temperature (27 ^{o}C). The media utilized is a modification to the LPM in which the phosphate concentration was increased to 100 mM. This was carried out to prevent the formation of bacterial blue aggregates. Cells were harvested by centrifugation at 10,000 g (Beckman at 4 ^{o}C for 10 min). After rinsing with deionized water twice, the pellets were resuspended in 20 mL of LPM giving an optical density measured at 600 nm of 1.0 absorbance value. In an effort to accommodate the variations in phosphate, molybdate, carbon sources, and pH conditions throughout the characterization works, appropriate modifications to the LPM was carried out. About 180 μL of the cellular suspension was transferred into the wells of a sterile microplate, and mixed with 20 μL of sterile glucose or other carbon sources from stock solutions. The final concentration of the carbon sources was 1.0 % (w/v). The microplates were covered (Corning® microplate), and then incubated at room temperature. Readings at 750 nm was periodically taken using a BioRad reader (Model No. 680, Richmond, CA). The molar extinction coefficient of 11,687 $M.^{-1}.cm^{-1}$ at 750 nm was utilized to quantify molybdenum blue production. This wavelength was chosen as it is the maximum filter available for the microplate unit (SHUKOR and SHUKOR, 2014). The effect of several heavy metals was studied utilizing Atomic Absorption Spectrometry calibration standard solutions from MERCK.

2.4. Detergents and hydrocarbons as carbon sources for bacterial growth

Preliminary works showed that none of the detergents and hydrocarbons tested could support molybdenum reduction. Hence, these compounds were then screened for bacterial growth in the microplate format as described by replacing glucose with these xenobiotics at the final concentration of 500 mg/L for detergents. Diesel and crude petroleum was initially added to the final concentration of 2 % (v/v) in 10 mL of the growth media and sonicated at 60 Hz (Biosonik 111TM, Bronwill Scientific, Rochester, N.Y) for 5 minutes. Then 200 μL of the media was added into the microplate wells, and mixed with 50 μL of bacterial cells (see above).

The microplate was incubated at room temperature (27 ^{o}C) for three days and the amount of molybdenum blue production was measured at 750 nm as before. The ingredients of the growth medium (1 L) were as follows: $(NH_4)_2.SO_4$ (22.7 mM), $NaNO_3$ (23.53 mM), $MgSO_4.7H_2O$ (2.03 mM), yeast extract (0.01 %), NaCl (85.56 mM), Na_2HPO_4 (50 mM) and 1 mL of trace elements solution. The trace elements solution composition (mM) was as follows: $CaCl_2$ (0.363), $FeSO_4{\cdot}7H_2O$ (0.076), $MnSO_4{\cdot}4H_2O$ (0.179), $ZnSO_4{\cdot}7H_2O$ (0.069), $CuSO_4{\cdot}5H_2O$ (0.02), $CoCl_2{\cdot}6H_2O$ (0.021), $Na_2MoO_4{\cdot}2H_2O$ (0.020). The media was adjusted to pH 7.0. Then 200 µL of the media was added into the microplate wells and incubated at room temperature (27 ^{o}C) for 72 hours. Bacterial growth was measured as the increase in optical density at 600 nm.

2.5. Methylene Blue Active Substances (MBAS) Assay

SDS degradation was quantified according to the MBAS assay JURADO *et al.* (2006). 100 µL of slightly acidic methylene blue reagent (pH of between 5 and 6) was added into 5 mL of the SDS calibration standards or samples (appropriately diluted) in a separating funnel followed by the addition of 200 µL of sodium tetraborate pH 10.5. The mixture was then mixed vigorously and then 4 mL of chloroform was added to the mixture and vigorously stirred for 1 minute. The mixture was left to separate and settle down for several minutes. The chloroform layer was separated and the absorbance was read at 650 nm in a glass cuvette.

2.6. Statistical analysis

Values are means ± standard deviation of three replicated experiments. Statistical analyses such as ANOVA and t-test utilized the Graphpad Prism software ver. 5.0 available from www.graphpad.com.

3. Results

3.1. Detergents as carbon sources for Enterobacter *sp. strain Neni-13 growth*

Utilization of detergents and hydrocarbons as potential carbon sources for *Enterobacter* sp. growth was determined after 72 hours of incubation. The data showed that the bacterium was able to grow on the detergent SDS (Fig. 1). However, it was discovered that none of the detergents and hydrocarbons tested in this experiment can support molybdate reduction. SDS degradation studies carried out at concentrations between 200 and 1400 mg/L under aerobic conditions showed that the fastest degradation occurred at 200 mg/L of SDS, with complete degradation occurring at day-4 of incubation. A 100 % of degradation was still observed at the SDS concentration of 800 mg/L. The lowest degradation after 12 days of cultivation occurred at 1400 mg/L showing 19.4 % degradation (Fig. 2).

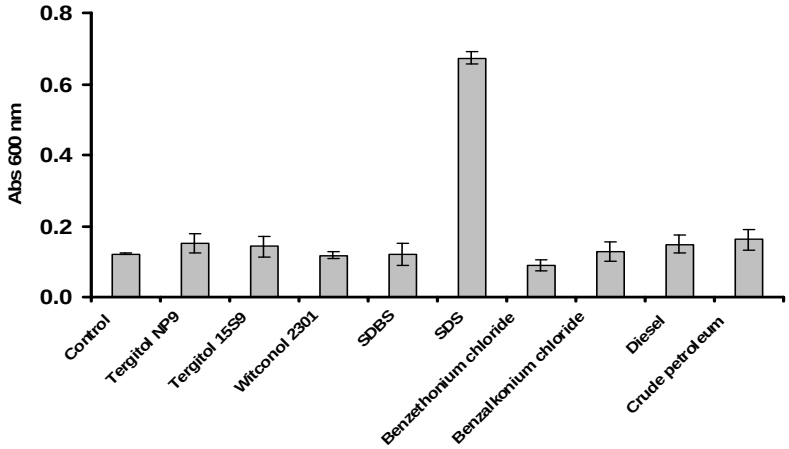

Fig. 1. Growth of *Enterobacter* sp. strain Neni-13 on xenobiotics as sole source of carbon and energy. Bars signify the standard deviation of triplicates.

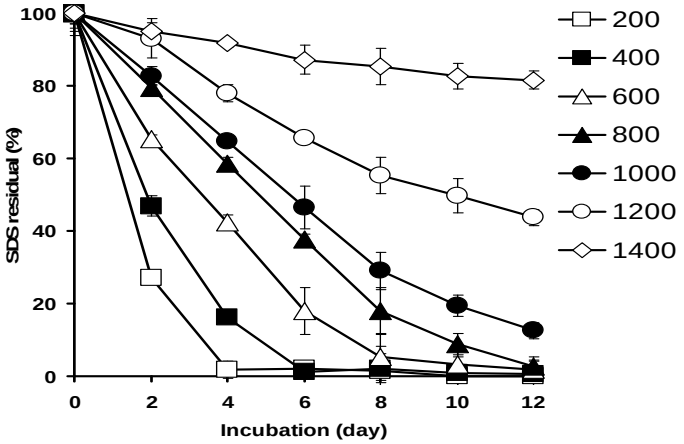

Fig. 2. SDS degradation by *Enterobacter* sp. strain Neni-13. The bacterium was grown at various concentrations of SDS in a volume of 50 mL in a 250 mL shake-flask on an orbital shaker at 120 rpm at room temperature (27 ^{o}C). Bars signify the standard deviation of triplicates.

3.2. Identification of molybdate-reducing bacterium

Strain Neni-13 was a Gram-negative, short rod-shaped, motile, and facultative anaerobic bacterium (Table 1). Identification of the bacterium through the ABIS online software suggested that the identity of the bacterium giving the highest similarity or homology score of 90 % and accuracy score of 100 % as *Enterobacter* sp. strain Neni-13. A more accurate molecular identification technique through phylogenetic analysis of the 16srRNA gene is currently being carried out to identify this species further. However, for now the bacterium is identified as *Enterobacter* sp. strain Neni-13 in honor of the late Dr. Neni Gusmanizar.

Table 1. Overview on the presence (+) or absence (-) of individual biochemical activities for *Enterobacter* sp. strain Neni-13.

Motility	+	Acid production from:	
Pigment	–	Alpha-Methyl-D-Glucoside	+
Catalase production (24 h)	+	D-Adonitol	+
Oxidase (24 h)	–	L-Arabinose	+
ONPG (beta-galactosidase)	+	Cellobiose	+
Arginine dihydrolase (ADH)	+	Dulcitol	+
Lysine decarboxylase (LDC)	–	Glycerol	+
Ornithine decarboxylase (ODC)	+	D-Glucose	+
Nitrates reduction	+	myo-Inositol	+
Methyl red	–	Lactose	+
Voges-Proskauer (VP)	+	Maltose	+
Indole production	–	D-Mannitol	+
Hydrogen sulfide (H_2S)	–	D-Mannose	+
Acetate utilization	+	Melibiose	+
Malonate utilization	+	Mucate	+
Citrate utilization (Simmons)	+	Raffinose	+
Tartrate (Jordans)	+	L-Rhamnose	+
Esculin hydrolysis	+	Salicin	+
Gelatin hydrolysis	–	D-Sorbitol	+
Urea hydrolysis	+	Sucrose	+
Deoxyribonuclease	–	Trehalose	+
Lipase (corn oil)	–	D-Xylose	+
Phenylalanine deaminase	–		
Growth on KCN medium	+		

3.3. Scanning absorption spectrum of molybdenum blue from Enterobacter *sp. strain Neni-13*

A scanning absorption spectrum of the molybdenum blue produced from this bacterium from the visible to the near infrared (IR) regions showed a peak with a maximum wavelength of 865 nm and a shoulder at approximately 710 nm (Fig. 3). The molybdenum blue spectra at 24-h and 48-h of incubations were similar.

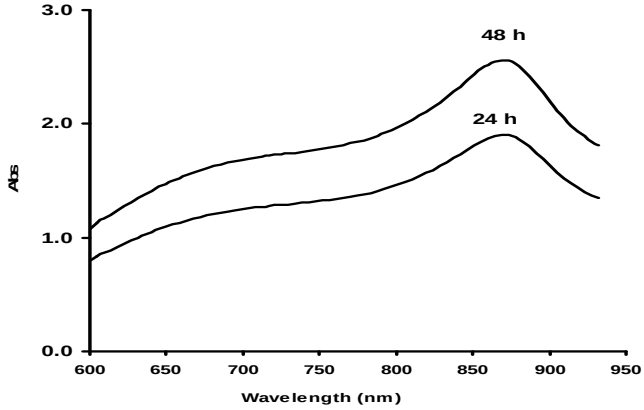

Fig. 3. Scanning absorption spectrum of molybdenum blue from *Enterobacter* sp. strain Neni-13 at different time intervals.

3.4. Carbon sources as electron donor for molybdenum reduction

Glucose was the best electron donor for supporting molybdenum reduction *Enterobacter* sp. strain Neni-13. This is followed in descending order of efficiency by sucrose, l-rhamnose, maltose, lactose, cellobiose, d-mannose, raffinose, mucate, d-mannitol, d-adonitol, melibiose, glycerol, d-sorbitol and l-arabinose (Fig. 4). The optimal concentration of glucose was 1 % (w/v) (data not shown).

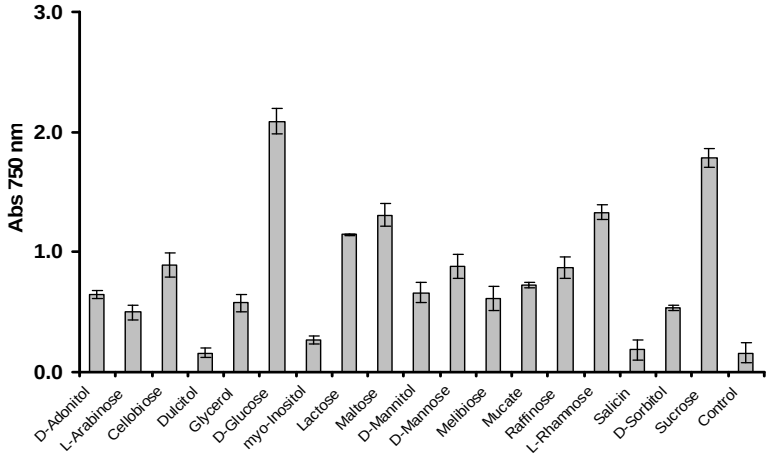

Fig. 4. The effect of various carbon sources on molybdenum blue production by *Enterobacter* sp. strain Neni-13. Bars signify the standard deviation of triplicates.

3.5. Molybdenum reduction at various phosphate and molybdate concentrations

The optimum concentration of phosphate occurred between 2.5 and 5.0 mM with higher concentrations were strongly inhibitory to reduction (Fig. 5).

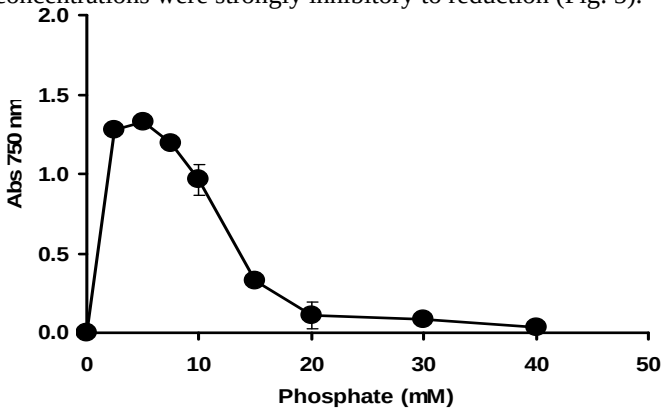

Fig. 5. The effect of phosphate concentrations on molybdenum blue production by *Enterobacter* sp. strain Neni-13. Bars signify the standard deviation of triplicates.

Molybdenum blue production at various sodium molybdate concentrations was studied by varying molybdate concentrations from 0 to 70 mM. The result shows that a lag period of about 10 hours was observed before molybdenum blue production started, and a maximum molybdenum blue production of 14.8 nmol molybdenum blue after 52 hours of incubation using 15 mM sodium molybdate was observed. An inhibition to molybdenum reduction was observed at molybdate concentrations higher than 30 mM (Fig. 6).

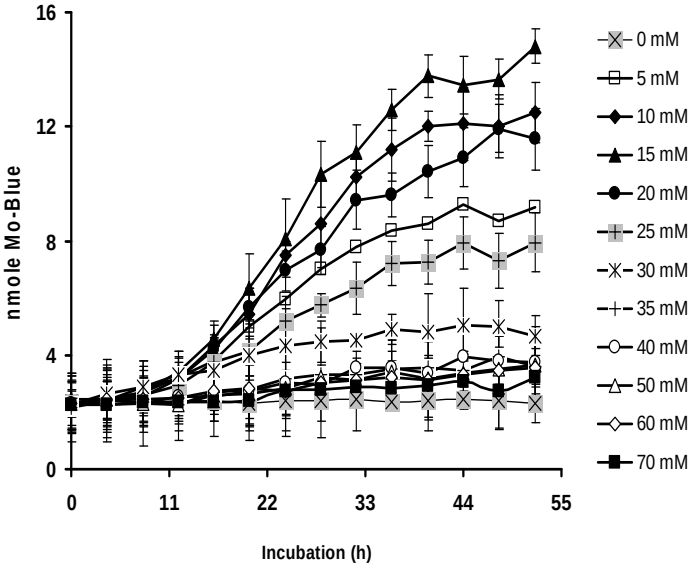

Fig. 6. The effect of molybdate on molybdenum blue production by *Enterobacter* sp. strain Neni-13. Bars signify the standard deviation of triplicates.

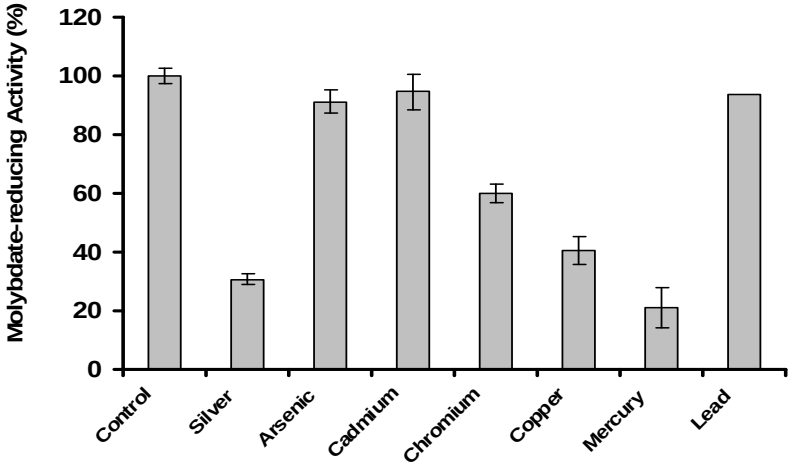

Fig. 7. The effect of heavy metals (2 ppm) on molybdenum blue production by *Enterobacter* sp. strain Neni-13. Bars signify the standard deviation of triplicates.

3.6. Effects of heavy metals on molybdate reduction

Molybdate reduction was inhibited by the heavy metals mercury, silver, copper and chromium with both mercury and silver showing a similar strong inhibition as judged by ANOVA analysis (Fig. 7).

4. Discussion

Although the phenomenon of molybdate reduction to molybdenum blue has been reported in *E. coli* more than a century ago (CAPALDI and PROSKAUER, 1896), the first detailed study on this phenomenon was carried out in *E. coli* K12 (CAMPBELL *et al.,* 1985). In 1993, the same phenomenon was reported in *Enterobacter cloacae* strain 48 (GHANI *et al.*, 1993), without citing previous works, indicating the rarity of similar studies. The potential for utilization in the bioremediation of molybdenum has first beent recognized by GHANI *et al.* (1993). To date many more Mo-reducing bacteria have been isolated (Table 2), including two strains that can grow on the detergent SDS (HALMI *et al.,* 2013b; MASDOR *et al.,* 2015). In addition, a psychrophilic Mo-reducing bacterium is also isolated from Antarctica. Previously, two molybdenum-reducing bacterium from this genus; *Enterobacter cloacae* strain 48 (GHANI *et al.,* 1993) and *Enterobacter* sp. strain Dr.Y13 (SHUKOR *et al.,* 2009) have been isolated.

Studies on the optimal conditions for molybdenum reduction utilizes the microtiter plate format with the use of whole or resting cells allowing a high throughput characterization to be carried out (SHUKOR and SHUKOR, 2014; MASDOR *et al.,* 2015). Usage of resting cells or whole cells was first started in the bacterium *Enterobacter cloacae* strain 48 (GHANI *et al.,* 1993). There are considerable reports on the utilization of resting cells in a microtiter plate format indicating the favorable use of this method. This include a study on polyalcohol ethoxylate biodegradation (SHARVELLE *et al.,* 2008). One of the advantages of resting cells is it can give cells tolerance to toxic xenobiotics especially in the beginning of the growth process.

The inhibition by heavy metals on molybdenum reduction was observed in nearly all of the molybdenum-reducing bacteria isolate to date (Table 2). The presence of these heavy metals at potential bioremediation site for molybdenum could present a problem if the concentrations of the metals are above 2 ppm. The inhibitory effects of heavy metals such as mercury and copper have been reported in bacterial chromate reduction, with the target of inhibition by these metal ions suggested as the sulfhydryl group of the chromate reductase (AHMAD *et al.,* 2013b). Several chemicals such as calcium carbonate, manganese oxide, and magnesium hydroxide can be added to bioremediation sites to alleviate the effect of toxic cations above as these chemicals can bind and immobilize the metal ions (HETTIARACHCHI *et al.,* 2000). However, the effect of the addition of these sequestering agents to molybdenum reduction remains to be studied. Another option to lessen the toxicity of these cationic heavy metals to bioremediation of molybdenum, especially in aquatic bodies is to entrap the molybdenum-reducing bacterium in dialysis tubing or membrane (HALMI *et al.,* 2014).

Table 2. Overview of Mo-reducing bacteria isolated to date.

Bacteria	Optimal C source	Optimal Molybdate (mM)	Optimal PO_4^{3-} (mM)	Heavy metal inhibitors	Reference
Klebsiella oxytoca strain Aft-7	glucose	5-20	5-7.5	Cu^{2+}, Ag^{+}, Hg^{2+}	MASDOR *et al.*, 2015
Bacillus pumilus strain lbna	glucose	40	2.5-5	As^{3+}, Pb^{2+}, Zn^{2+}, Cd^{2+}, Cr^{6+}, Hg^{2+}, Cu^{2+}	ABO-SHAKEER *et al.*, 2013
Bacillus sp. strain A.rzi	glucose	50	4	Cd^{2+}, Cr^{6+}, Cu^{2+},Ag^{+}, Pb^{2+}, Hg^{2+}, Co^{2+},Zn^{2+}	OTHMAN *et al.*, 2013
Pseudomonas sp. strain DRY2	glucose	15-20	5	Cr^{6+}, Cu^{2+}, Pb^{2+}, Hg^{2+}	SHUKOR *et al.*, 2010
Pseudomonas sp. strain DRY1	glucose	30-50	5	Cd^{2+}, Cr^{6+}, Cu^{2+},Ag^{+}, Pb^{2+}, Hg^{2+}	AHMAD *et al*., 2013a
Enterobacter sp. strain Dr.Y13	glucose	25-50	5	Cr^{6+}, Cd^{2+}, Cu^{2+}, Ag^{+}, Hg^{2+}	SHUKOR *et al.*, 2009
Enterobacter cloacae strain 48	sucrose	20	2.9	Cr^{6+}, Cu^{2+}	GHANI *et al.*, 1993
Escherichia coli K12	glucose	80	5	Cr^{6+}	CAMPBELL *et al.*, 1985
Klebsiella oxytoca strain hkeem	fructose	80	4.5	Cu^{2+}, Ag^{+}, Hg^{2+}	LIM *et al.*, 2012

Literature search showed a variation in the efficiency of bacterial isolates on SDS degradation. SDS-degrading bacteria are potential agents for aquatic and soil bioremediation of SDS. To date, several SDS-degrading bacteria have been isolated and characterized including cold-adapted, psychrophilic or psychrotolerant SDS-degrading (MARGESIN and SCHINNER, 1998; HALMI *et al.*, 2013a). A bacterial consortium of *Pantoea agglomerans* and *Acinetobacter calcoaceticus* is able to degrade 4000 mg/L of SDS in approximately 5 days (ABBOUD *et al.*, 2007). The SDS-degrading bacteria *Pseudomonas betelli* and *Acinetobacter johnsonii* degrades 500 mg/L SDS within 5 days of incubation (HOSSEINI *et al.* 2007). *Klebsiella oxytoca* srain DRY14, degrades 80 % of 2 000 mg/L of SDS within 4 days of incubation (SHUKOR *et al.*, 2009), while *Pseudomonas aeruginosa* sp. degrades 100 % of 1 000 mg/L of SDS within 2 days of incubations (SYED *et al.*, 2010). One of the most efficient SDS-degrading bacterium isolated is a mutated strain of *Pseudomonas aeruginosa MTCC* 10311 that degrades 1 500 mg/L of SDS within two days of incubation (AMBILY and JISHA, 2012). At high concentrations, SDS disrupts cellular membrane integrity. This leads to disturbances to the ion gradients resulting in the leakage of bacterial cytosolic contents (CHATURVEDI and KUMAR, 2011). Another mechanism of SDS toxicity is through surface protein and enzymes denaturation (AMBILY and JISHA, 2012).

The identity of the molybdenum blue produced from bacterial reduction of molybdenum has never been identified since it was first mentioned in 1896. CAMPBELL *et al.* (1985) were the first to suggest that the molybdenum blue found in *E. coli* K12 is likely a reduced form of phosphomolybdate. They primarily based this evaluation on the molybdenum blue spectrum acquired. Phoshomolybdate (molybdophosphate) was not mentioned in the works on *Enterobacter cloacae* strain 48 by GHANI *et al.* (1993). Instead, they proposed that the Mo-reducing enzyme to

molybdenum (Mo^{5+}) reduces molybdenum in the form of molybdate (Mo^{6+}) enzymatically to Mo^{5+} before the reaction with phosphate anion formed molybdenum blue. Nonetheless, there are a few problems with this mechanism. Firstly, molybdenum exist as $[MoO_4]^{2-}$ or molybdate anions, and does not exist as Mo^{6+} in solution. Secondly, an acidified solution of molybdate above 1 mM will be instantaneously converted to polyions such as $Mo_8O_{26}^{4}$, $Mo_7O_{24}^{6-}$, and $Mo_{12}O_{37}^{2-}$. Thirdly, these polyions combine with phosphate and form the heteropolymolybdate molybdophosphate or phosphomolybdate. Fourthly, enzymes such as aldehyde and xantine oxidases can catalytically reduce phosphomolybdate to molybdenum blue but not molybdate to molybdenum blue (GLENN and CRANE, 1956). Lastly, almost all of the bacterially created molybdenum blue display spectra that demonstrate near resemblance of the molybdenum blue generated from the phosphate determination method (PDM) (SHUKOR *et al.,* 2007; MASDOR *et al.,* 2015). The heteropolymolybdate species of molybdenum blue produced in the PDM is phosphomolybdate, the latter displays a scanning spectrum with a characteristics shoulder of from 700 to 720 nm, and a peak maximum from 870 nm to 890 nm (CLESCERI *et al.,* 1989). In line with all of this, we suggested a new hypothesis on the standard mechanism of molybdenum blue production in bacteria. In this new mechanism, we propose that initially, a phoshomolybdate intermediate is formed from molybdate. Phosphomolybdate is then reduced by the Mo-reducing enzyme to molybdenum blue in bacteria (SHUKOR *et al.,* 2007). When we use phosphomolybdate as the electron acceptor substrate instead of the original molybdate in the Mo-reducing enzyme assay, we obtain an increase in enzyme activity of about two hundred times. Utilizing this compound, the Mo-reducing enzyme is purified for the first time after the phenomenon was first documented in 1896 (SHUKOR *et al.,* 2014).

The existence of an intermediate species in molybdenum blue production is likewise noticed during heavy metal reduction of chromate. In the bacteriaum *Shewanella putrefaciens* (now known as *S. oneidensis*) (MYERS *et al.,* 2000), biological reduction of chromate from the 6+ to 3+ shows the occurrence of the intermediate species Cr^{5+}. This has been proven via spectroscopic and paramagnetic resonance works. This indicates that the presence of an intermediate species is not unique to molybdenum. Spectroscopic analysis of the resultant molybdenum blue as a method to distinguish between existing heteropolymolybdates including silicomolybdate, sulphomolybdate and phosphomolybdate is a simple and acceptable method, and is routinely used to characterize phoshomolybdate or to differentiate between the numerous heteropolymolybdate (SIMS 1961). However, further investigations making use of nuclear magnetic resonance and electron spin resonance are needed for detail identification of the possible lacunary species of phosphomolybdate observed in bacterial reduction of molybdenum to molybdenum blue (SIMS, 1961).

Carbon sources such as glucose and sucrose are efficient substrate for growth, energy or electron donors for the reduction of heavy metals. As bioremediation sites are often electron donor deficient, studies on the best electron donor are very useful as an important additive in bioremediation sites. At very low concentration of cells,

carbon sources are often utilized as carbon sources for energy, as the cell concentration increases, bacterial cells begin to produce excess reducing equivalents such as NADPH and NADH that are true electron donors for metal reduction. In resting or whole cells preparation, carbon sources are converted to reducing equivalents in a much greater amount, which makes metal reduction increases in efficiency. Glucose is also one of the best electron donors in many metal reductions such as chromate (LLOVERA *et al.*, 1993) and selenate (LOSI and FRANKENBERGER JR., 1997). Mo-reducing bacteria isolated so far either prefer glucose or sucrose as efficient electron donor for supporting molybdenum reduction to molybdenum blue (Table 2). Metabolic pathways such as glycolysis, Kreb's cycle and the electron transport system are the sites where these carbon sources are converted to the reducing equivalents NADH and NADPH. Both of these reducing equivalents are substrates for the molybdenum reducing-enzyme (SHUKOR *et al.*, 2014).

Molybdenum reduction is impacted by phosphate concentrations, with high phosphate concentrations significantly decreasing its production in the course of bacterial reduction of molybdenum. As an example, GHANI et al. revealed that phosphate concentrations greater than 2.9 mM inhibited reduction (GHANI *et al.*, 1993), whilst levels greater than 5 mM inhibited most of the Mo-reducing bacteria isolated up to now (Table 1). Previous studies (GLENN and CRANE, 1956) have demonstrated that high phosphate concentrations negatively affecting phosphomolybdate structure by means of sustaining the pH at neutral. The phosphomolybdate complex is extremely unstable at this pH, and it is solely stable under acidic environments (GLENN and CRANE, 1956).

Mo-reducing bacteria isolated so far can tolerate and reduce molybdate at the optimal concentrations ranging from 5 to 80 mM (Table 2). As molybdenum concentrations may achieve up to 900 mg/L in waters and 6,500 mg/Kg in soils (STONE and STETLER, 2008), Mo-reducing bacteria represent a meaningful tool for remediation processes. Another essential prerequisite is the soil phosphate concentrations should be about 5 mM but not exceeding 20 mM for reduction to occur. Fortunately, phosphate concentrations are hardly ever observed exceeding beyond these range in regular type of soils (CHAI *et al.*, 2011).

5. Conclusions

A molybdate-reducing bacterium with the ability to grow on SDS as a sole carbon source has been isolated. Molybdenum blue characterization results indicate a good tolerance to high concentration of molybdenum, a requirement for low concentration of phosphate. Glucose, one of the most assimilable carbon sources was the best electron donor for molybdenum reduction. The absorption spectrum of molybdenum blue indicates that molybdenum blue is likely a reduced phosphomolybdate. Molybdenum reduction was inhibited by several toxic heavy metals indicating future bioremediation.

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